The Interaction of DNA with Hoechst 33258 Studied by Fluorescence Spectroscopy

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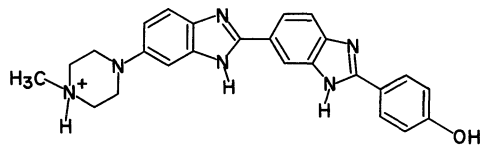
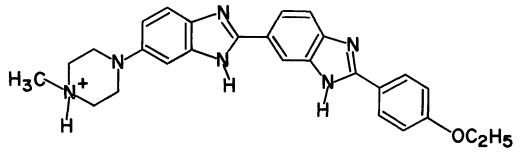
The interaction of DNA with the bi-1*H*-benzimidazole dye Hoechst 33258 at a high molar ratio of DNA phosphate to dye has been studied by means of steady-state and nanosecond time-resolved emission spectroscopy. The spectral shifts and lifetime data, which can be interpreted in terms of the solvent-solute relaxation, are consistent with the existence of two types of bound species. One type, less hydrated bound species, predominates at high ionic strength, whereas the other, more hydrated bound species, occurs at low ionic strength.

The bi-1*H*-benzimidazole dye Hoechst 33258 [H33258; 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole; Fig. 1] has been extensively used as a base-specific fluorescent probe for metaphase chromosomes[1,2] and DNA.[3-9] This fluorochrome, which is only weakly fluorescent in aqueous solution, gives an intense fluorescence especially when bound to AT-rich regions in DNA and chromation.[3-9] Spectroscopic and hydrodynamic studies show that H33258 does not bind to DNA by intercalation, but rather binds externally to the double helical structure of DNA.[3-10] Two types of binding modes have been proposed.[4,5,8-10] The primary (strong) binding mode occurs at a high molar ratio of DNA phosphate to dye (P/D>5—20) with binding constant (10^6—10^7) dm^3 mol^{-1}, exhibits a strong specificity for AT base pairs, and shows little dependence on ionic strength. The secondary (weak) binding mode occurs at low P/D ratios with a weaker binding constant of (10^4—10^5) dm^3 mol^{-1}, shows little base specificity, causes quenching of the fluorescence, and is weakened with increasing ionic strength. Footprinting studies[9,11,12] have shown that the strong binding site of H33258 spans three to five base-pairs which predominantly involve AT pairs and the minor groove of B-DNA has been implicated as the site of binding by competition studies with other known groove-binding molecules such as netropsin. Recent X-ray crystallographic studies of the complex of H33258 with B-DNA dodecamers d(CGCGAATTCGCG)[13,14] and d(CGCGATATCGCG)[15] have revealed that the binding is indeed through the minor groove of B-DNA.



H33258



H33342

Fig. 1. Chemical structures of H33258 and H33342.

In order to better understand the microenvironment of bound H33258, we applied steady-state and nanosecond time-resolved fluorescence spectroscopy and investigated the physical properties of the binding of H33258 to natural DNAs and synthetic polynucleotides at high P/D ratios. Among the results, it is revealed that there are two types of bound species depending on the degree of hydration.

Experimental

The following DNAs and synthetic polynucleotides were commercial products: calf thymus DNA (CT DNA, 42% GC; Worthington), *Micrococcus lysodeikticus* DNA (ML DNA, 72% GC; Sigma), poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC)(P-L Biochemicals), poly(rA) and poly(rU) (Yamasa Shoyu). Double-stranded poly(rA)·poly(rU) was prepared by mixing aliquots of poly(rA) and poly(rU) solutions and equilibrating the mixtures for two days at 4 °C to complete complex formation. H33258 and Hoechst 33342 [H33342; 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole; Fig. 1], chromatographically pure, were obtained from Sigma. Analytical grade reagents and double-distilled water were used for the preparation of all aqueous solutions.

Absorption and circular dichroism spectra were recorded with a Hitachi U-3200 spectrophotometer and a JASCO J-20 spectropolarimeter, respectively. Steady-state fluorescence spectra were recorded with a Hitachi MPF-2A spectrophotofluorometer. Observed fluorescence spectra were corrected for the unequal wavelength sensitivity of the detector system. Fluorescence quantum yields were determined by comparing the area under the fluorescence spectrum with the corresponding area of the fluorescence standard; quinine sulfate was used for the standard.[16,17] Fluorescence decay curves and nanosecond time-resolved fluorescence spectra were measured with an Ortec nanosecond emission spectrophotometer. Further details and the analysis of data are described elsewhere.[18]

Flow dichroism measurements were made as described elsewhere.[19]

All measurements were made in phosphate buffer (pH 6.9) at 25 °C; ionic strength was controlled by adding NaCl solution. Dye concentrations and P/D values for fluorescence measurements were $(1.8—2.0)\times10^{-6}$ mol dm^{-3} and 200—300, respectively. The equilibrium dialysis experiments showed that the amount of unbound dye is negligibly small at high P/D values (P/D>50).

Results

The absorption spectrum of H33258 is well known to undergo changes in both intensity and maximum wavelength in the presence of DNA.[3-7] To gain a deeper insight into the binding interaction, we first reexamined absorption and CD spectra of DNA-H33258 systems as a function of P/D under various salt concentrations. Figures 2 and 3 show typical results obtained with CT DNA–H33258 systems. The free dye exhibits an absorption maximum at 338 nm, whereas the spectrum at a low P/D value shows a marked hypochromicity which is accompanied by a red shift in the absorption maximum. There are a progressive increase in absorption intensity and a continued red shift with increasing P/D until there is no further change at a high P/D (P/D>50). In agreement with the previous findings,[5-7] CD spectra of DNA-H33258 complexes exhibit a broad positive band with a maximun around 335 nm at a low P/D value. As the P/D value increases, this is progressively replaced by an intense positive band at 335—360 nm. There is no negative band at wavelengths above 300 nm at any P/D value. The CD band around 335 nm at a low P/D value has been assigned to the dye aggregates which causes quenching of the fluorescence;[5] this band was found to decrease with increasing ionic strength. Careful inspection of Figs. 2 and 3 reveals some interesting features in absorption and CD spectra. (1) Both absorption and CD spectra approach limits at high P/D values (P/D>50). (2) The absorption spectrum at a high P/D value is almost identical with the corresponding CD spectrum. (3) The apparent peaks of both spectra shift to shorter wavelengths with increasing ionic strength. (4) The narrowing of both absorption and CD bands occurs at high ionic strength when compared to those at low ionic strength. In view of findings (1) and (2), it is quite likely that the spectra at high P/D values result from isolated monomeric bound species.

In order to avoid the complication due to the presence of different binding processes, the following fluorescence studies were performed at sufficiently high P/D values (P/D>200) where strong binding predominates and concentration of free dye is negligible.

In agreement with the findings previously reported,[3-8] fluorescence maxima (λ^{f}_{max}) of DNA–H33258 complexes at high P/D values are blue-shifted as compared to that of free H33258 (λ^{f}_{max}=510 nm). More-

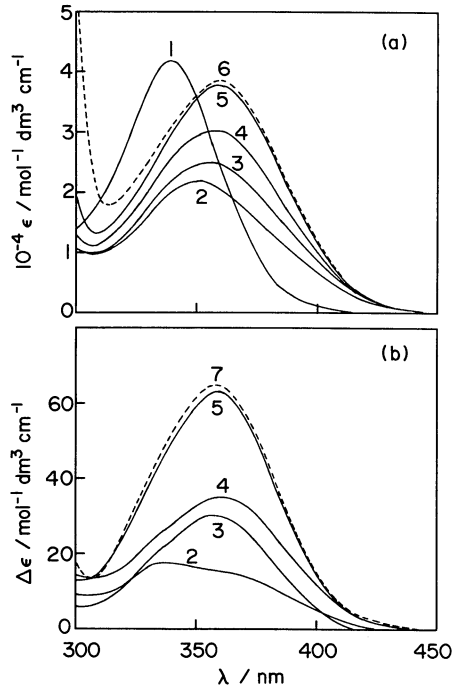


Fig. 2. (a) Absorption and (b) CD spectra of CT DNA–H33258 complexes in 0.005 mol dm^{-3} phosphate buffer. P/D: (1) 0; (2) 4.9; (3) 9.6; (4) 19; (5) 48; (6) 201; (7) 226. The dye concentrations were 1.3×10^{-5} mol dm^{-3} for (1)—(5), 6.3×10^{-6} mol dm^{-3} for (6), and 2.6×10^{-6} mol dm^{-3} for (7).

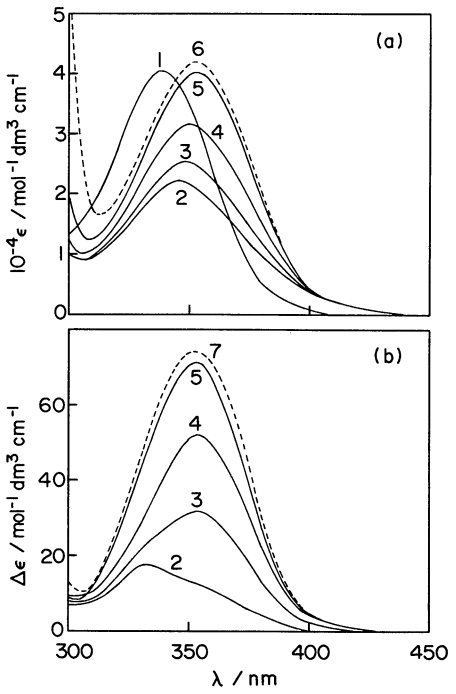


Fig. 3. (a) Absorption and (b) CD spectra of CT DNA–H33258 complexes in 0.005 mol dm^{-3} phosphate buffer, 0.5 mol dm^{-3} NaCl. Legends to each spectrum are as described for Fig. 2.

Table 1. Fluorescence Decay Parameters and Quantum Yields for H33258 Bound to Various Polynucleotides[a]

Polynucleotide	[NaCl][b]	λ^{a}_{max}	λ^{f}_{335}[c]	τ_1	α_1	τ_2	α_2	Φ_F
	mol dm^{-3}	nm	nm	ns		ns		
CT DNA	0	359	470	2.1	0.43	4.1	0.57	0.75
	0.04	354	464	2.4	0.70	4.1	0.30	0.77
	0.2	352	462	2.1	0.78	4.0	0.22	—
	0.5	352	460	2.3	0.83	4.2	0.17	0.76
ML DNA	0	360	482	2.3	0.58	4.5	0.42	0.37
	0.5	351	464	2.5	0.89	4.7	0.11	0.40
Poly(dA-dT)·poly(dA-dT)	0	359	482	2.1	0.32	4.2	0.68	0.82
	0.5	351	468	2.4	0.85	4.7	0.15	0.78
Poly(dG-dC)·poly(dG-dC)	0	360	488	2.4	0.65	4.7	0.35	0.27
	0.5	347	480	1.9	0.86	4.6	0.14	0.19
	4.0[d]	350	488	—	—	3.4	1.0	0.21
Poly(rA)·poly(rU)	0	367	484	—	—	4.9	1.0	0.72

a) Buffers used were 0.005 mol dm^{-3} for DNAs and synthetic polydeoxynucleotides and 0.01 mol dm^{-3} for poly(rA)·poly(rU). The excitation wavelength was 335 nm and the decay was observed at each apparent emission peak. The amplitudes (α's) are normalized to unity. The Φ_F values for free H33258 were 0.03 and 0.05, respectively, in 0.005 mol dm^{-3} phosphate buffer and 0.005 mol dm^{-3} phosphate buffer, 0.5 mol dm^{-3} NaCl. b) The concentration of NaCl in buffer solutions. c) λ^{f}_{335} denotes the fluorescence maximum upon the excitation at 335 nm. λ^{a}_{max} and λ^{f}_{335} of free H33258 were 338 nm and 510 nm. d) Poly(dG-dC)·poly(dG-dC) exists as a left-handed helix, which was confirmed by circular dichroism measurements.[20]

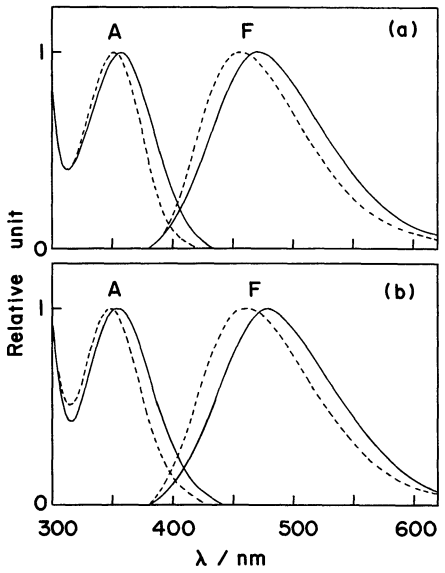


Fig. 4. Absorption (A) and fluorescence (F) spectra of (a) CT DNA–H33258 and (b) ML DNA–H33258 complexes in 0.005 mol dm^{-3} phosphate buffer (—) and 0.005 mol dm^{-3} phosphate buffer, 0.5 mol dm^{-3} NaCl (-----). Fluorescence spectra were observed upon excitation at 335 nm.

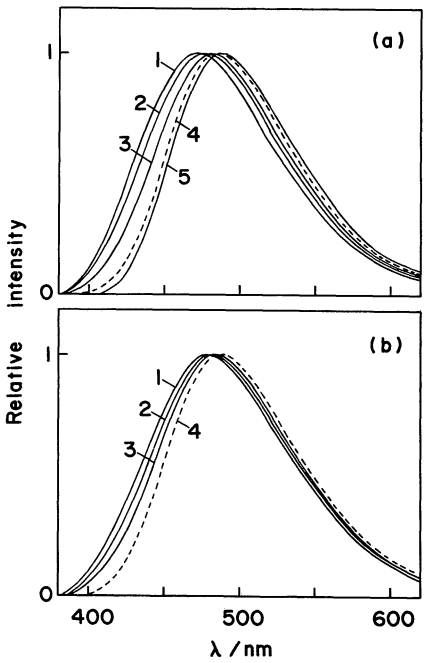


Fig. 5. Fluorescence spectra of (a) CT DNA–H33258 and (b) ML DNA–H33258 complexes in 0.005 mol dm^{-3} phosphate buffer as a function of excitation wavelength. The spectra were corrected for distortions by scattering and are shown for excitation wavelengths of (1) 335 nm, (2) 365 nm, (3) 380 nm, (4) 395 nm, and (5) 410 nm.

over, it was found that fluorescence spectra of H33258 bound to DNA as well as absorption spectra are markedly affected by ionic strength. As shown in Fig. 4 and Table 1, both absorption maxima (λ^{a}_{max}) and λ^{f}_{max} shift toward shorter wavelengths with increasing ionic strength. In order to make clear the spectral shifts dependent on ionic strength (Figs. 2—4), fluorescence and fluorescence-excitation spectra of complexes were measured as a function of the excitation wavelength and emission wavelength, respectively. As typical results are shown in Fig. 5, it was found that λ^{f}_{max} is red-shifted with an increase in the excitation wavelength. It was also observed that the apparent peak of the fluorescence-excitation spectrum shifts to the longer wavelength with increasing the emission

Table 2. Fluorescence Decay Parameters for H33258 Bound to CT DNA and ML DNA as a Function of the Excitation (λ_{ex}) and Emission (λ_{em}) Wavelengths[a)]

DNA	λ_{ex}	λ_{em}	τ_1	α_1	τ_2	α_2
	nm	nm	ns		ns	
CT DNA	335	430	2.5	0.92	4.7	0.08
		450	2.5	0.65	4.4	0.35
		460	2.1	0.43	4.1	0.57
		470	2.4	0.45	4.6	0.55
		490	2.3	0.36	4.1	0.64
	380	460	2.5	0.38	4.3	0.62
	405	460	2.1	0.36	4.3	0.64
ML DNA	335	440	2.7	0.90	5.1	0.10
		465	2.3	0.58	4.5	0.42
		490	2.2	0.55	4.6	0.45
	380	465	1.9	0.51	4.3	0.49

a) The solvent was 0.005 mol dm^{-3} phosphate buffer. The amplitudes (α's) are normalized to unity.

wavelength (data not shown). These spectral shifts, observed anew in this study, were pronounced at low ionic strength and for DNA with high AT content.

To further clarify the phenomenon of red shifts of fluorescence spectra upon red-edge excitation, we measured the dependence of fluorescence decays upon the excitation and emission wavelengths. It was found that the fluorescence decay curves of the DNA–H33258 complexes can be well resolved into two-exponential components corresponding to the short (τ_1) and long (τ_2) lifetimes (Tables 1 and 2). Table 1 summarizes fluorescence decay parameters and quantum yields (Φ_F) upon binding of H33258 to various polynucleotides, where the excitation wavelength was 335 nm and the decay curves were observed at each apparent fluorescence peak. It can be clearly seen from Table 1 that the amplitudes (α's) depend on both the base composition of DNA and ionic strength. Table 1 also shows that Φ_F values of DNA–H33258 complexes are almost constant irrespective of ionic strength, suggesting that the contribution of the weak binding mode which results in quenching of the fluorescence is negligible at high P/D values. Table 2 shows that the fluorescence decay parameters for H33258 complexed with CT DNA and ML DNA depend on both the excitation and emission wavelengths; the amplitude (α_2) corresponding to the long lifetime (τ_2) increases with increasing excitation and emission wavelengths.

From determinations of the time-resolved emission spectra of DNA–H33258 complexes, it was found that large time-dependent spectral shifts are observed (Fig. 6); this behavior was pronounced at low ionic strength. Inspection of Figs. 5 and 6 shows that the fluorescence spectrum at a shorter time range (<4 ns) resembles the steady-state fluorescence spectrum excited at a wavelength shorter than the absorption maximun, while the fluorescence spectrum at a longer time range (>10 ns) is similar to that excited at a wavelength longer than it.

We next examined absorption, CD, and fluorescence

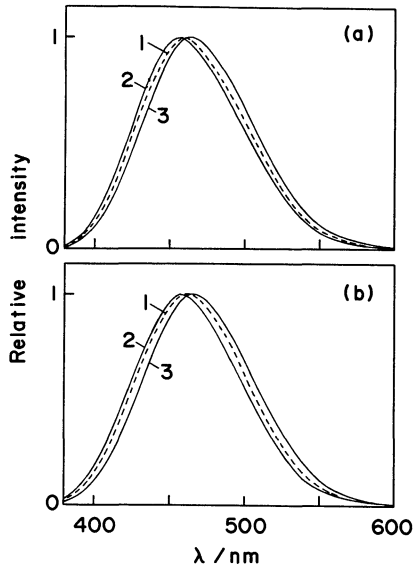


Fig. 6. Nanosecond time-resolved fluorescence spectra of (a) CT DNA–H33258 and (b) ML DNA–H33258 complexes in 0.005 mol dm^{-3} phosphate buffer. The excitation wavelength was 335 nm and the spectra were not corrected for instrumental wavelength response. (1) Total fluorescence (t=0—90 ns); (2) t=0—4 ns; (3) t=10—90 ns. t denotes the time after the initial rise of the nanosecond flash lamp.

properties using H33342 which has a structure very similar to H33258 (Fig. 1). It was found that H33342 exhibits almost the same optical behavior as that of H33258. Here, Fig. 7 shows typical steady-state and time-resolved fluorescence spectra obtained with the CT DNA–H33342 complex. Fluorescence lifetimes and quantum yields of the CT DNA–H33342 complex in 0.005 mol dm^{-3} phosphate buffer were as follows: τ_1=2.4 ns, τ_2=4.7 ns, and Φ_F=0.76.

To obtain some information concerning the orientation of the dye bound to DNA, we lastly investigated the flow dichroism of the CT DNA–H33258 and CT DNA–H33342 complexes. The flow dichroism for the

Table 3. Flow Dichroism of CT DNA–H33258 and CT DNA–H33342 Systems[a]

Dye	[NaCl][b] / mol dm^{-3}	P/D	λ / nm	$(\Delta\varepsilon/\varepsilon)_{3040}$	$B(\alpha)$[c]	α[c] / °
H33258	0	66	360	0.223	0.60	46.9
	0.5	99	355	0.206	0.58	47.2
H33342	0	99	360	0.140	0.49	48.3
	0.5	99	355	0.130	0.44	49.0

a) The buffer used was 0.005 mol dm^{-3} phosphate buffer and dye concentrations were (2.0—2.3)×10^{-5} mo dm^{-3}. b) The concentration of NaCl in buffer solutions. c) The uncertainties in $B(\alpha)$ are estimated to be ±0.05, corresponding to approximately ±1° in α.

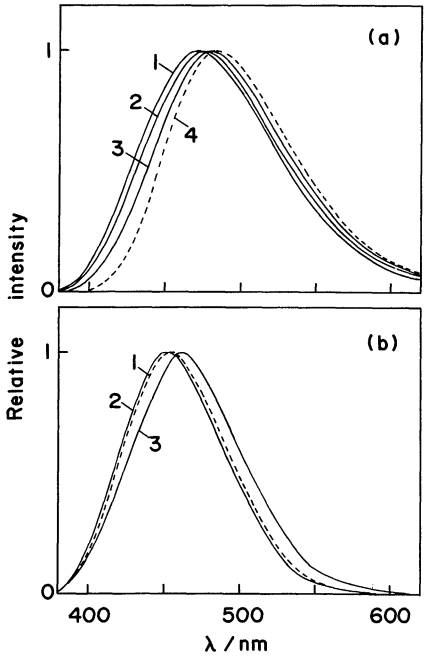


Fig. 7. (a) Fluorescence and (b) nanosecond time-resolved fluorescence spectra of CT DNA–H33342 complexes in 0.005 mol dm^{-3} phosphate buffer. Legends to each spectrum are as described for Figs. 5 and 6.

absorption region of DNA bases, 220—280 nm, was negative as expected for double-stranded DNA with the planes of the bases oriented more or less perpendicularly to the DNA helix. The flow dichroism for the dye absorption band, 320—400 nm, was positive, in contrast to the intercalators such as acridine dyes which show negative flow dichroism.[19] The reduced dichroism at a velocity gradient of 3040 s^{-1}, $(\Delta\varepsilon/\varepsilon)_{3040}$, was found to be −0.222—−0.284 at 260 nm and 0.130—0.223 at the absorption maxima (355—360 nm) (Table 3); here, $\Delta\varepsilon$ is the difference between the molar absorption coefficient for the light polarized along to the flow line and that polarized perpendicularly to it, and ε is the molar absorption coefficient of the sample. The reduced dichroism at a perfect orientation, $B(\alpha)$, can be estimated from the dependence of the reduced dichroism on the velocity gradient and is given by[21]

$$B(\alpha) = 3(3\cos^2\alpha - 1)/2 \quad (1)$$

where α is the angle between the direction of electronic transition moment of the chromophore and the DNA helix axis. Table 3 summarizes the values of $B(\alpha)$ and α, demonstrating that the orientation of the bound H33258 and H33342 is more parallel than perpendicular to the DNA helix.

Discussion

The present data of absorption, CD and fluorescence quantum yields confirm that the strong binding mode predominates at high P/D values. The previous optical and hydrodynamic studies claim that the strong binding mode shows little dependence on ionic strength.[5,8] However, the present fluorescence studies of DNA–H33258 complexes clearly indicate that the bound H33258 at high P/D values exists in at least two distinct species. One type (Type I) predominates at high ionic strength, while the other (Type II) occurs at low ionic strength. Optical properties obtained with CT DNA are summarized as follows (Table 1): for Type I, λ^a_{max}=325 nm, λ^f_{335}=460 nm and τ=2.1—2.4 ns; for Type II, λ^a_{max}=359 nm, λ^f_{335}=470 nm and τ=4.0—4.2 ns. Here, λ^f_{335} denotes the fluorescence peak upon excitation at 335 nm.

We also observed that H33258 in polar viscous solvents exhibits large time-dependent spectral shifts as well as 8-anilino-1-naphthalenesulfonate (ANS),[22] 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS),[23] and *N*-acetyl-L-tryptophanamide.[24] This phenomenon has been ascribed to the dipole–dipole interaction between the solvent and excited-solute molecules.[22-24] Therefore, the present observation suggests a large increase in the dipole moment of H33258 upon electronic excitation; this means that the excited state of H33258 can be stabilized through the solvent-solute relaxation, that is, through the reorientation of solvent molecules around H33258 subsequent to excitation. Furthermore, it is found that ANS and TNS bound to proteins or membranes[23,25] and tryptophan residues in proteins[26,27] also show both time-dependent spectral shifts and fluorescence shifts upon red-edge excitation. These phenomena depend on the binding-site environ-

ment or to what extent chromophores are exposed to the surrounding solvent. If the rate of the dipole–dipole relaxation is too slow or too fast compared to the excited lifetimes of chromophores, no time-dependent spectral shifts are observed.[23,25–27] Such a case results from chromophores that exist in the non-polar environment, namely, in the interior of proteins or chromophores that are much exposed to the rapidly relaxing aqueous solvent. Thus, we attribute the fluorescence shifts upon red-edge excitation (Fig. 5) and the wavelength-dependent lifetimes (Table 2) to the reorientation of the solvent around the excited state of the bound H33258. On the red edge of the fluorescence spectra (490 nm), the longer lifetime is predominant because the relaxed emission is formed from the initially excited unrelaxed state. On the blue edge of the spectra (430—440 nm), the shorter lifetime is mainly observed because the blue emission is formed by the transition from the unrelaxed excited state to the ground state.

X-Ray crystallographic studies[13–15] show that the strong binding mode occurs through the groove binding and the bound H33258 is buried deep within the minor groove. This idea is consistent with the data obtained from chemical footprinting experiments.[11,12] Furthermore, the interior of the minor groove is more negative which could explain the stronger binding in this site.[28] The reduced flow dichroism at a perfect orientation indicates that the H33258 transition moment is inclined by about 47° from the helix axis (Table 3), consistent with the idea that H33258 binds inside and parallel to the DNA groove. The same angle (43±3°) is also reported for netropsin and distamycin, typical groove-binding drugs.[29]

In view of these results, it seems reasonable to conclude that Type I is ascribed to H33258 buried deep in the DNA minor groove, less hydrated bound species, whereas Type II is attributed to the dye bound not deep in the DNA grooves (minor or major grove), more hydrated bound species, which is exposed to some extent to the surrounding solvent molecules. This conclusion explains that the bound species Type I is not completely relaxed by the solvent–solute interaction during the excited lifetime, while the bound species Type II fluoresces from the relaxed state and thus shows time-dependent spectral shifts. The present data also indicates that H33342 having a chemical structure very similar to H33258 can exist in two bound species. A comparison of chemical structures between H33258 and H33342 (Fig. 1) suggests that the ethylation of the phenyl group may not exert a significant effect upon the binding nature.

Carrondo et al.[15] have found that the conformation of H33258 bound to the B-DNA dodecamer d(CGCGATATCGCG) considerably differs from that found in the other dodecamer d(CGCGAATTCGCG).[13,14] This suggests that the base sequence may play an important role in the binding mode. In agreement with this, it is observed that the fractions of Types I and II (α_1 and α_2) at low ionic strength depends on the GC content of DNA (Table 1). It is also conceivable that a number of conformational states are in constant equilibrium, depending on the base sequence. Therefore, our results may be interpreted as indicating that the bound species are classified into two main categories, Types I and II.

Table 1 shows that the bound species Type I predominates at high ionic strength. It is expected that an increase in ionic strength decreases the binding of dye molecules and affects the conformations of the DNA-dye complexes.[30,31] For the above finding, therefore, there are two possible explanations. One is that the bound species Type II, the more outside-bound species, may be readily dissociated and the relative population of Type II may be decreased with increasing ionic strength, because the outside binding is primarily an electrostatic interaction. The other is that increasing salt concentration may change the conformation of the DNA-dye complex to one where a local motion of the bound dye would be somewhat restricted,[7,15,31] disturbing the solvent–solute relaxation and hence the bound species Type I may occur predominantly at high ionic strength. Both the time-dependent fluorescence anisotropy and static fluorescence anisotropy (0.31±0.01 upon excitation at the absorption maximun) of the DNA–H33258 complexes were found to be almost independent of ionic strength, suggesting that the local mobility of the bound dye may be insensitive or only slightly sensitive to ionic strength.[7] Judging from this finding, the former explanation appears more acceptable.

Interestingly, for H33258 when bound to double-stranded poly(rA)·poly(rU) and left-handed form of poly(dG-dC)·poly(dG-dC), the fluorescence decay kinetics obeys a single exponential decay law (Table 1) and almost no time-dependent spectral shifts are observed. This means that there exists only one bound species in the cases of poly(rA)·poly(rU) and left-handed DNA (Z-DNA). Poly(rA)·poly(rU) has a conformation with a deep major groove and a shallow minor groove compared to B-DNA and both grooves are a little narrow for H33258 to lie sideways in the grooves.[31] Therefore, it is expected that the binding affinity of H33258 to poly(rA)·poly(rU) is weak, compared with B-DNA; in fact, the binding of H33258 remarkably decreased with increasing ionic strength. On the other hand, the major groove of Z-DNA is wide, but is filled with cytosine C_5 and guanine N_7, C_8 atoms and its minor groove is deep and narrow.[31] Taking into account the conformational structures of poly(rA)·poly(rU) and Z-DNA, it is concluded that H33258 bound to these polynucleotides is exposed to a large extent to the surrounding solvent molecules and thus the emission from the completely relaxed state is

observed.

The fluorescence quantum yield (Φ_F) of bound H33258 decreases with increasing GC content of DNA (Table 1); this suggests that there occurs the specific interaction between a GC pair and the bound H33258 as well as some aminoacridines.[18,32] Further study concerning the specific interaction is in progress.

In conclusion, this study indicates that H33258 bound to DNA can be described as consisting of at least two different types of bound species at high P/D values. It may be that time-resolved emission spectroscopy can contribute to an understanding of the binding mechanism of other fluorescent groove-binding drugs.

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