Salt Effects on Fluorescence Spectral Shifts of DNA-Bound Hoechst 33258 and Reaction Volumes of the Minor Groove Binding

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In this study, salt effects on the interaction of Hoechst 33258 (2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride) with several synthetic polynucleotide duplexes were studied by fluorescence spectral shifts and reaction volumes derived from the pressure dependence of binding equilibria. Both the excitation and emission spectra of Hoechst 33258 saturated by polyd(A)·polyd(T) or poly[d(A-T)]·poly[d(A-T)] shifted to shorter wavelengths as NaCl concentrations increased. The peak intensities first increased ([NaCl] < 100 mM) then started to decrease (> 100 mM NaCl). Salt dependent blue shifts were also observed in their absorption spectra. These results were in contrast to what occurred to the drug complexed with poly[d(G-C)]·poly[d(G-C)] or poly[d(A-G)]·poly[d(C-T)]. The pressure dependence of the association equilibrium constants (K_p) of the drug and two A·T polymers was examined at several salt concentrations; the results helped to derive reaction volumes. In the case of the A-T copolymer, negative volume changes were found at all ionic concentrations studied. In contrast, in the case of the homopolymer, negative volume changes were found at lower salt concentrations (10 and 50 mM) while positive ones were found at higher concentrations (100 and 200 mM NaCl). These results are discussed in terms of the local structural deformation and hydration rearrangement of DNA.

The polymorphism and flexibility of DNA structure are important for various biological functions such as DNA-protein/ligand interactions and chemical reactions involving related sequences of DNA. The polymorphism of DNA structure is a complicated, multidimensional phenomenon, and is highly dependent upon environmental conditions.[1] Changes in DNA conformations by environmental conditions can be probed by the application of steady-state and time-resolved fluorescence spectroscopy on sequences containing fluorescent base analogs or external fluorophores.[2] Many aspects of locally steady and dynamic properties of DNA structure, such as the interior diffusivity and viscosity, the polarity in the major/minor groove, the motion, flipping, and opening of base pairs, and their dependence on environmental conditions have been studied by time-resolved fluorescence polarization,[3] fluorescence spectral shifts,[4,5] and Stokes shifts.[6,7]

Interactions of DNA with a benzimidazole derivative, Hoechst 33258 (2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride), which are strongly dependent upon DNA sequences and environmental conditions, have been extensively studied by structural resolution, thermodynamic determinations, and biological applications.[8—13] No major modification in the structural parameters of DNA is induced by the minor groove binding. An edge-insertion with an average orientation angle of 45 degrees relative to the DNA helix axis was suggested for the binding to poly[d(A-T)]·poly[d(A-T)].[14] A significant reorganization in the flexible structure of Hoechst 33258, such as adopting a more planar structure and a more restricted intramolecular mobility than the free drug in the solution, was suggested to take place in the binding process.[11,14,15] Reconstitution in the drug conformation together with changes in the local environment properties can be characterized by a number of spectroscopic techniques.[12,14,17]

Previously, we have made a physico-chemical characterization of the interaction of Hoechst 33258 with polynucleotides and plasmid DNA and we have quantified volume effects involved in the complexation at a defined salt concentrations.[18] In this study, salt effects on the drug–DNA interactions were examined by the salt effects on pressure dependence of the DNA–drug binding equilibrium and on the fluorescence spectroscopy of DNA–drug complexes. The observed salt effects on absorption and emission spectral shifts as well as on reaction volumes of the complex formations between the drug and A-T polymers are discussed on the basis of the environment dependence of local geometry and hydration of DNA.

Materials and Methods

Poly(dA)·poly(dT) was purchased from Sigma; poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly[d(A-G)]·poly[d(C-T)] were purchased from Pharmacia. The concentrations of these polynucleotide duplexes were determined according to their respective absorption extinction coefficients per nucleotide: ε_{258} ($cm^{-1}\,M^{-1}$) = 6600 (poly(dA)·poly(dT)), 6000 (poly[d(A-T)]·poly[d(A-T)]), 6800 (poly[d(G-C)]·poly[d(G-C)]), and 5700 (poly[d(A-G)]·poly[d(C-T)]), respectively (1 M = 1 mol dm^{-3}).

Hoechst 33258 was purchased from Sigma. It was dissolved in 10 mM Tris-HCl buffer containing 40% dimethyl sulfoxide. Its concentration was determined by monitoring the absorption at 342 nm with ε_{342} ($cm^{-1}\,M^{-1}$) = 42000. Salt concentrations were varied by adding concentrated solutions of corresponding salts.

Hoechst 33258 prefers to bind to the minor groove of A-T-rich sequences of B-DNA with a high binding affinity of 10^7—10^8 M^{-1}.[9,19] The fluorescence spectroscopy of Hoechst 33258, free or bound to DNA in the solution, was monitored through a RF5000 spectrofluorometer (Shimadzu) at a defined temperature (25 °C). The concentration of Hoechst 33258 used was 10 or 100 nM, which is far less than that may arouse self-aggregation or self-absorption.[9] The relative amount of DNA and Hoechst 33258 for observations of spectral shifts without significant disturbance from the free drug molecules was selected based on preliminary fluorescence titrations. On this basis, the ratio of DNA to drug (R = [nucleotides]/[drug]) was determined to be higher than 100/1 for A-T polymers, and the ratio was 400/1 for another two non A-T polymers.

The averaged primary spectra were plotted through several scans of the samples and smoothed by using the software affiliated with the spectrofluorometer. The smoothed spectra were then used to determine and compare the peak wavelengths of Hoechst 33258 free or bound to the DNA.[6] To determine the peak wavelength of an emission spectrum, Hoechst 33258 bound to DNA was excited at 360 nm, below which the red-edge excitation effect was insufficient (< 1 nm) even at 10 mM NaCl. The red-edge excitation effect was significantly diminished by higher salt concentrations. To determine the peak wavelengths of fluorescence excitation spectra independent on the emission wavelength, the dye was emitted at 460 nm (for the two A-T polymers) or 480 nm (for the other two non-A-T polymers). The band-paths for excitation and emission were 5 and 10 nm, respectively.

Fluorescence spectral shifts can be transformed into Stokes shifts (cm^{-1}) as $\nu = 1/\lambda_{ex} - 1/\lambda_{em}$, It is known that Stokes shifts are powerful for reflections of static and dynamic properties of local environments. Furthermore, movements in fluorescence spectra of Hoechst 33258 were also evaluated by the center of spectral mass (< COM > $= \int F_i / \int \lambda_i F_i$), where F_i is the fluorescence intensity at wavelength λ_i. Such data transitions did not alter the conclusion of salt effects of fluorescence spectroscopic properties of DNA-bound Hoechst 33258.

Volume changes involved in Hoechst 33258-DNA interactions were estimated from the pressure dependence of equilibrium properties (K_p) of the complexation by monitoring fluorometric titrations of the drug with DNA. The apparatus, data collection, and the method of data analysis were described in our previous paper.[18] Fluorescence intensities were measured at λ_{ex} = 360 nm and λ_{em} = 460 nm with band-paths of 5 and 10 nm, respectively.

Results

Salt-Dependence of Fluorescence Spectroscopy of Hoechst 33258 Binding to polyd(A)·polyd(T) or poly-[d(A-T)]·poly[d(A-T)]. By selectively binding to A-T-rich sequences, the weak fluorescence of Hoechst 33258 can be largely enhanced (by 150 times in our case). The absorption and fluorescence spectra undergo large red and blue shifts from original positions around 336 and 490 nm, respectively, reflecting changes in the environment of the drug brought about by the event of DNA binding. To probe the environmental effect on DNA conformation by changes in the spectroscopic signals of Hoechst 33258, the portion of the free drug in the solution relative to its binding target was reduced to the minimum by largely increasing the DNA phosphates/drug ratio (R) based on preliminary tests. Although values of R to attain the fluorescence titration equilibrium (as reflected by the fluorescence intensity) of the drug–DNA complexation are dependent upon the original drug concentrations, due to the decrease in the binding affinity by lowering the concentration of Hoechst 33258,[9] the quite high R values used (above 150) and the large binding affinity made

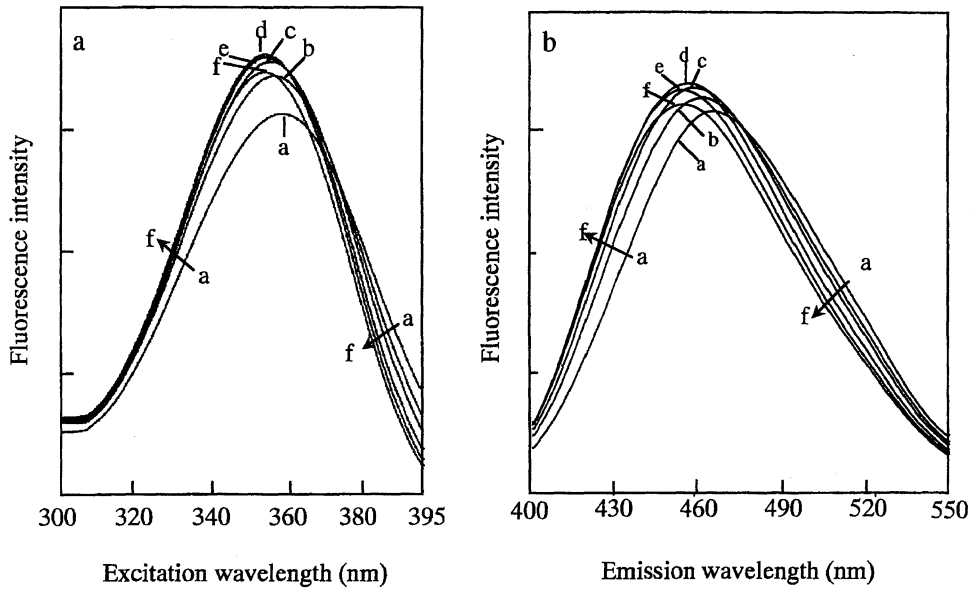

Fig. 1. Fluorescence spectroscopy in excitation (a) and emission (b) of Hoechst 33258 (10 nM) bound to poly[d(A-T)]·poly-[d(A-T)] (2 μM in nucleotides) at various salt concentrations. The NaCl concentrations (mM) corresponding to the spectra in the buffer of 10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA are a, 10; b, 30; c, 50; d, 100; e, 200; and f, 300; respectively. Temperature is 25 °C.

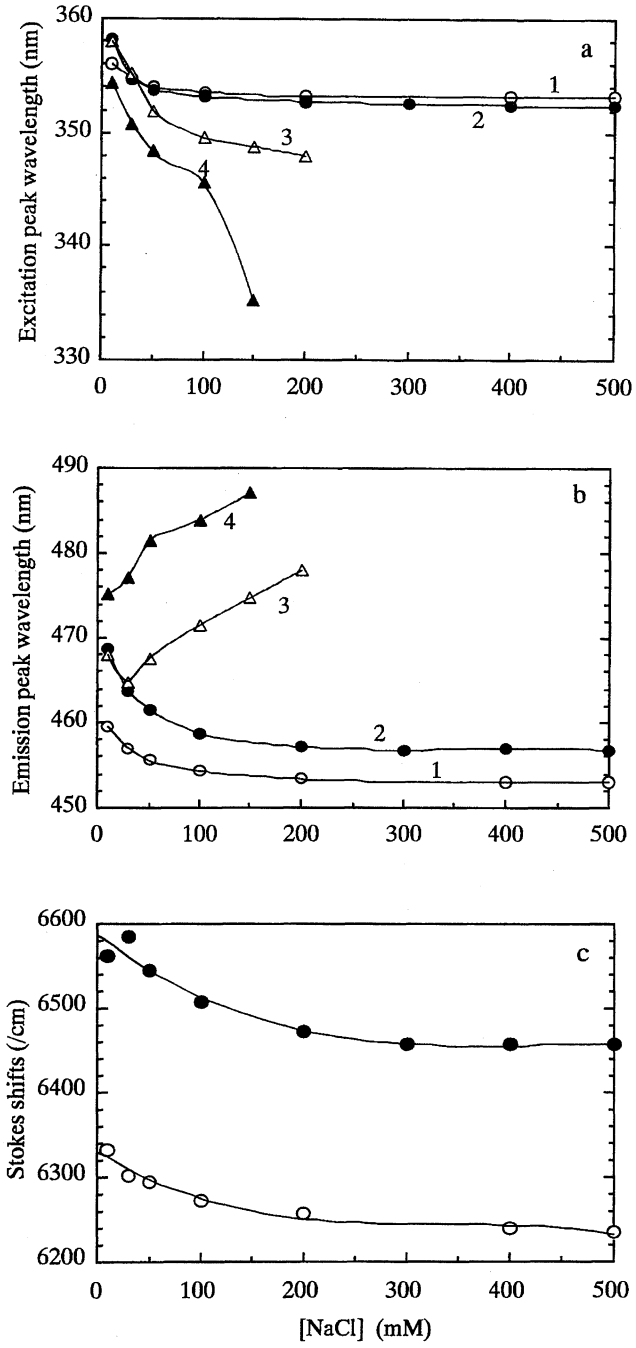

Fig. 2. Salt dependence of fluorescence peak wavelengths in excitation (a), emission (b), and of Stokes shifts (c) of Hoechst 33258 bound to DNA. DNA molecules are poly(dA)·poly(dT) (○), poly[d(A-T)]·poly[d(A-T)] (●), poly[d(A-G)]·poly[d(C-T)] (△), and poly[d(G-C)]·poly-[d(G-C)] (▲), respectively.

spectral contributions from the dissociated ligand negligible.

As shown in Figs. 1, 2a, and 2b, increasing the salt concentration by additions of NaCl caused remarkable movement in the peak wavelengths of both fluorescence excitation and emission spectra of Hoechst 33258 saturated by poly-[d(A-T)]·poly[d(A-T)] or poly(dA)·poly(dT) towards shorter wavelengths. Clearly, the spectral shifts in both excitation and emission spectra toward the same direction induced by increasing the ionic concentrations can not be accounted for by the events of complex association/dissociation. In addition, a notable shift in the peak positions will require a large magnitude of changes in R values since the fluorescence spectroscopy (peak wavelengths) of the drug bound to DNA is insensitive to variations of DNA concentrations within the high region of R values. Small increases in the salt concentrations could not yield enough changes in the binding affinity/extent of the drug and DNA.[9] No isosbectic points appeared among the salt-shifted spectra, implying an evolution of multiple conformers of DNA–dye complexes rather than simple two-state transitions. It seems that at least two types of salt-induced elution in the fluorescence spectra could be resolved. The first type is seen during the NaCl concentration from 10 to about 100 mM, where sharp spectral shifts take place in the overall spectra, and the peak intensities (emission) are increased by ca. 10% (Fig. 1a). The spectral shifts in this region could be fit to an exponential function of the NaCl concentrations. The second is seen during a further increase in the NaCl concentrations from 100 mM, above which the peak positions continue to be blue-shifted by a small but measurable magnitude until a platform is reached. Simultaneously, the emission peak intensities start to go down after the attainment of the highest values. Spectral shifts which occurred in this salt region may be more clearly seen by the calculated shifts in the center of spectral mass ($< \mathrm{COM} >$) to lower wavelengths (Fig. 3). Shifts in the $< \mathrm{COM} >$ clearly showed that the salt effect on moving peak wavelengths was accompanied by changes in the overall fluorescence spectra. The shifts in peak wavelengths are followed by an asymmetrical shrinkage of the spectral contour from the red edge. The resulting reduction of spectral widths may be promoted by a reduction in the conformation (therefore spectral) heterogeneity of drug–DNA complexes, and may be combined with intramolecular motions of drug molecules.[20] The salt dependence of the fluorescence peak position and of spectral widths therefore gives information on the local dynamics in the proximity of the chemophore in the matrix of minor grooves.

The salt effects on the peak wavelengths of fluorescence spectra can be reflected by Stokes shifts (Fig. 2c). It is known that Stokes shifts are strongly responsive to local static and dynamic properties, while they are weakly sensitive to collective internal relaxation and overall deformations of the helix conformations.[5,21] The presence of salt-dependent Stokes shifts of DNA–drug complexes therefore suggests the salt effects on reconstituting the local structure of DNA–drug complexes. Previously, Stokes shifts of Hoechst 33258 bound to poly[d(A-T)]·poly[d(A-T)] were used to quantify the polarity in the minor groove occupied by the drug to be near 20 D.[6] Dielectric constants in the groove estimated from the connection with fluorescence Stokes shifts are probably dependent on environment conditions.[7] By referring to the literature curves that were used to connect the Stokes shifts of Hoechst 33258 and dielectric constants in the minor groove, increasing the NaCl concentrations from 10 to 500 mM therefore might have caused the minor groove of

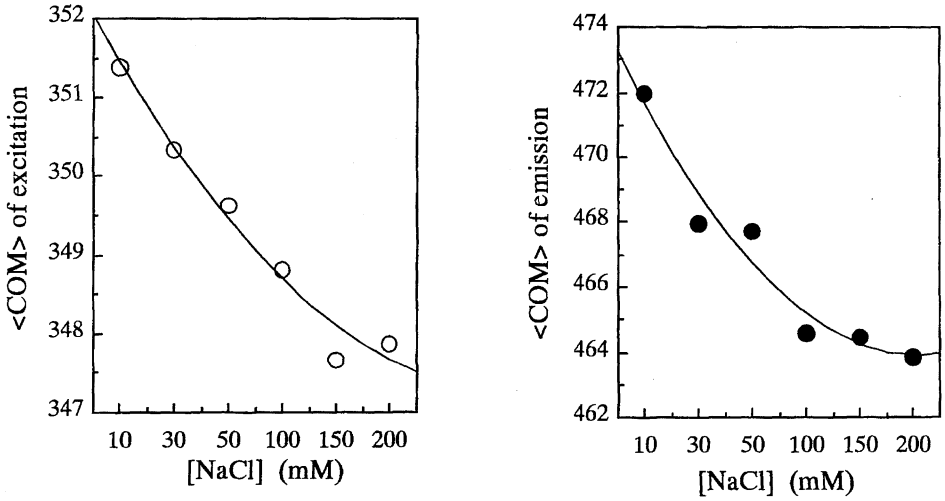

Fig. 3. Salt dependence of centers of spectral mass, < COM > in fluorescence excitation (left) and emission (right) of Hoechst 33258 bound to poly[d(A-T)]·poly[d(A-T)]. < COM > was calculated from the data shown in Fig. 1.

the A-T polymer to be more nonpolar, given that changes in the fluorescence Stokes shifts are exclusively due to the hydration changes. Meanwhile, complexes of Hoechst 33258 with the A-T homo- and co-polymers appear to be distinguished by fluorescence emission peak positions and Stokes shifts of Hoechst 33258 separately attached with their minor grooves (Fig. 2). This is also applicable to binding of the drug with other two polymers, poly[d(A-G)]·poly[d(C-T)] and poly[d(G-C)·poly[d(G-C) (see curves 3 and 4 in Figs. 2a and 2b). The ability of Hoechst 33258 to discriminate the residual groups in the minor groove may also been found by other spectral, chemical or thermodynamics studies.[9,19,22,23]

Replacement of Na^+ with Li^+, K^+, or Cs^+ made minimal differences in the magnitude of spectral shifts of DNA-bound Hoechst 33258, while the respective emission peak wavelengths (data not shown), and changes in fluorescence intensities appear to be slightly different (Fig. 4). Generally, models for the stabilization of the secondary structure of B-DNA by alkali metal ions suggested that these ions play positive roles either by direct ion-base interactions in the minor groove according the order of $Cs^+ > K^+ > Na^+ > Li^+$ or by indirect hydration energies ($Li^+ > Na^+ > K^+ > Cs^+$).[24,25] The counterion-induced reorganization of DNA hydration and structure by alkali metal ions such as Cs^+ was observed with absorption and acoustical titration of A-T sequences.[26]

Corresponding to changes in fluorescence properties, absorption spectra of DNA-bound Hoechst 33258 underwent the salt-dependent changes. As shown in Fig. 5, in the presence of an excess of poly[d(A-T)]·poly[d(A-T)], visible absorption peaks are blue-shifted by 5 nm and peak strengths were increased with gradual additions of concentrated NaCl. The high absorbance of DNA–dye complexes at 260 nm was reduced by about 3% (Fig. 5. Inset) when the ionic concentration increased to 200 mM. The hypochromicity around 260 nm during the salt titration may be due to conformational changes of drug–DNA complexes. Perhaps some general stacking effects on DNA double helix by increasing the ionic strengths also exist in this salt titration process.

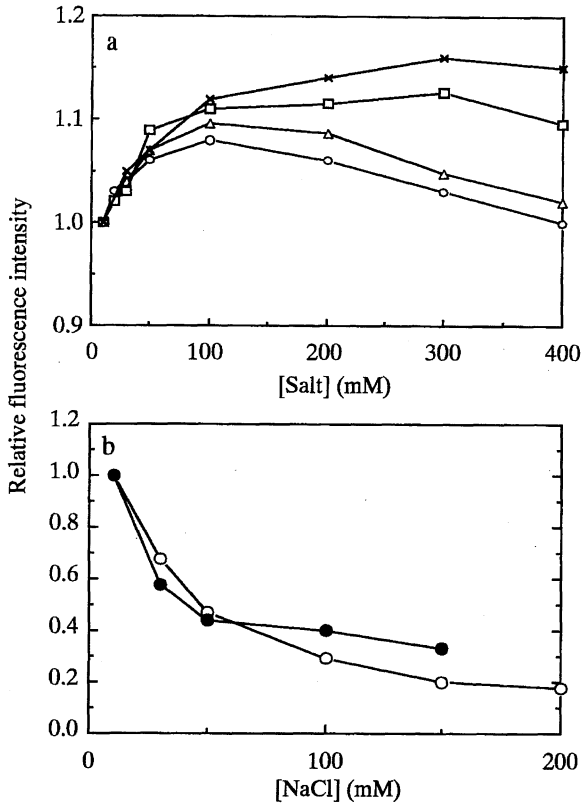

Fig. 4. Salt dependence of fluorescence peak intensities in emission spectra of Hoechst 33258 bound to (a) poly[d(A-T)]·poly[d(A-T)], or to (b) poly[d(A-G)]·poly[d(C-T)] (○) or poly[d(G-C)]·poly[d(G-C)] (●). Salts used in (a) are LiCl (○), NaCl (△), KCl (□), and CsCl (×), respectively. The salt used in (b) is NaCl. The buffer is 10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA at 25 °C. [Hoechst] = 10 nM and R = 200/1 in (a) or 400/1 in (b). The dilution effects from additions of salt have been corrected.

As a control, fluorescence and absorption properties of free Hoechst 33258 were invariable upon varying NaCl concentration (Ref. 6, and data not shown). Additions of organic

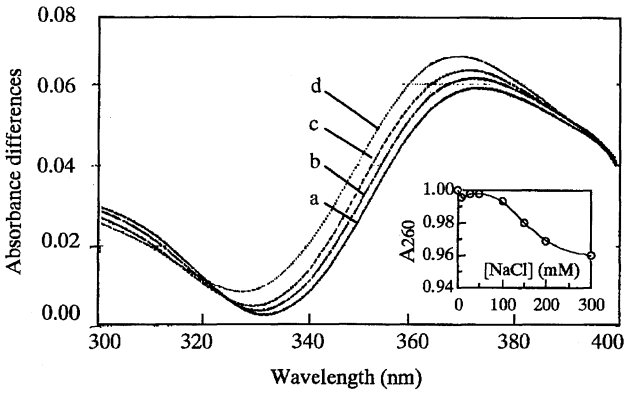

Fig. 5. Differential absorption spectra of Hoechst 33258 bound to poly[d(A-T)]·poly[d(A-T)] at various salt concentrations. The NaCl concentrations (mM) increase from 10 mM to a, 30; b, 50; c, 100; and d, 200, respectively. The buffer is 10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA. R = [phosphate]/[drug] = 100. Temperature: 25 °C.
Inset: Changes in the absorbance at 260 nm of DNA–drug complexes as NaCl concentrations were increased.

solvents such as ethanol progressively increased the fluorescence intensity and mainly moved the emission spectra of free Hoechst 33258 in the solution (Ref. 14, and data not shown). Such results imply that hydration arrangements alone may not be enough to account for changes in the fluorescence spectroscopy of Hoechst 33258-DNA complexes.

Salt Dependence of Fluorescence Spectroscopy of Hoechst 33258 Bound to poly[d(G-C)]·poly[d(G-C)] and poly[d(A-G)]·poly[d(C-T)]. It has been concluded that the preference for A-T-rich sequences of drugs like Hoechst 33258, distamycin, and netropsin is essentially determined by the presence of a strong steric obstacle from the protruding NH_2 of guanosine into the minor groove.[16] Recently, however, Hoechst 33258 was suggested to be able to form complexes with G-C stretches though with a relatively low affinity.[14,17] This may be obtained by intercalation with bases of G-C sequences as a dimer stacking in the major groove or by nonspecific attachment to phosphate groups of the backbone. Relative to a large spectral shift and enhancement in the fluorescence of the drug upon binding to A-T sequences, the fluorescent increments aroused by binding to poly[d(G-C)]·poly[d(G-C)] or poly[d(A-G)]·poly-[d(C-T)] were relatively small (by 6 and 14 times in the present case, respectively), and the emission spectral peaks of the drug complexes were also in the region of longer wavelengths (Figs. 2a and 2b). Increasing the NaCl concentrations strongly quenched the fluorescence of these Hoechst 33258-DNA complexes and moved the peak wavelengths toward their original positions before the binding took place, which seemed to be in agreement with a progressive dissociation of the DNA-drug complexes (lines 3 and 4 in Figs. 2a and 2b, Fig. 4b). The high sensitivity in fluorescence spectral properties of the drug complexes with the two non-A-T sequences to ionic conditions strongly contrasted to what had occurred to the drug complexes with two A-T polymers, and suggested an important role of electrostatic interactions in maintaining the stability of complexes.

Salt Effects on Reaction Volumes of the Complex Formations between Hoechst 33258 and A-T Polymers. The salt effects on interactions of DNA and ligands may be reflected by changes in other physical parameters such as the equilibrium association constants (K). Based on the early work,[18] we addressed this problem by evaluating the pressure dependence of K (denotes as K_p) at various salt concentrations. The effects of salt concentrations on the equilibrium binding of Hoechst 33258 with poly(dA)·poly(dT) or poly[d(A-T)]·poly[d(A-T)] were examined by fluorometric titrations under several elevated pressures, as described previously.[18] Figure 6 gives experimental and simulation curves of the fluorometric titration conducted at 10 mM

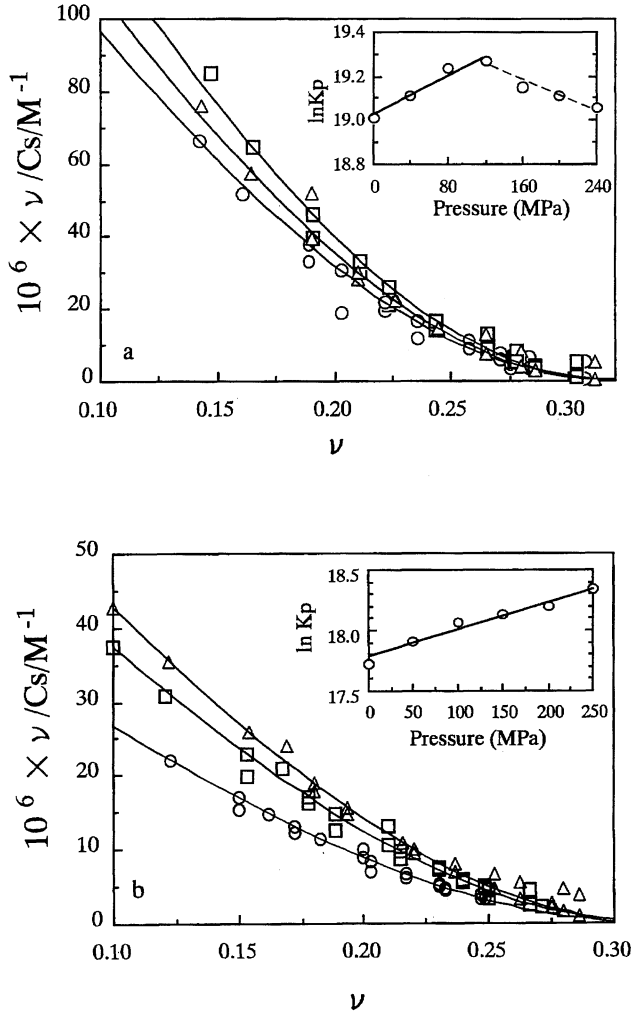

Fig. 6. Typical binding isotherms for Hoechst 33258 with poly(dA)·poly(dT) (a) or poly[d(A-T)]·poly[d(A-T)] (b) at 25 °C. The solid curves are simulation results according to the exclusion-site model. Pressure conditions are 0.1 (○), 120 (□), and 240 MPa (△) in (a), respectively, and 0.1 (○), 100 (□), and 200 MPa (△) in (b), respectively.
Inset: Relationship between pressure and the apparent association constant of Hoechst 33258 with the two A-T polymers, respectively. In the inset in (a), only the data not more than 120 MPa (filled line) were used to estimate the value of volume changes.

NaCl and 25 °C and under elevated pressures. By assuming a linear relationship between the apparent binding constants and the pressures, as well as the nearly incompressibility of the DNA structure under the studied range of high pressure, one can calculate volume changes involved in the drug–DNA complexation from the slopes of these linear plots (Fig. 6. Inset). Pressure elevations were found to progressively increase the binding affinity of Hoechst 33258 with poly[d(A-T)]·poly[d(A-T)], whereas pressure effects on the binding of the drug with poly(dA)·poly(dT) appeared to become complicated by the appearance of distortions in $\ln K_p$–P curves at 10 and 50 mM NaCl and 120 MPa (Fig. 6a). The origin of this effect is still unknown. Perhaps the anomaly of pressure effects on the drug binding with the homopolymer at lower salt concentrations partly correlates to the peculiar characteristics of the A-T homopolymer in structure, stability, and dynamics. It is known that the A-T homopolymer exhibits extremely large hydration, which associates with its B′-type structure.[27—30] Thus the noncanonical B-type structure could convert to a normal B-type one with altered environment conditions (e.g., temperature), or upon drug binding with dehydration.[31,32] It is also assumed that the minor groove binding of drugs that selectively targets canonical B-DNA probably brings about such structural recovery.[12] The pronounced effect of premelting of the homopolymer is sensitive to drug binding, increased ionic strengths, or changes in other thermodynamic parameters, and ligand binding.[4,31—34] Perhaps the equilibrium of ligand binding is mixed with these processes, and perhaps these processes are sensitive to pressure increases. The validity of these discussions calls for further theoretical and experimental investigations.

Data of volume changes obtained at various salt concentrations are shown in Table 1. Obviously, volume changes upon Hoechst 33258-DNA complexation were affected by salt conditions, probably reflecting the binding-induced changes of counterion associations with DNA and their effects on DNA–drug complexes, coupling to changes in hydration changes throughout the complexation.[18,35—38] Differences in the volume effects relative to the two A-T polymers upon the drug binding are investigated with several drugs and interpreted by original hydration differences of the drug-free polymers together with the differences of drug–DNA complexes.[18,24,38,39]

Effects of counterions were also considered on the spectral shifts of DNA-bound drug under pressure. Raising pressure up to 200 MPa aroused small red shifts mainly in the emission spectra of drug complexes with A-T polymers (about 2 and 3 nm at 200 and 400 mM NaCl, respectively), while the excitation peaks remained nearly unmoved (< 1 nm), implying that the salt-dependent effect of pressure on the DNA–drug structure may be trivial and limited on restructuring the hydration atmosphere which probably extends to the drug's binding sites in the DNA minor groove.

Table 1. Volume Changes (ΔV, $cm^3\,mol^{-1}$) Involved in Hoechst 33258-DNA Interactions in Several NaCl Concentrations

[NaCl] (mM)	poly(dA)·poly(dT)	poly[d(A-T)]·poly[d(A-T)]
10	-3.0 ± 0.9[a)]	-5.7 ± 1.2
50	-3.5 ± 0.4[a)]	-8.5 ± 1.6
100	$+6.1\pm1.2$[b)]	-3.4 ± 0.3[b)]
200	$+8.9\pm1.9$	Not determined

a) ΔV was estimated from the early linear stage of pressure dependence of changes in K_p as displayed in Fig. 6a, inset. b) Data from Ref. 18.

Discussion

The binding of Hoechst 33258 with DNA arouses multiple changes in fluorescence and absorption spectroscopic properties that are explained by various models.[6,12,14] A large enhancement in the fluorescence intensity can be obtained by transferring the drug to a nonpolar environment; the resulting reduction of proton exchange helps, as does an inhibition in the intermolecular rotation by the minor groove contour. A significant red shift in the absorption spectrum, which is often regarded to be typical for the intercalation, instead indicates structural deformations of drugs including Hoechst 33258 which bind to the minor grooves of A-T sequences by a nonintercalative way.[12,14,39] Probable binding-induced deformation in the drug structure includes an improvement in the coplanarity of the phenol and the benzimidazole chromophores whose conjugations in the electronic dipole transitions probably determine the long absorption band.[15,40] Environment-induced (e.g., temperature) changes in the dihedral angles between the aromatic rings of Hoechst 33258 bound to the minor groove have been proved.[41] The solvent composition dependence of the fluorescence and absorption frequencies (wavelengths) and of the spectral widths of the bound rather than free drug molecules therefore indicate the local changes in structure/hydration in the proximity of the chromophore in the minor grooves.

Furthermore, by considering the structural flexibility of drugs including Hoechst 33258 and 4,6-diamidino-2-phenylindole with free rotational bonds between aromatic rings[40] and its effects on spectral characteristics, one concludes that a previous assertion defining an exclusive connection between the fluorescence Stokes shifts of Hoechst 33258 and the polarity in the minor groove[6] needs to be modified. In that assertion, effects of the structural deformability of Hoechst 33258 on their spectral properties were neglected, by analogy with a highly rigid fluorophore. This is against not only UV absorption and fluorescence of Hoechst 33258 but also other spectroscopic properties such as the induction of CD signals and the multiple NMR states which support alterations in the drug conformation upon the binding to preferred sequences.[10—14,17]

Here, a reevaluation of the data of Stokes Shifts of Jin and Breslauer[6] that were formerly used to exclusively correlate with the polarity was performed. By doing so, we inferred a possible relationship between Stokes shifts and the polarizability, a parameter that reflects dispersion interactions (van der Waals) of the fluorophore and the microenvironment that would shift the excitation transition,[5,21] of the environment in

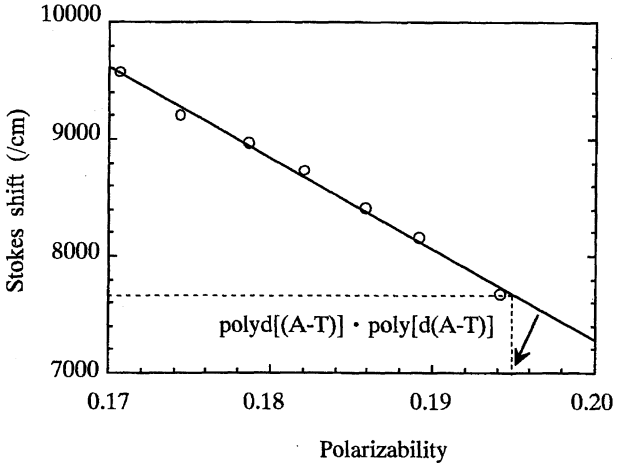

Fig. 7. Suggested relationship between the Stokes shifts of Hoechst 33258 and the polarizability in corresponding environments. The data of Stokes shift (circle) are from Jin and Breslauer's work (Ref. 6) which instead specified an exclusive correlation with the polarity of corresponding environments. The dotted line expressed the Stokes shifts upon binding to poly[d(A-T)]·poly[d(A-T)] (from Ref. 6) (R = 100/1, [Hoechst] = 300 nM). Note their data of Stokes shifts with drug–DNA complexes are larger than ours, possibly because of different measuring conditions.

the minor groove (Fig. 7). The excitation maximum/Stokes shift would correlate with the polarizability of the solvent as measured by the appropriate function of the solvent index of refraction (n). For the drug molecules intercalated with DNA, their fluorescence Stokes shifts may be enhanced by a polar/nonpolar reorganization stemming from movements of neighboring groups, for instance, base motions in DNA sequences.[5] Figure 7 that showed the result was obtained by plotting the fluorescence Stokes shifts of Hoechst 33258 free in the solvent with different refractive index (n) as a function of calculated values of $(n^2-1)/(2n^2+1)$. The abscissa denotes the calculated polarizability. Original data of n and Stokes shifts were taken from Jin and Breslauer's work (Table 1).[6] If one uses the same method that inserts the Stokes shift data of drug–DNA complexes (at constant ionic strengths in the phosphate buffer) into the assumed linear line as did by Jin and Breslauer, one can find correlations of the fluorescence spectral shifts of the drug to changes in the polarizability in the matrix of the minor groove (as shown by the arrow). This contradicts to the assertion that attempted to establish an exclusive relationship between fluorescence spectral properties of the drug and the polarity in the matrix. Furthermore, it suggests that hydration changes alone cannot, at least in the present case, provide all the basis on which the fluorescence of Hoechst 33258 when bound to the DNA minor groove could be completely interpreted.

In the light of considerations above, the observed changes in fluorescence properties are interpreted as reflections of environment-dependent structural features of DNA–dye complexes. The structural polymorphism of DNA is a complicated, multidimensional phenomenon, including influences of sequence context, salt and drug binding, temperature and pH, and the content of water.[1] Even for simple ions, their effects on DNA structures are multiple and on various levels. Generally, increasing salt concentrations improves the stability of base pairing and intends to induce a more compact and bent form of DNA; another effect is the induction of solvation rearrangement of DNA in the solution. It was reported that moderate increases in the NaCl concentrations (e.g., from 10 to 30 mM) effectively inhibited a large scale opening particularly of A-T base pairs.[41] Although the primary distribution of simple monovalent ions is widely accepted to be delocalized along the DNA backbone, recent NMR observations and theoretical simulations indicated a dynamic distribution of simple monovalent ions such as Na^+ preferably in the minor groove of A-A and A-T pockets.[42—44] Sodium ions can partially occupy the primary hydration spine in the minor groove, but not in the second spine.[42] The site-specific bindings of counterions (sodium ions) in the minor groove probably have many effects on sequence-dependent features of local structures, including further bending helical axis, effectively narrowing the widths of minor grooves, and rearranging the hydration structure;[42—44] such factors inevitably affect the ligand conformation in the minor groove.[10,11] Hydration reorganization in the primary layer that acts as the template of the second hydration layer may bring about large subsequent changes in the whole hydration structure, since the secondary hydration layer is directly connected with the bulk solvent. This was suggested to be responsible for the yielding of large volume changes in the minor groove binding of netropsin with A-T polymers.[42]

The heterogeneity in the binding of Hoechst 33258 to native DNA or synthetic polynucleotides was often seen at small R values. The salt inhibition of fluorescence spectral shifts induced by increasing the drug to chromatin DNA ratios was previously illustrated by transitions between several binding modes.[45] Such an explanation may be reasonable because of the highly heterogeneity in chromatin DNA sequences to which the drug binds with various affinities and modes. In addition, it was also suggested that complexes of Hoechst 33258 with poly[d(A-T)]·poly[d(A-T)] formed at moderate [nucleotides]/[drug] ratios might consist of two types of complex forms with different penetration depths of the drug into the minor groove.[46] According to this model, parts of the drug molecules might deeply enter into the minor groove and be well separated from the solvent, while some others might permit more access of the solvent. Present results with absorption and fluorescence spectral changes still found difficulties to clearly exclude or accommodate this hypothesis, though our explanations have included the possibility of hydration changes as parts of counterion effects while in the meantime, they have rejected the possibility that it acts as a whole role. Perhaps it is more suitable to propose that drug molecules attached in the minor groove, while sample various environments, experience different conformational and solvent-exposure states.

The spine of hydration residing in the minor groove of B-DNA molecules is generally regarded to be destroyed and replaced by a minor groove binding drug.[11,47,48] The

replacement of original hydration water constitutes an important source for the resulting volume changes.[35—37] Part of volume changes may have other origins such as conformational changes of DNA.[36] Although electrostatic interaction contributions are not the driving force in determining the DNA–Hoechst 33258 association affinity,[11] they appeared to affect the pressure dependence of equilibrium binding constants as suggested by the salt concentration dependence of pressure effects. The appearance of "curved" pressure dependence of the equilibrium constant of Hoechst 33258–homopolymer complex formation at lower salt concentrations might reflect DNA structural/hydration peculiarity and their changes in response to environment conditions.[27—30] While the B′ structure of poly(dA)·poly(dT) in the fiber form was found not to undergo a methanol-induced conversion to the A form,[49] it can be converted into a normal B-type one by drug intercalation or by increasing the temperature above the premelting point.[31,32] Meanwhile increases in ionic concentrations to a higher extent induces a disproportionation reaction of the A-T homopolymer to gradually yield a triplex form of T·A·T and a single strand of poly(dA).[50] At least two forms of such DNA triplex, S-like and B-like, were suggested and their relative stability is in dispute.[51] The DNA triple helix structure was stabilized by elevated pressure[46] while it was destabilized by additions of Hoechst 33258.[47] Furthermore, the A-T homopolymer exhibits a broad, solid-state structural premelting transition, and whether the A-T copolymer also undergoes a similar premelting remains not very clear.[31,32,35,36] The premelting of DNA is expected to be sensitive to environmental conditions, e.g., temperature, salt condition, pH, and other thermodynamic parameters (perhaps including pressure), and drug binding.[32—34] Although we so far can not identify which process really predominates the production of a "bent" curve for pressure effects on the Hoechst 33258–homopolymer complexation, the present results do show distinctively "abnormal" behavior of the A-T homopolymer in ligand binding as compared to the "normal" one of the copolymer.[54] Previously, we have discussed the significant discrepancy in the reaction volumes of drug complexation of poly(dA)·poly(dT) and poly-[d(A-T)]·poly[d(A-T)],[18] and we believe that it is still effective in general in spite of the present complexity which appears with the A-T homopolymer at lower salt concentrations. Differences in the pressure effects on the DNA-binding of the A-T homopolymer and copolymer with Hoechst 33258 appeared in line with the previous findings that attribute the different volume changes upon ligand binding to the sequence-dependent features of DNA hydration and conformation.[18,35—38] In fact, sequence-dependent differences in the volume effects of A-T polymers exist not only in the ligand binding but even in the duplex formation.[52] In many cases, the minor groove drug complexes of A-T homopolymer were found to be less solvated than the drug complexes of A-T copolymer,[18,35—38] indicating a larger loss of hydration water from the homopolymer upon the drug binding, since the homopolymer free in solution holds a larger amount of hydration water than the copolymer.[28—30] Differences in the hydration changes around the homopolymer and the copolymer of A-T upon ligand binding may also be reflected by investigations of their distinguishable fluorescence emission spectra and Stokes shifts. The present disclosure of different reaction volumes seemed not strange. We have added information to a large body of differences in physical characteristics (e.g., helical repeat, axial rise, groove width, hydration, and conformational flexibility)[27—30] and chemical ones (e.g., ligand binding)[18,35—38,54] of the homopolymer and the copolymer of A-T found over the past decades.

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