Actinomycin D–deoxynucleotide interactions: Binding isotherms at the benzenoid and quinoid portions of the drug

(thermodynamic parameters/circular dichroism)

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ABSTRACT  Titrations of actinomycin D (AMD) with dG and with dG-dC were monitored by circular dichroism at 380 nm and 470 nm. These wavelengths are sensitive predominantly to nucleotide binding processes at the benzenoid and quinoid portions, respectively, of the phenoxazone ring of the drug chromophore [Auer, H. E., Pawlovski-Konopnicki, B. E., Chiao, Y. C. C. & Krugh, T. R. (1978), Biopolymers, 17, 1891–1911]. The temperature dependence of these isotherms was analyzed by the van't Hoff equation to obtain values for the enthalpy and entropy changes. For dG these are about −11 kcal mol⁻¹ and −20 cal mol⁻¹ deg⁻¹, respectively, for complex formation at both the benzenoid and quinoid sites (1 cal = 4.184 J). The enthalpy and entropy changes for complex formation with dG-dC remain unchanged at the benzenoid site, but both values are more negative at the quinoid site. These results indicate that the additional process of binding C in the intercalated AMD–(dG-dC)₂ complex, with respect to the simply stacked AMD–dG₂ complex, has distinctive properties at the two sites, reflecting their structural differences. The ability to resolve binding processes at the two sites by circular dichroism has permitted us to suggest assignments for the two ³¹P magnetic resonance lines from the phosphodiester groups observed in the AMD–(pdG-pdC)₂ complex [Patel, D. J. (1976) Biochim. Biophys. Acta 442, 98-108].

Actinomycin D (AMD, Fig. 1) is an antibiotic used in the chemotherapy of certain forms of cancer. It binds to double-helical DNA by intercalation between successive base pairs (1) with a strong preference for guanine-containing sequences (2–4). In the crystal structure of an AMD–dG₂ complex (5), it was observed that the G bases are stacked diametrically opposed to one another over the benzenoid and quinoid portions, respectively, of the phenoxazone ring of the drug; the selectivity for G is apparently due to the formation of strong hydrogen bonds between the 2-amino groups of the bases and the threonyl carbonyls of the drug. Based on this structure it was proposed (6) that in native DNA a class of tight binding sites for AMD intercalation is comprised of self-complementary -dG-dC- sequences. This hypothesis has led to numerous studies of complexes formed between AMD and simple model deoxynucleotides (7–17). It has emerged that dGMP, pdG-dN (N, not C), and pdN-dG form stacked complexes with AMD in solution with the stoichiometry of AMD/dG = 1:2, whereas pdG-dC binds with the same molar ratio in a cooperative fashion forming an intercalated complex. These observations are consistent with the findings of Sobell and Jain (6).

We have recently been studying the properties of AMD–deoxynucleotide complexes by using circular dichroism (CD) spectroscopy (18, 19). Based on differing spectral responses observed under various experimental conditions, we concluded that the broad, relatively featureless absorption band of AMD centered at 440 nm in fact comprised of three electronic transitions. One, occurring at about 370 nm, is localized to a large degree on the benzenoid section of the phenoxazone ring, whereas a second, located at about 490 nm, is predominantly centered on the quinoid portion of the chromophore. These wavelengths monitor nucleotide binding processes of high and low affinity, respectively. The third transition, located in the vicinity of the absorption maximum at 425–440 nm, is more extensively delocalized across the entire chromophore, and therefore it presents an optical mixture of binding processes at both sites. Most studies reported in the literature have been carried out in the vicinity of this absorption maximum because the greatest sensitivity to the formation of complexes is found there.

Our studies indicate that detailed comparisons of nucleotide binding to the benzenoid and quinoid sites of AMD can be conveniently carried out in dilute solutions by using CD spectroscopy. This contrasts with other methods, such as NMR spectroscopy, that require high drug concentration, because under such conditions the ratio of concentrations of drug to free nucleotide is not propitious for the resolution of the two binding processes. As the first application of this new potential, we report here a comparative study of the binding isotherms for dG and dG-dC monitored at 380 nm and 470 nm. We used the latter wavelength instead of 490 nm in order to attain sufficient spectral sensitivity for the titration profiles. We examined the temperature dependence of these titration curves in order to obtain thermodynamic parameters for each binding process. The two binding sites were found to manifest readily distinguishable properties, which may be anticipated from the difference in their chemical constitution.

MATERIALS AND METHODS

AMD (Grade I) and dGMP were from Sigma. dG and dG-dC were purchased from P-L Biochemicals. Solutions were prepared in 5 mM potassium phosphate, pH 7.

Experimental procedures were similar to those previously described (19). Initial AMD concentrations were 20–25 µM, at which spectral complications arising from dimers of free AMD could be avoided. Under these conditions of high dilution, sufficient spectral sensitivity to the changes occurring at both wavelengths was attained by the use of a jacketed 4.90-cm cell.

The temperature was measured with a calibrated YSI thermometer probe inserted directly into the cell.

RESULTS

The CD spectrum of monomeric AMD displays a negative extremum at 570 nm and weak negative ellipticity centered at 445 nm (17–24). Upon complex formation of AMD with deoxynucleotides, the 370-nm band intensifies and shifts slightly to the red. Titration with dGMP produces an intense positive

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extremum at 445 nm with a long tail extending to 550 nm. The addition of pdG-dC to a solution of AMD leads to the formation of a weak negative band at 470 nm (17, 19).

dG. CD spectra of the complex formed by dG and AMD closely resemble those reported for the dCMP–AMD adduct (19). Profiles of CD titrations of AMD with dG monitored at 380 nm and 470 nm are shown in Fig. 2 for 1.4° (upper) and 31.8° (lower). These are presented as normalized titration curves, obtained from the changes in ellipticity by dividing by the maximum change observed. Additional experiments were carried out at 11.7° and 20.2°. The curve obtained at 380 nm at 1.4° is slightly biphasic in character. This phenomenon is not apparent at any of the other temperatures studied here. A similar observation was made in the case of dCMP at 0.1° (19) and was ascribed to an effect arising from the binding of nucleotide at the low-affinity binding site on the CD profile of the high-affinity process monitored at this wavelength. This effect is much less pronounced with dG than with dCMP, because the value of Δθ/Δθ_{max} at saturation is about 0.93 with dG but decreases to about 0.75 with dCMP at 0–1°. With dCMP, the biphasic character grows progressively weaker at 10.9° and 22.2°, and is absent at 29.6°. These results show that the binding curves for dG are more susceptible to analysis than are those for dCMP.

Fig. 2 demonstrates that the titration curves shift to higher nucleotide concentrations as the temperature increases, indicating a decreased stability for the drug–nucleotide complexes. In order to obtain estimates for the association constants K_a from these curves, we followed the rationale presented in ref. 19. The titrations are plotted against the total concentration of nucleotide added. Therefore, for a given binding curve the ratio of concentrations of added nucleotide to drug at half-saturation, p_{1/2} = (N_0)/(D_0), must be corrected for the nucleotide bound at both sites in order to arrive at the ratio of free nucleotide to drug. This is done by subtracting ½, the fractional saturation from the monitored curve, as well as the fractional saturation r from the second binding isotherm at p_{1/2}. This gives

$$K_a = \frac{(D_0) \cdot (p_{1/2} - \frac{1}{2} - r)}{N_0}. \quad [1]$$

Van’t Hoff plots of the results for dG are shown in Fig. 3. The values of ΔH and ΔS are presented in Table 1. They may be compared with the results of earlier studies (25-28).
action between the binding of the two nucleotide molecules diminishes with increased temperature. This is not in accord with earlier studies, in which complete cooperativity was reported at 8° (17) and at 25° (10).

The curve for 470 nm at 21.3° passed through negative values of $\Delta \theta / \Delta \theta_{\text{max}}$ at low nucleotide concentrations (Fig. 4). A similar but less pronounced effect was observed at 15.2°, but not at 8.3°. Because formation of the AMD-(dG-dC) complex led to enhanced negative ellipticity at this wavelength, a negative fractional change arose from a positive deflection of the CD amplitude in this portion of the titration. The CD spectrum of the AMD-(dGMP) complex is positive in this region of the spectrum and has a somewhat greater intensity than does the negative extremum of the AMD-(dG-dC) complex (19, 21).

![Figure 4](image)

**Fig. 4.** Fractional change of the ellipticity of AMD with added dG-dC followed at two wavelengths. The titration was carried to 0.708 mM dG-dC at 0.1° (Upper) and to 0.886 mM dG-dC at 21.3° (Lower). A, 380 nm; O, 470 nm.

![Figure 5](image)

**Fig. 5.** Van’t Hoff plot of data obtained from the titrations with dG-dC according to Eq. 1. A, 380 nm; O, 480 nm.

Therefore, this effect may arise from the formation of a binary AMD-dG-dC complex in which the G is stacked at the quinoid binding site, and the C is probably not stacked on the opposite face of the phenoxazone ring. This binary complex could have existed early in the titration, at which time a significant fraction of the benzenoid sites would have remained free. As the concentration of added dinucleotide is increased, the population of this binary species is expected to decline because it should pass to the ternary complex. Specifically, at $\Delta \theta / \Delta \theta_{\text{max}}$ near 0.5 on the 470-nm curve, little or no binary complex is expected to remain, so that this point on the binding isotherm should accurately reflect the half-saturation point $p_{1/2}$.

Values of $K_a$ were obtained at each wavelength and temperature, as outlined above for the dG titrations. Van’t Hoff plots prepared from the results are presented in Fig. 5, and the values of $\Delta H$ and $\Delta S$ are given in Table 1. The values of $\Delta H$ and $\Delta S$ manifest different trends at the two binding sites: no changes occurred at the benzenoid site with respect to binding dG, whereas complex formation with dG-dC at the quinoid site was more exothermic and led to a more negative value of $\Delta S$ than was found with dG.

**DISCUSSION**

The circular dichroic isotherms obtained in this work monitor the binding of deoxynucleotides by AMD in a way governed by the optical properties of the phenoxazone chromophore. It has been noted that the electronic transitions used to follow these titrations resolve the binding processes at the two binding sites in an imperfect fashion (19). That is, $\lambda_0(380 \text{ nm})$ and $\lambda_0(470 \text{ nm})$ do not reflect exclusively nucleotide binding at the benzenoid and quinoid sites, respectively. Rather, each contains contributions from the second process.

The binding of two nucleotide molecules to the drug may be depicted as follows:

$$
\begin{align*}
&\text{A} \\
&\text{K}_a \quad \text{NA} \\
&\text{K}_b \quad \text{AN} \\
&\text{K}_c \quad \text{NAN} \\
&\kappa \text{ is a cooperativity parameter which is set equal to unity in the}
\end{align*}
$$

* $\Delta H$ in kcal mol$^{-1}$; $\Delta S$ in cal deg$^{-1}$ mol$^{-1}$.

**Table 1.** Thermodynamics of formation of AMD-deoxynucleotide complexes

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>Nucleotide</th>
<th>Benzenoid, 380 nm</th>
<th>Quinoid, 470 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H$</td>
<td>dG</td>
<td>-11.1</td>
<td>-10.3</td>
</tr>
<tr>
<td></td>
<td>dG-dC</td>
<td>-11.0</td>
<td>-14.7</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>dG</td>
<td>-20.2</td>
<td>-20.2</td>
</tr>
<tr>
<td></td>
<td>dG-dC</td>
<td>-19.7</td>
<td>-33.4</td>
</tr>
</tbody>
</table>

* *AH* extremem negative followed *dG-dC*.
absence of cooperative effects. Because the monitoring wave-
lengths do not resolve the binding processes in a perfect
fashion, the saturation curve at each wavelength describes the optical
contributions manifested at the wavelength in question due to
complex formation at both binding sites. The fraction of the
drug with a given site occupied is taken as the sum of the
fractions of singly and doubly liganded species obtained by
following the upper or lower path of scheme [2], respective-
ly.

We attempted to fit this model to titrations of AMD with
gMP which were also studied during the course of this work,
using the MLAB curve fitting system (29) operating on a
DEC10 computer. As noted above, the 380-nm titrations at the
lowest three temperatures of the four temperatures examined
were biphasic. Moderate success was achieved in fitting these
data. Analysis of the dG-dC titrations by this model, however,
did not produce convergence. This suggests that the model may
be inappropriate, perhaps in the way the spectroscopic inter-
actions between the binding sites were treated. No other models
were devised for analysis. Plotting biphasic titration curves
according to the Benesi–Hildebrand equation (50) yields neg-
ative intercepts, demonstrating that this procedure is also in-
applicable. For the reasons we have analyzed our results ac-

cording to Eq. [1]. It must be recalled (19) that, because the
optical resolution of the binding processes monitored at the two
wavelengths is imperfect, the distinctions in thermodynamic
properties found here represent only partial approaches to the
correct values.

The thermodynamic parameters for reaction with the
deoxynucleotides studied in this work (Table 1) show different
trends at the two nucleotide binding sites of the drug. At
the benzenoid site the values of both $\Delta H$ and $\Delta S$
remain essentially unchanged upon passing from the stacking reaction of dG to
the intercalation process of dG-dC. The reaction of dG-dC at the
quinoid site, however, is more exothermic and leads to a
greater decrease in entropy than does complex formation with
dG. The differing patterns in the thermodynamic parameters
cannot be attributed to contributions from the cooperativity of
the binding of the two dG-dC molecules. This originates in
the hydrogen bonding between the G-C base pairs. It is likely
to be exothermic and to have the same intensity for binding at
both sites; indeed it is not easy to assign one or another set of
hydrogen bonds to a given binding site.

The distinctions in behavior between the binding sites must
rather be sought in their chemical differences. The primary
 distinction between the binding of dG and of dG-dC is thought
to be the stacking of C on the face of the phenoxazine ring
opposite to that on which G is bound (12–15). Van der Waals

290.
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Natl. Acad. Sci. USA 57, 1036–1042.
Quantitative Chemistry and Biochemistry II, 394–410.
3983.
1914.
1782.
4425.
4922.
4438.
FEBS Lett. 73, 167–170.
Biochem. 6, 106–113.
7, 996–1001.

complex is quite large and not likely to be strongly dependent
on temperature, whereas the dissociation rate constant is small,
characteristic of a significant energy of activation (17).
Therefore the relative destabilization at the quinoid site with
increasing temperature probably comes about by the en-
heancement of the dissociation rate constant rather than by a
decrease in the rate of formation of the complex. Less drastic
effects of temperature are proposed for the benzenoid site. In
$^3$H magnetic resonance studies of the AMD-(pdG-dC)$_2$
complex, two peaks were resolved for the phosphodiester bridges
of the two dinucleotides, at 1.905 ppm and 2.465 ppm upfield
from trimethylphosphate (15). The former resonance was
broadened, indicating a shortened lifetime, at lower temper-
ature than was the latter. Our results suggest that the 1.905-ppm
phosphodiester resonance be assigned to the dinucleotide
stacked at the quinoid site, and the 2.465-ppm peak to that
complexed at the benzenoid site. In proton magnetic resonance
experiments on the same system, Patel also distinguished two
lines arising from the guanine N$_2$H protons engaged in base-
pairing hydrogen bonds with cytosine (15). These were found
to exchange with the H$_2$O solvent to different extents as the
temperature was increased. His assignments of these resonances
are in accord with our observations.