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Pure Spectra of Actinomycin D Bound to DNA at Various Ionic Strengths Extracted by the Principal-component-analysis Method[1)]

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The interaction between actinomycin D (AMD) and native and heat-denatured DNA was studied spectrophotometrically at 25 °C. By applying the principal-component-analysis (PCA) method to the series of observed spectra, the number of light-absorbing components was determined. Two spectroscopically different species of bound AMD, *i.e.*, AMD–DNA complexes, were formed when an aqueous AMD solution was titrated with either the native or the denatured DNA solution over a wide range of the nucleotide residue-to-AMD mixing ratios (P/D) between 0 and 30. The pure spectra of AMD bound to DNA were extracted in the P/D range 0—4 by the extended PCA method with an appropriate binding scheme. These bound-AMD spectra were affected by the DNA conformation but not by the addition of NaCl (1—500 mmol dm^{-3}). The fractions of bound AMD were calculated as a function of P/D.

Actinomycin D (AMD)[2-4)] is an antibiotic that inhibits DNA-dependent RNA synthesis by binding to DNA;[5,6)] the interactions between AMD and DNA and other related compounds have been studied by many physicochemical methods.[5-22)] For example, two classes of AMD–DNA complexes with different binding constants have been reported;[5-7)] the first class is the "strongly" binding complex and the second is the "weakly" binding complex. Although the strongly binding complex has been studied extensively, few attempts have been made to clarify the weakly binding complex. In particular, the absorption spectrum of the AMD–DNA complex, free from the spectrum of unbound AMD, has not been unmasked because of the strong overlap between the two spectra in the visible wavelength region.

The pure spectrum of the AMD–DNA complex or the *bound*-AMD spectrum is important in interpreting the optical behavior and the binding mode of AMD in the AMD–DNA complex. In this paper, therefore, the interaction between AMD and DNA was studied spectrophotometrically, and the results were analyzed with a new technique——the principal-component-analysis (PCA) method.[1,23-27)] This method is useful, because no additional measurement such as equilibrium dialysis is required. By applying the PCA method to a series of observed spectra of AMD–DNA solutions, the number and the pure spectra of light-absorbing components in the system can be determined. The binding curve of AMD can also be evaluated by using an appropriate equilibrium equation.

Experimental

Materials. Actinomycin D was a gift of Merck Sharp and Dohme, Inc. (USA) and used without further purification. The aqueous solution of AMD was filtered through a sintered glass filter and stored in the refrigerator under dark. The concentration of AMD was determined to be 1.81×10^{-5} M (1 M = 1 mol dm^{-3}) with the molar absorption coefficient ε_{441} of 24800 cm^{-1} M^{-1}.[7)] Calf thymus DNA was purchased from Worthington Biochemical Corp.[1)] The concentration of the stock native DNA (nDNA) solution was 9.35×10^{-4} M, which was determined by using the absorption coefficient ε_{260} of 6400 cm^{-1} M^{-1}.[8)] Heat-denatured DNA (dDNA) in 1 mM NaCl was prepared as before.[1,8)] The hyperchromicity of the DNA solution was *ca.* 34% in 1 mM NaCl. Spectrophotometric titration was carried out at 25 °C in a 1 or 2 cm long cuvette cell by the successive addition of the stock DNA solution in the same manner as before.[1)]

Analysis. The absorption spectra of the AMD–DNA solution were analyzed by the PCA procedure, for which the following equilibrium equation was used (Eq. 2 of Ref. 1):

$$K_a = \frac{[DP^*]}{[D][P]}\left(\frac{[P]}{[D]_0}\right)^{1-\alpha}, \quad (1)$$

where K_a is the apparent equilibrium (binding) constant, the brackets denote the equilibrium concentration, DP* is the bound AMD or the AMD–DNA residue complex, D is the free AMD, P is the unoccupied DNA residue (in the nucleotide unit), and $[D]_0$ is the initial AMD concentration. The adjustable parameter α has been introduced to reproduce the sigmoidal change of optical titration curves for various dye-polymer combinations.[1,24,26,27)] When $\alpha=1$, Eq. 1 reduces to the commonly postulated equilibrium scheme, $K'=[DP^*]/[D]\cdot[P]$. When $\alpha>1$, Eq. 1 represents a sigmoidal change in the titration curve.

Results and Discussion

Observed Spectra of AMD–DNA Systems and Number of Light-absorbing Components. Figure 1 shows representative absorption spectra of AMD solutions obtained from the successive titration with (a) nDNA and (b) dDNA in the presence of 1 mM NaCl. The molar absorption coefficients, ε, of the spectra of AMD–DNA solutions are almost the same at 461 nm for nDNA and at 457 nm for dDNA (the isosbestic points) in the low P/D range. The spectral change near 380 nm at higher P/D values, together with the gradual deviation from the isosbestic points, indicates that more than two independent, light-absorbing components are present in each system. (The absorbance of the DNA component is negligibly small above 370 nm.) The exact number of these components may be determined by comparing the relative magnitudes of the eigenvalues, which are obtained from a family of the observed AMD–DNA spectra by the PCA method.[1,23-27)]

The eigenvalues calculated for the AMD–nDNA system at 1 mM NaCl are shown in Fig. 2(a). According to the previously set criteria,[1,23-27)] the significant number of light-absorbing components is determined

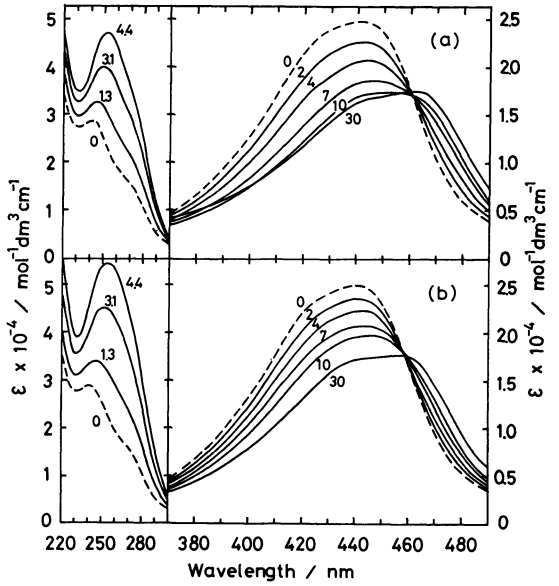

Fig. 1. Representative solution spectra of AMD observed in the presence of nDNA (a) and dDNA (b) at 1 mM NaCl. The dashed curves ($P/D=0$) are the spectra of AMD in the absence of DNA. The numerals denote values of P/D. The apparent molar absorption coefficient, ε, is expressed with the concentration of AMD in aqueous solution which is 1.81×10^{-5} M throughout this work.

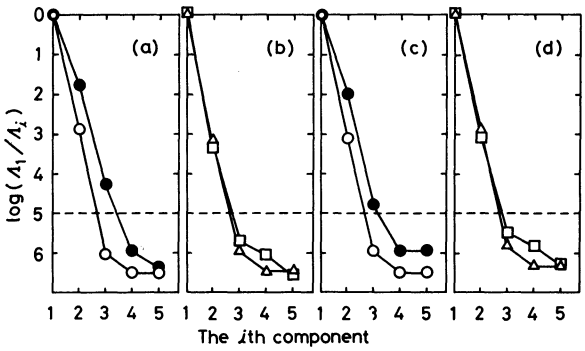

Fig. 2. Variations in eigenvalue Λ_i ($i=1$, 5) with the ith light-absorbing component for the AMD–nDNA solutions (a) and (b) and for the AMD–dDNA solutions (c) and (d). The concentrations of NaCl in those solutions are 1 mM (○), 100 mM (△), and 500 mM (□). The ordinate is expressed in terms of the ratios of the eigenvalue of the first component, which is the largest, to that of the ith component. The dashed lines indicate the criteria set for the significant component. Open symbols are used for the observed spectra which are limited to the low P/D range between 0 and 4, and the closed circle is used for the observed spectra whose P/D values range between 0 and 30.

to be three in a wide P/D range of 0—30. One of these species must be the free AMD and, hence, two differently bound AMD species may exist in agreement with previous reports that calf thymus DNA possesses two classes of binding sites for AMD (strong and weak binding sites).[6,7] The number of absorbing components reduces to two, if the P/D region is limited between 0

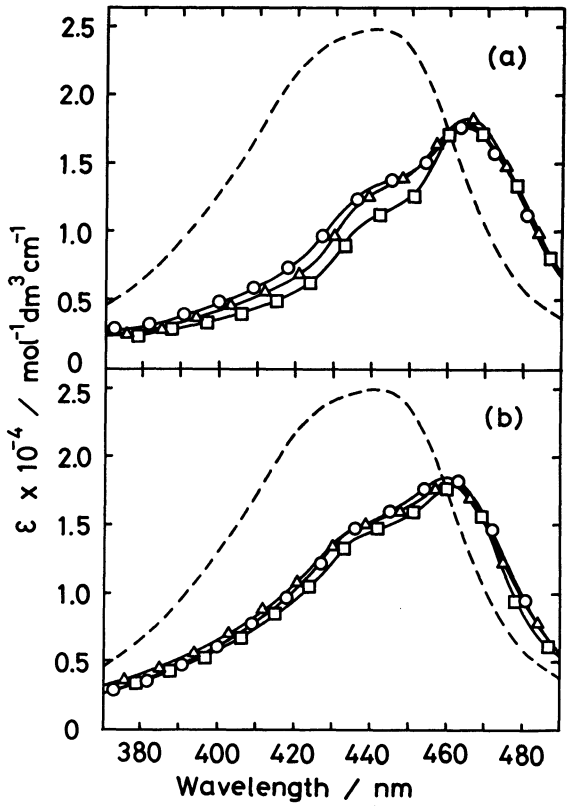

Fig. 3. The pure spectra of AMD bound to nDNA (a) and to dDNA (b). Concentrations of NaCl are 1 mM (○), 100 mM (△), and 500 mM (□). The spectrum of free AMD is shown by the dashed curve for comparison.

and 4; therefore, it may be concluded that the strongly bound AMD almost disappears in the low P/D region and that the dominant species are the weakly bound AMD and the free AMD. The eigenvalues for the AMD–nDNA system at higher ionic strengths (100 and 500 mM NaCl) are shown in Fig. 2(b). The result is the same as in the case of 1 mM NaCl; namely, free AMD and weakly bound AMD are present in the low P/D region.

The eigenvalues for the AMD–dDNA system are shown in Figs. 2(c)—(d). The results are similar to those of the AMD–nDNA system; three light-absorbing components, free AMD and two differently bound AMD, in the wide P/D range 0—30 and two components in the low P/D range 0—4. It is clearly shown that the number of light-absorbing components can be estimated in a straightforward manner by the PCA method without relying on the delicate isosbestic points.

Pure Spectra of AMD Bound to nDNA and dDNA at Various Ionic Strengths. Figure 3(a) shows the pure absorption spectra of AMD, which is weakly bound to nDNA, at three different concentrations of added NaCl. Each bound-AMD spectrum could be extracted by the extended PCA method from a family of observed (five to eight) spectra in the low P/D region 0—4. The binding constant K_a and the parameter a were also determined and are listed in Table 1. Three pure bound-AMD spectra of AMD–nDNA complexes in Fig. 3(a) are nearly identical regardless of the NaCl concen-

Table 1. The apparent binding constants, K_a and K', and the parameter α evaluated by the PCA method

[NaCl] mM	AMD–nDNA			AMD–dDNA		
	α	$K_a \times 10^{-3}$	$K' \times 10^{-3}$	α	$K_a \times 10^{-3}$	$K' \times 10^{-3}$
1	1.2	6.0	7.7	1.0	5.3	5.3
100	1.0	4.4	4.4	1.2	6.3	8.2
500	1.1	2.7	3.0	1.1	5.7	6.3

The dimension of K_a is in $(\text{mol/dm}^3)^{-1}$. $K' = K_a([P]/[D]_o)^{\alpha-1}$ and was calculated at $P/D=4$.

trations. This result is surprising in that a five hundred-fold change in ionic strength does not affect the bound-AMD spectrum and, hence, the binding mode. The three bound-AMD spectra all show the absorption maximum at 465 nm and a distinct shoulder at 440—430 nm, being bathochromic and hypochromic relative to the free-AMD spectrum. In addition, the spectral profile is similar to that of the free AMD observed in less polar organic solvents such as benzene and ethanol.[2]

Figure 3(b) shows the pure absorption spectra of AMD, which is weakly bound to dDNA, in the low P/D region at different concentrations of NaCl. Values of K_a and α are given in Table 1. Three bound-AMD spectra at different NaCl concentrations are again almost identical with each other, indicating that nearly the same AMD–dDNA complexes are formed as in the case of the AMD–nDNA system. The bound-AMD spectra of the AMD–dDNA system show both bathochromism and hypochromism relative to the free-AMD spectrum, but the spectral characteristics are less prominent than those of the AMD–nDNA system. Since a bound-AMD molecule would occupy several nucleotide base pairs,[7,9] the difference could be attributed to the presence or absence of the stable double-helical conformation over several base pairs, where the AMD molecule is bound, and also to the extent of the exposure of the AMD-bound site to the environmental water.

The usefulness of the PCA method is clear; a well-defined bound-AMD spectrum (Fig. 3) could be extracted from the broad, nearly featureless experimental spectra of AMD–DNA solutions (Fig. 1). The bound-AMD spectra in Fig. 3 are in excellent accord with the ORD[2] or circular dichroism (CD)[2,12] data previously reported for the AMD–nDNA complex. The CD spectra show three negative bands at 465 nm, 440 nm, and 385 nm in the visible region. The locations of the first two CD bands correspond exactly to the peak and the pronounced shoulder of the bound-AMD absorption spectrum [Fig. 3(a)]. Since the latter reveals a slight hump in the 400—370-nm region, a third, weak-intensity band probably exists there. This absorption band may be related to a large negative CD band at 385 nm. Auer *et al.*[12] have assigned the 465-nm CD band to the quinoid and the 385-nm band to the benzenoid portion of the phenoxazone ring of AMD. We may assign the 440-nm shoulder to a vibrational component of the 465-nm electronic transition on the basis of the spectral profile of the bound-AMD spectrum.

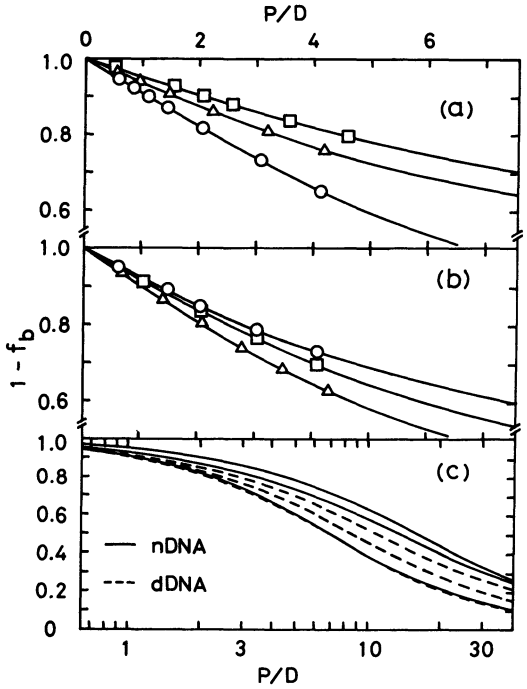

Fig. 4. Fractions of AMD, f_b, bound to nDNA (a) and to dDNA (b) as a function of P/D. Concentrations of NaCl are 1 mM (○), 100 mM (△), and 500 mM (□). Binding curves (solid lines) are expressed in terms of the fraction of the free AMD, $(1-f_b)$. In (c) shown are the curves which are calculated with values of K_a and α over a wider P/D range.

Binding Curves of AMD toward Native and Denatured DNA. Figure 4 shows plots of the mole fraction of free AMD, $(1-f_b)$, against P/D (solid lines).[1] Values of $(1-f_b)$ were calculated with values of K_a and α given in Table 1, and also directly from the **C** matrix by the PCA procedure (symbols).[24-27] Less than 50% of AMD are probably bound to DNA at P/D values 6—15, but more than 90% of AMD may become bound at P/D values >50 for dDNA and >200 for nDNA [Fig. 4(c)], provided that the binding scheme can be described by Eq. 1 throughout the P/D range 0—200. The values of K_a show a trend to decrease with increasing salt concentrations for AMD–nDNA, while they are nearly constant for AMD–dDNA. The small effects of added sodium chloride on K_a and on the bound-AMD spectra (Fig. 3) suggest that the binding mode of AMD toward the DNA site is nonelectrostatic. In the low P/D range, the apparent binding constants, K_a, are in the order of 10^3 for both AMD–nDNA and –dDNA systems (Table 1). In the high P/D range, the values of K_a for the strong binding mode have been reported to be in the order of 10^6 for AMD–DNA.[7,9,10] Thus, it is now clear that the weak binding between AMD and DNA is less by an order of three.

In a number of dye-synthetic polyelectrolyte systems, optical titration curves change sigmoidally in the very low P/D region.[1,24-27] This sigmoidal change has been reproduced well with the adjustable parameter α, if its value is larger than unity.[24,26,27] In the AMD–DNA systems, the fraction of free AMD decreases almost linearly with respect to P/D, as reflected by the values

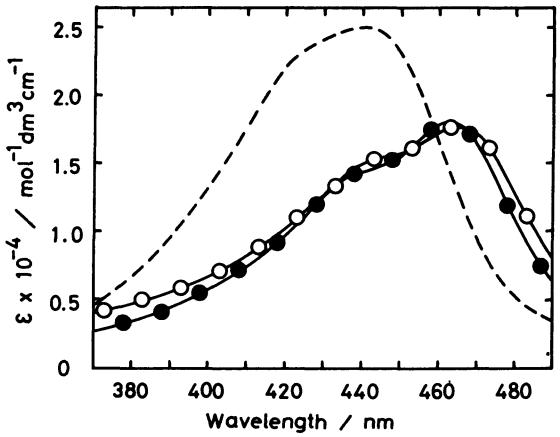

Fig. 5. Mixed-bound spectra of AMD at $P/D=31$ in the presence of 1 mM NaCl. AMD–nDNA (○) and AMD–dDNA (●). The spectrum of free AMD is shown by the dashed curve.

of α which are close to unity (Table 1). It is worth notice that the values of α are also close to unity in other dye–DNA complexes such as crystal violet, trypaflavine, and ethidium bromide.[1,24] This fact supports the use of the commonly postulated binding scheme, *i.e.*, $K'=[DP^*]/[D]\cdot[P]$ $[=K_a([P]/[D]_o)^{\alpha-1}]$, for the binding equilibrium between DNA and small ligands.

Mixed Bound-AMD Spectra in AMD–DNA Systems. At P/D about 30, where both strongly and weakly bound AMD species may coexist (*cf.* Fig. 2), the absorption spectrum of bound AMD results from the mixture of these two bound AMD species. Such a spectrum has been termed the mixed bound-spectrum, expressed with ε_{mix} at a given wavelength,[25] which can be obtained if the equilibrium scheme (Eq. 1) holds for AMD–DNA systems at $P/D \fallingdotseq 30$:

$$\varepsilon_{mix} = \varepsilon_f + (\varepsilon_{obsd}-\varepsilon_f)/f_b, \qquad (2)$$

where ε_f and ε_{obsd} are the molar absorption coefficients of AMD in the free-AMD solution and the AMD–DNA mixture, respectively, and f_b is the mole fraction of bound AMD at a given P/D (*cf.* Fig. 4). Figure 5 shows the mixed bound-AMD spectra for AMD–nDNA and –dDNA complexes at a P/D of 31. These mixed bound-AMD spectra possess the absorption peak and the prominent shoulder almost at the same wavelengths as those of the pure bound-AMD spectra in Fig. 3. Hence, the pure spectrum of the strongly bound AMD in a high P/D range may not differ too markedly from that of the weakly bound AMD in the low P/D range.

Bound-AMD Spectra in the UV Region. Since both AMD and DNA have absorption bands in the ultraviolet region,[2] the AMD–DNA solution also shows a resultant spectrum (Fig. 1). The pure ultraviolet absorption spectrum of weakly bound AMD species, ε_b, can be calculated as

$$\varepsilon_b = \varepsilon_f + (\varepsilon_c-\varepsilon_f)/f_b. \qquad (3)$$

The sum total of the molar absorption coefficients of free and bound AMD, ε_c, is given as

$$\varepsilon_c = (A_{obsd}-A_{DNA})/[D]_o l, \qquad (4)$$

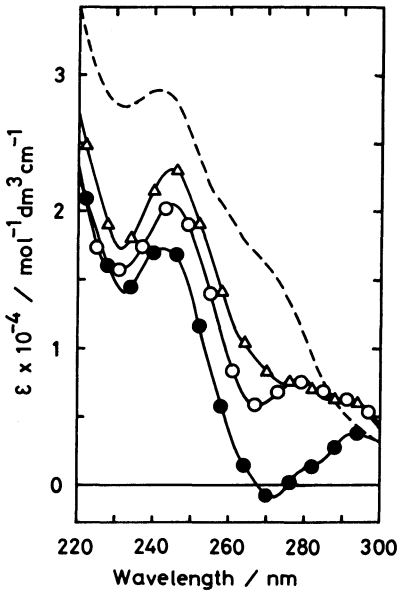

Fig. 6. Pure UV spectra of AMD bound to DNA at 1 mM NaCl calculated from the observed spectra of AMD ($P/D=0$) and of AMD–DNA solutions ($P/D=4.4$), which are shown in Fig. 1. AMD–nDNA (○) and AMD–dDNA (●), both of which were calculated with Eqs. 3 and 4. AMD–dDNA (△) is calculated with Eqs. 3 and 5. The spectrum of free AMD is shown by a dashed curve for comparison.

where l is the optical path length, A_{obsd} is the observed absorbance of the AMD–DNA solution, and A_{DNA} is the absorbance of the DNA component in this solution. For the A_{DNA} of AMD–nDNA solution, the absorbance of nDNA in an AMD-free solution may be assumed (the concentrations of DNA should be the same in the two solutions), considering that the possible conformational change of nDNA by binding of AMD would be small, if any. The procedure, which was utilized for nDNA, was likewise adopted for dDNA of the AMD–dDNA system.

Figure 6 shows the bound-AMD spectra in the ultraviolet region which were calculated on the above assumption. The pure spectrum of AMD bound to nDNA (open circles) seems reasonable, but the pure spectrum of AMD bound to dDNA (closed circles) is unrealistic, because a part of the spectrum has a negative absorbance near 270 nm. This may indicate that the conformation of dDNA in the AMD–dDNA complex (hence, the absorbance of dDNA in Eq. 4) differs from the disordered, single-stranded coil without AMD. Recovery of some kind of partially ordered structure in the AMD–dDNA complex is possible as reported before.[7] If the binding of AMD to dDNA induces an ordered structure, Eq. 4 may be modified as

$$\varepsilon_c = \frac{1}{[D]_o l}\{A_{obsd}-[f_{ord}\cdot A_{ord}+(1-f_{ord})A_{dDNA}]\}, \qquad (5)$$

where f_{ord} and A_{ord} are the fraction and the absorbance of the ordered portion of the dDNA in the AMD–dDNA complex, and A_{dDNA} is the absorbance of dDNA in the absence of AMD.

The pure spectrum of AMD bound to dDNA can be calculated from Eqs. 3 and 5 with appropriate values for f_{ord} and A_{ord}. The ordered structure of dDNA in the AMD–dDNA complex may be approximated as the same as, or close to, the double-helical structure of nDNA. The molar absorption coefficient of the ordered portion of dDNA, ε_{ord}, is then equal to that of nDNA, ε_{nDNA}. The fraction of the ordered structure, f_{ord}, may range between 0 and 1, a unique value being difficult to assign. On the assumption that four nucleotide bases per bound AMD molecule are needed to induce the ordered structure in the AMD–dDNA complex, the following relation may be written as $f_{ord}=4f_b(P/D)^{-1}$; then, $f_{ord}=0.26$ at $P/D=4.4$ ($f_b=0.29$, read from the binding curve in Fig. 4). With these A_{ord} and f_{ord}, the absorbance of the bound AMD in the AMD–dDNA complex was calculated. It now becomes reasonably positive in the 290—260-nm region, as shown in Fig. 6 (open triangles). Although these pure UV spectra of AMD bound to nDNA and dDNA were calculated for the purpose of illustration and can never be claimed unique, they appear to be different from the free-AMD spectrum and hypochromic relative to it.

Conclusion

By using the PCA method, the number of bound AMD species in each of the AMD–nDNA and –dDNA complexes was determined to be one in a low P/D range (0—4) and two in a wide P/D range (0—30). The pure visible absorption spectra of bound AMD were extracted, for the first time, in the weak-binding region. They show a distinct absorption peak at 465 nm (bound to nDNA) and at 460 nm (bound to dDNA) together with a pronounced shoulder near 440 nm. These spectral features are in excellent accord with the measured CD spectra. The variation of the fraction of bound AMD with P/D was calculated from the apparent binding constant K_a which was determined by the extended PCA method. The effect of added sodium chloride on K_a is modest; hence, the binding of AMD to DNA is probably nonelectrostatic. The pure ultraviolet spectrum of AMD bound to dDNA indicates the partial recovery of "nativeness" to the AMD–dDNA conformation. The PCA method is a very useful technique to extract the pure spectra of light-absorbing components from a series of experimentally measured absorption spectra.

References

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