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Retroviral Nucleocapsid Protein Specifically Recognizes the Base and the Ribose of Mononucleotides and Mononucleotide Components†

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ABSTRACT: The interaction of the retroviral nucleocapsid protein (NC) with nucleic acids forms the basis of its varied roles in the replication cycle, which include binding and condensing the viral RNA within the virion, stimulation of the early steps in reverse transcription, and dissociation from RNA in the replication complex. As part of an investigation of the NC binding site and of the forces that drive its interaction with nucleic acids, the relative affinities of NC from avian myeloblastosis virus were determined for a series of mononucleotides and mononucleotide components using a competitive displacement assay utilizing the extrinsic fluorescent probe bis-ANS [Secnik, J., Wang, Q., Chang, C.-M., & Jentoft, J. E. (1990) *Biochemistry 29*, 7991-7997]. The estimated binding affinities were unexpectedly similar for nucleotides, nucleosides, and bases ($K_a > 10^6$ M^{-1}). AMP, UMP, GMP, and CMP bound to NC with essentially equal affinity, indicating that NC does not discriminate between bases. This is consistent with its role in coating, condensing, and packaging the RNA within virions. Nucleosides, bases, riboses, and ribose phosphate bind to NC with 1000-fold higher affinity than inorganic phosphate, indicating that the NC binding site includes elements that recognize nucleotide base and ribose components in addition to phosphate ions. However, the binding affinities of components are not additive, i.e., the K_{app} values for adenine and deoxyribose are very similar to that for deoxyadenosine, indicating that the interaction between the NC subsite and the base and the sugar components is complex. The stoichiometry of the complex between bis-ANS and NC was established to be NC·(bis-ANS)$_3$. The data are consistent with a model in which bis-ANS or nucleotide ligands can compete on a one-for-one basis for binding at three NC subsites, where binding at each site is independent of binding at the other two sites. Taken together, these findings allow us to predict that when NC binds to RNA or DNA, it directly interacts with three nucleotides.

The nucleocapsid (NC) protein is a small basic protein that is an essential, multifunctional component of replication competent retroviruses. During the early stages of viral assembly, sequences within the NC play a role in the specific recognition of genomic viral RNA (Jentoft et al., 1988; Gorelick et al., 1988; Meric & Spahr, 1986). Within viral particles, the 2000 or so copies of this small, basic protein bind to the dimeric genomic RNA (Jentoft et al., 1988; Fleissner, 1971; Davis & Rueckert, 1972) indicating a histone-like role for NC in packaging viral RNA. NC also appears to have a role in the dimerization of the genomic RNA (Prats et al., 1988). NC is generally considered to be a component of the in vivo transcription complex (Fuetterer & Hohn, 1987; Brown

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et al., 1987) consistent with the observation in vitro, that NC promotes the initiation of reverse transcription (Barat et al., 1989).

The requirement for NC in replication competent retroviruses establishes the importance of understanding its biochemical properties, particularly those related to its interaction with nucleic acids. Presently, few details are known about the specificity or forces involved in these interactions. NC derived from avian sarcoma/leukosis viruses [which include avian myeloblastosis virus (AMV) and Rous sarcoma virus] has been used as a model NC for genetic (Fu et al., 1988a,b), biochemical, and biophysical characterization (Fu et al., 1985; Jentoft et al., 1988; Katz & Jentoft, 1989; Secnik et al., 1990). Nucleic acid binding studies have established that the occluded site size of AMV NC is five or six nucleotides and that cooperativity is small ($\omega = 25$) (Jentoft et al., 1988). A K_a of 1×10^5 M^{-1} was obtained for NC binding to poly(ethenoadenylic acid) (Jentoft et al., 1988), consistent with the result of Karpel et al. (1987). Similar binding constants were reported for both viral RNA and heterologous DNA using an indirect fluorescent method (Smith & Bailey, 1979). Although both Smith and Bailey (1979) and Karpel et al. (1987) found no difference between RNA and DNA binding interactions with NC, differences in the pH dependence of these interactions were suggested by filter binding experiments (Leis & Jentoft, 1983). Binding of AMV NC to poly(ethenoadenylic acid) was found to be independent of the presence or absence of exogenous zinc(II) (Jentoft et al., 1988), and binding of the NC from Moloney murine leukemia virus to this fluorescent nucleic acid was found to be unaffected by modification of its three cysteine residues (Karpel et al., 1987). These results suggest that zinc(II) is not required for NC activity, despite the suggesting that NC is a zinc finger protein (Berg, 1986). However, these studies taken together provide an incomplete picture of NC interactions with nucleic acids, in part because detailed, quantitative data on, e.g., the base and ribose specificity of NC interactions with nucleic acids, have not been reported.

A quantitative determination of the binding specificity of NC for nucleic acids is fundamental to a full understanding of the biochemical bases for the biological functions of this protein. NC is widely presumed to be specific for single-stranded nucleic acids (Roberts et al., 1988; Casas-Finet et al., 1988), and it has been proposed, on the basis of cross-linking data, to have high-affinity binding for specific regions of viral RNA (Darlix & Spahr, 1982). Since specificity should be largely determined by interactions in the binding site between groups on the nucleic acids and NC amino acid side chains, we have determined the relative affinity of AMV NC for mononucleotides and mononucleotide components using a fluorescent method established previously (Secnik et al., 1990).

Materials and Methods

Materials. The extrinsic fluorescent probe 4,4′-bis(phenylamino)-(1,1′-binaphthalene)-5,5′-disulfonic acid (bis-ANS) was obtained from Molecular Probes, Inc., Eugene, OR. D-Ribose 5′-phosphate, 2-deoxy-D-ribose, 5′-CMP, and adenosine were obtained from Sigma; 5′-GMP and deoxyadenosine were obtained from ICN Pharmaceuticals; adenine was obtained from Aldrich; 5′-AMP was obtained from Schwarz/Mann; 5′-UMP was obtained from P-L Biochemicals. Buffers and other chemicals were of the highest available commercial grade.

Isolation of NC. NC protein was isolated from pelleted AMV as described previously (Johnson et al., 1983; Jentoft et al., 1988). The protein was homogeneous as judged by SDS–PAGE and Western blot analyses. NC concentrations were measured spectrophotometrically, using an $A^{1\%}$ of 8.4 at 280 nm (Katz et al., 1986). NC contained undetectable amounts of Zn(II) (less than 0.05 ppm), as determined by plasma emission analysis (Jentoft et al., 1989) of 30 ppm NC samples.

Fluorescence Measurements. Fluorescence spectroscopy was performed on a Perkin-Elmer LS-5B spectrofluorometer equipped with a xenon lamp, variable slits, and microprocessor-controlled photomultiplier gain. All measurements (unless otherwise indicated) used fluorescence excitation and emission wavelengths of 298 nm and 495 nm, respectively, an excitation slit width of 3 nm, and an emission slit width of 10 nm. Inner filter effects were insignificant at the excitation wavelength of 298 nm. A 0.5×0.5 cm quartz cuvette with a sample size of ≥ 250 μL was used for all experiments. Fluorescence was stable with time under all conditions.

Ligand competition experiments were performed without dilution of NC or bis-ANS by including 1 μM NC and 3 μM bis-ANS in both the initial and ligand stock solutions. All experiments were performed at 27.0 ± 0.2 °C in 40 mM buffer adjusted to the appropriate pH. The binding titration data were recorded as relative fluorescence values at each concentration of added ligand. In all experiments, the data were consistent with a competitive displacement of bis-ANS by the added ligand, since fluorescence emission from bis-ANS decreased as added nucleotide ligand displaced bis-ANS from NC until, at saturation, the fluorescence was quenched to the level expected for free bis-ANS in solution. Since bis-ANS fluorescence was excited indirectly by energy transfer from Trp 80 of NC, it is more likely that this quenching is caused by displacement of bis-ANS from the protein than by a conformational change of the NC·bis-ANS complex. The fact that the excitation maximum was constant during the competition experiments [see Secnik et al. (1990)] is also consistent with ligand-induced displacement of bis-ANS from NC.

Determination of Stoichiometry for bis-ANS Binding to NC. The stoichiometry and dissociation constant for the bis-ANS interaction with NC was determined using the model of Wang and Edelman (1971) (Horowitz & Criscimagna, 1985) for a fluorophore (bis-ANS) binding to n independent sites on a protein, given by

$$\text{bis-ANS} + \text{NC} \overset{K_d}{=} \text{NC·bis-ANS} \qquad (1)$$

where K_d is the dissociation constant for the binary complex and NC represents the concentration of NC sites. For the case where the NC·bis-ANS fluorescence predominates, then for an arbitrary concentration of bis-ANS, [bis-ANS], and a fixed total concentration of NC, $[NC_o]$, it follows that

$$I/I_{max} = (\psi[\text{NC·bis-ANS}])/(\psi n[NC_o] = [\text{NC·bis-ANS}])/n[NC_o] = f \qquad (2)$$

where I is the corrected fluorescence intensity, I_{max} is the maximum fluorescence intensity (when all NC is present as the complex), the quantity $n[NC_o]$ is the total concentration of NC sites, ψ is a proportionality constant relating the fluorescence intensity to the concentration of complex, and f is the fraction of NC sites complexed with bis-ANS. By applying the law of mass action, and appropriate substitutions, the following equation can be obtained:

$$1/I = 1/I_{max} + (K_d/I_{max})(1/[\text{bis-ANS}]) \qquad (3)$$

K_d can be obtained from a double-reciprocal plot of $1/I$ versus $1/[\text{bis-ANS}]$ under conditions where bis-ANS is in excess.

The stoichiometry of the complex is obtained by combining the K_d determined as above with the value K_d/n obtained from a second series of titrations at constant bis-ANS and changing NC, under which conditions eq 3 can be written as

$$1/I = 1/\psi[\text{bis-ANS}] + K_d/\psi[\text{bis-ANS}](n[NC_o] - [\text{NC·bis-ANS}]) \quad (4)$$

When $[NC_o] \gg [\text{NC·bis-ANS}]$, this equation reduces to the simpler form

$$1/I = 1/(\psi[\text{bis-ANS}]) + K_d/(\psi[\text{bis-ANS}])(n[NC_o]) \quad (5)$$

and K_d/n and ψ[bis-ANS] can be obtained from the double-reciprocal plot of $1/I$ versus $1/[NC_o]$.

Since these experiments required a broad range of concentrations of NC, bis-ANS, and NC·(bis-ANS)$_3$, they were performed in 40 mM MES at pH 5.5 since the fluorescence enhancement from bound bis-ANS is maximal under these conditions (J. Secnik, unpublished observations).

Determination of Apparent Inhibition Constants, K_{app}, in the Displacement Titration. Values for K_{app} were determined by a direct fit to a hyperbola of the change (decrease) in relative fluorescence of bound bis-ANS as a function of total added ligand concentration. A Marquardt gradient analytical search procedure was used in iterating to the best parameter fit by least-squares analysis (program BASICFIT written by K. Neet, personal communication).

Models of the Displacement of bis-ANS by Nucleotides and Nucleotide Components. All binding experiments involved the displacement of bis-ANS from NC by nucleotide-type ligands. Equation 2 was used to determine the fraction (f) of total NC sites complexed to bis-ANS from the fluorescence data. To test the hypothesis that the displacement reaction involved a competition by bis-ANS and a nucleotide-based ligand (MN) for the same site on NC (Secnik et al., 1990), the data were tested against a model for the interaction whose assumptions are (i) that ligand binding to NC occurs at n (n = 3) independent sites and (ii) that each bis-ANS is competitively displaced by a single ligand. The reactions which describe these events are shown in eq 1 and in the following equation:

$$\text{NC} + \text{MN} \overset{K_{MN}}{=} \text{NC·MN} \quad (6)$$

except that K_{bis} is now defined as the equilibrium *association* constant for eq 1 and K_{MN} is the equilibrium association constant for the competing reaction. To test the data against the model, the equilibrium expressions for the competing reactions were solved for [NC] and set equal to each other, since the reactions occur simultaneously, giving

$$[\text{NC·bis-ANS}]/(K_{bis}[\text{bis-ANS}]) = [\text{NC·MN}](K_{MN}[\text{MN}]) \quad (7)$$

The expressions were rearranged to separate the independent variable (the free nucleotide ligand concentration) and dependent variables (the fluorescence-dependent parameters of [NC·bis-ANS], [NC·MN], and [bis-ANS]), as shown in

$$[\text{NC·bis-ANS}]/\{[\text{NC·MN}][\text{bis-ANS}]\} = (K_{bis}/K_{MN})(1/[\text{MN}]) \quad (8)$$

Under the conditions of our experiments, bis-ANS binding to NC is stoichiometric, and the amounts of NC and bis-ANS are adjusted so that only the complex is present in significant quantity when the experiment begins. Thus, the only significant source of free bis-ANS is that released when the ligand MN displaces it from an NC site. Therefore, the concentration of free bis-ANS will be equal to the concentration of bound MN, as given by

$$[\text{bis-ANS}] = [\text{NC·MN}] = (1 - f)n[NC_o] \quad (9)$$

Substituting from eqs 2 and 9 into eq 8 and rearranging, we obtain

$$f/(1 - f)^2 = \{n[NC_o]K_{bis}/K_{MN}\}(1/[\text{MN}]) \quad (10)$$

where the expression in brackets is a constant for each experiment. If the data are consistent with the simple competition mechanism, a plot of $f/(1 - f)^2$ versus $1/[\text{MN}]$ should be linear and should extrapolate through the origin. The ratio of K_{bis}/K_{MN} can then be calculated from the slope, since n and $[NC_o]$ are known.

A second model of n independent NC sites in which a single bis-ANS is displaced by two molecules of MN was also considered. For the purposes of analyzing this model, the competing reaction is defined by

$$\text{NC} + 2\text{MN} \overset{K^*_{MN}}{=} \text{NC·MN}_2 \quad (11)$$

where K^*_{MN} is the overall association constant for two noninteracting MN ligands binding at a NC site. The expressions for the competing reactions were solved for [NC], set equal to each other, and treated as above, making the assumption that

$$[\text{bis-ANS}] = [\text{NC·MN}_2] = (1 - f)n[NC_o] \quad (12)$$

Substitution gives

$$f/(1 - f)^2 = \{n[NC_o]K_{bis}/K^*_{MN}\}(1/[\text{MN}]^2) \quad (13)$$

If the data are consistent with this model, a plot of $f(1 - f)^2$ versus $1/[\text{MN}]^2$ should be linear and should extrapolate through the origin. The ratio of K_{bis}/K^*_{MN} would then be determined from the slope, since n and $[NC_o]$ are known.

Results

The extrinsic fluorophore bis-ANS binds to NC with a large enhancement of its intrinsic fluorescence emission (Secnik et al., 1990). This enhancement was also observed for the NC·bis-ANS complex upon excitation of the protein tryptophan fluorescence, indicating that energy was transferred from the tryptophan to the bound bis-ANS. Significantly, the addition of either anions or RNA to a solution containing the NC·bis-ANS complex causes complete quenching of the bis-ANS fluorescence, indicating that binding of a second ligand displaces bis-ANS from NC. It was also demonstrated that quenching of bis-ANS fluorescence can be used to follow binding of other ligands to NC (Secnik et al., 1990).

In this study, the stoichiometry of the interaction of NC and bis-ANS is more fully established and the fluorescence quenching of bound bis-ANS is used as a probe to investigate the interaction between NC and mononucleotide-type ligands.

Stoichiometry of the NC·bis-ANS Complex. Under conditions of stoichiometric binding (40 mM buffer), we previously estimated that there were between 2 and 10 bis-ANS sites per NC molecule, with a bias toward 2–3 sites on the basis of an analysis of a Job's plot (Secnik et al., 1990; Job, 1928). In the present study, we determined the number of sites on NC for the bis-ANS complex under conditions of equilibrium binding, i.e., in the presence 200 mM NaCl.

The double titration approach (Wang & Edelman, 1971; Horowitz & Criscimagna, 1985) was used to determine K_d and n, the number of sites for the NC bis-ANS interaction, as described in Materials and Methods. Under conditions where the fixed concentration of free NC (0.1–1.5 μM) was much greater than that of the complex, an averaged value for K_d of 11.6 ± 0.6 μM was obtained for the NC·bis-ANS complex. Under conditions of excess bis-ANS (fixed con-

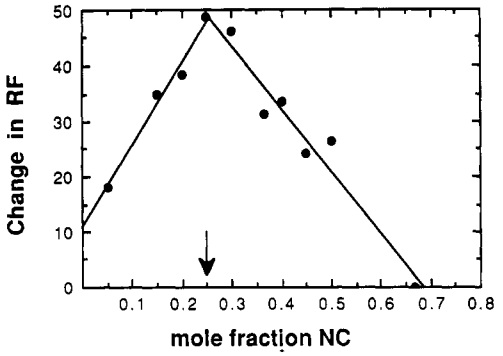

FIGURE 1: Job's plot of the corrected change in relative fluorescence as a function of changing mole ratios of NC and bis-ANS. The sum of the concentrations of NC and bis-ANS was held constant at 2.0 μM. Experiments were performed at pH 5.5, 27 °C, in 40 mM MES, 200 mM NaCl.

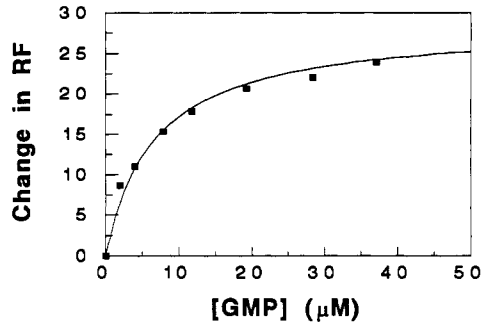

FIGURE 2: The change (decrease) of the relative fluorescence of bis-ANS in the NC·bis-ANS complex as a function of added GMP, under standard conditions of 1 μM NC, 3 μM bis-ANS, 40 mM MOPS, pH 7.0, and 27 °C, using fluorescence excitation and emission wavelengths of 298 nm and 495 nm, respectively. The calculated K_{app} for the competitive displacement of bis-ANS in this experiment is 6 μM.

centrations of 0.2–1.0 μM), an averaged value of K_d/n of 3.8 ± 0.7 μM was obtained. The value of n, the number of independent sites for bis-ANS on NC, was determined to be 3 from the ratio of the two constants.

The stoichiometry of the NC·bis-ANS complex was also determined by the method of continuous variation. The results are displayed in a Job's plot (Job, 1928) in Figure 1. The maximum in the plot occurs at a mole ratio of 0.25, corresponding to 3 sites for bis-ANS on NC.

These results allow us to conclude that NC has 3 independent sites for bis-ANS. This value is consistent with the earlier estimate of 2–3 sites on NC for bis-ANS, determined from a Job's plot in the same buffer, but in the absence of salt (Secnik et al., 1990).

Binding of Nucleotide-Type Ligands. The interaction of nucleotide monophosphates with NC is shown by the ability of GMP to quench the fluorescence emission of bis-ANS in the NC·bis-ANS complex, as illustrated in Figure 2. GMP completely quenches the bis-ANS fluorescence to background levels (data not shown), a result similar to the effect of poly(A) and anions upon the NC·bis-ANS complex (Secnik et al., 1990). The data was analyzed by a fit to a hyperbola. The solid line through the data points in Figure 2 corresponds to a K_{app} of 6 μM for GMP displacing bis-ANS from NC.

The base specificity of NC was quantified by measuring the relative ability of AMP, UMP, GMP, and CMP to displace bis-ANS from the NC·bis-ANS complex. As shown in Table I, the K_{app} values for the four ribonucleotide monophosphates are similar, indicating that NC binding is largely nonspecific with respect to nucleotide bases.

Table I: K_{app} Values for the Displacement of bis-ANS from the NC·bis-ANS Complex for Various Nucleotide-Type Ligands

ligand	K_{app}[a] (μM)	ligand	K_{app}[a] (μM)
PO_4^{3-}[b]	4000	ribose 5-phosphate	3.1 ± 0.2
AMP	8.7 ± 0.6	deoxyadenosine	3.0 ± 0.3
UMP	5.1 ± 0.2	adenine	6.9 ± 0.9
GMP	6.0 ± 0.6	adenosine	7.6 ± 0.8
CMP	5.6 ± 0.4	deoxyribose	1.3 ± 0.1

[a] Unless otherwise indicated, all experiments were performed at 27 °C and pH 7.0 in solutions containing 1 μM NC, 3 μM bis-ANS, and 40 mM MOPS. K_{app} was determined from the direct fit to a hyperbola of fluorescence quenching versus total ligand concentration data, as described in Materials and Methods. The reported uncertainty in K_{app} is a measure of the goodness of the fit of the theoretical line through the ≥10 experimental points. [b] From Secnik et al. (1990).

The contribution of the phosphate, ribose, and adenine components to the apparent binding affinity measured for AMP was assessed by measuring the relative ability of various nucleotide-based ligands to displace bis-ANS. The K_{app} was determined for ribose 5-phosphate to assess which nucleotide components are required for the large increase in affinity noted in going from inorganic phosphate to AMP. As indicated in Table I, the K_{app} of NC for ribose 5-phosphate was indistinguishable from that for AMP. Thus, the addition of the sugar to the phosphate group appears to be sufficient for an increase of nearly 3 orders of magnitude in the relative ligand affinity for NC.

To further establish the relative contributions of base and sugar to the total nucleic acid binding affinity, the interaction was studied for nucleotide bases, sugars, and nucleosides. The K_{app} values for these ligands were similar, as shown in Table I. Thus, either the base or the ribose component is sufficient to cause a large increase in the affinity of these ligands for NC, relative to the affinity of inorganic phosphate. In addition, these data indicate that the phosphate group is not essential for the higher affinity binding.

To determine if NC shows differential affinity for RNA and DNA components, the K_{app} was determined for adenosine and deoxyadenosine. The K_{app} values are very similar, as indicated in Table I, suggesting that NC does not discriminate between DNA and RNA at the level of the individual mononucleotide ligand.

Models of the Interaction. The mononucleotide data was tested for consistency with two models of competitive binding between bis-ANS and a nucleotide-type ligand at 3 independent sites on NC, as presented in Materials and Methods. For these analyses, the free concentration of mononucleotide ligand, [MN], was calculated from the total concentration minus bound MN, where the amount of bound MN is dependent upon the model.

The first model assumes a one-for-one displacement of bis-ANS by ligand (MN) at a NC site, where the relationship between the fluorescence data and the concentration of free MN is given by eq 10. The fit of the GMP displacement data to this model is shown in Figure 3A. The data points fall on a least-squares line constrained to intersect the origin, as required by the mechanism, indicating that the data are consistent with the model. The data for all the mononucleotide ligands gave good fits to this model.

Since the occluded site size of NC is 5 or 6 nucleotides, the mononucleotide displacement data were also tested against a second model which assumed three independent bis-ANS sites on NC, where 2 molecules of ligand were required to displace each bis-ANS (see Materials and Methods). In this case, the relationship between fluorescence data and free MN concentration is given by eq 13. The fit of the GMP data to this

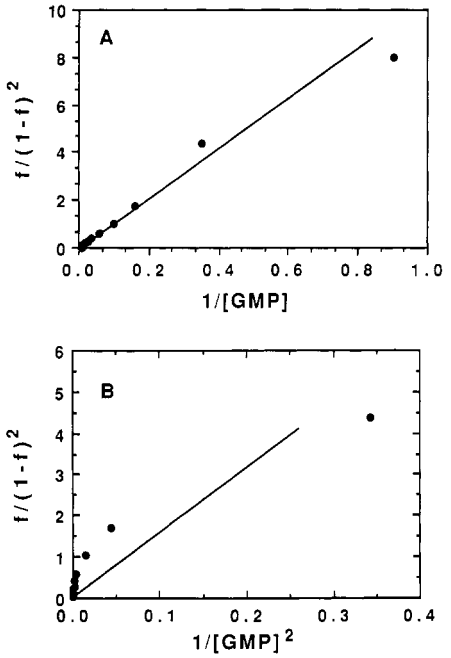

FIGURE 3: Tests of the data for GMP to the models of mononucleotide and bis-ANS binding to NC. Panel A: The raw data from Figure 2 plotted in the form of eq 10 (Materials and Methods), which was derived for a competitive displacement mechanism in which the ligand (GMP) displaces bis-ANS from 3 independent binding sites on NC. The data are consistent with this mechanism, by the criteria that the data points fall on a straight line that intersects the origin. A ratio of K_{bis}/K_{GMP} of 3.1 (for association constants) was derived for these data (see Table II), indicating that bis-ANS binds 3 times tighter to NC than GMP. Panel B: The raw data from Figure 3 plotted in the form of eq 13 (Materials and Methods), which was derived for a competitive displacement mechanism in which 2 mol of ligand displace each mole of bis-ANS from 3 independent binding sites on NC. The data points describe a curve and are thus inconsistent with this mechanism, since eq 13 describes a straight line that intersects the origin.

model is shown in Figure 3B. The data points show curvature rather than linearity as predicted by the model, indicating that the data are not consistent with the model. All the ligand displacement data showed curved rather than linear plots when tested against this model.

Thus, the displacement data are consistent with the simplest model incorporating competitive displacement of bis-ANS by nucleotide ligands at 3 independent sites on NC. The ratio K_{bis}/K_{MN} was calculated from the fits to eq 10 of the displacement data for all of the nucleotide ligands, as shown in Table II. K_{bis}/K_{MN}, a quantitative measure of the difference in binding affinities for bis-ANS and the specific nucleotide ligand, varies from 0.4 to 6.2, a range of only 15-fold, indicating that the bis-ANS and the nucleotide ligands bind to NC with similar affinities. However, bis-ANS binds more tightly to NC than most of the nucleotide ligands, with the sole exception of deoxyribose. To emphasize the similarities in binding affinities, the differences in free energies of the interaction for bis-ANS and the various nucleotide ligands are shown Table II. To show the similarities between nucleotide ligands, the ratio of affinities for each nucleotide-type ligand relative to that for GMP is also shown in Table II.

Table II: Results of the Fit of Binding Data to the Model for Competitive, Single-Displacement Binding of bis-ANS and Nucleotides at Three Independent sites on NC

ligand	K_{bis}/K_{MN}[a]	$\Delta\Delta G$[b]	K_{GMP}/K_{MN}
PO_4^{3-}	667	−3.9	0.005
GMP	3.1	−0.7	1
AMP	4.6	−0.9	0.7
UMP	2.6	−0.6	1.2
CMP	2.4	−0.5	1.3
ribose 5-phosphate	1.1	−0.1	2.8
deoxyadenosine	1.1	−0.1	2.8
adenine	1.5	−0.2	2.1
adenosine	6.2	−1.1	0.5
deoxyribose	0.4	+0.5	7.8

[a] The ratio K_{bis}/K_{MN} is derived from the slope divided by the total concentration of sites. All data were consistent with an independent sites model for competing equilibria in which each bis-ANS is replaced by a single mononucleotide-type ligand (eq 2). [b] $\Delta\Delta G = -RT \ln K_{bis}/K_{MN}$, in kilocalories per mole.

Discussion

The interactions of the retroviral NC with RNA are critical to infectivity at several stages in viral replication (Jentoft et al., 1988; Gorelick et al., 1988; Meric & Spahr, 1986; Fleissner, 1971; Davis & Rueckert, 1972; Barat et al., 1989). The recognition of viral genomic RNA by NC sequences within the *gag* gene precursor polyprotein, $Pr76^{gag}$, requires specific recognition of a sequence and/or structure. In contrast, NC histone-like packaging activity must necessarily be nonspecific in nature. The nature and specificity of the interaction of NC that leads to stimulation of reverse transcription are presently undefined, as is the nature of the forces that induce the dissociation of the NC from the genomic RNA during transcription. A detailed elucidation of the nucleic acid binding properties of NC is a thus a prerequisite to the understanding of the biochemical basis of these biological properties. In this study we establish the specificity of NC for the component parts of nucleic acids and thus begin to define the nature of the NC binding interactions.

Previously, we showed that poly(A) could displace bis-ANS from NC, with a $K_{app} < 10^{-6}$ M, in 40 mM MES, pH 5.5, consistent with a competition between poly(A) and bis-ANS for the same binding region in NC (Secnik et al., 1990). In fact, the fluorescence from bound bis-ANS is quenched completely in the presence of 6 equiv of poly(A) bases, confirming that the occluded site size of NC is 6 nucleotides. The data from this study were consistent with a competitive model for bis-ANS and nucleic acids binding to NC. This model was expanded to include anion binding to the same region when it was observed that anions such as inorganic phosphate could also competitively displace bis-ANS from the NC·bis-ANS complex. The K_{app} for all the anions was about 4 mM (Secnik et al., 1990), indicating an unusually high affinity for these ligands, probably because the highly basic NC has clusters of basic residues in or near its binding site. The bis-ANS displacement assay was used in the present study to investigate the interaction of nucleotide ligands with NC to assess the relative contribution to the binding affinity of the base, sugar, and phosphate portions of the nucleic acid under conditions when the polyelectrolyte effect was not a complicating factor.

The most significant result from the present investigation is that mononucleotide ligands displace bis-ANS from NC 3 orders of magnitude more effectively than anions. The K_d for these ligands is estimated to be $\leq 10^{-7}$ M, on the basis of similar estimates for the K_d of bis-ANS (Secnik et al., 1990). This indicates that electrostatic interactions between the protein and the phosphate backbone of the nucleic acid are not required for the interaction between nucleotides and NC. The relatively tight binding of mononucleotides to NC was unexpected, since in general the driving force of the interaction between DNA binding proteins and nonspecific DNA has been the entropic effect caused by the displacement of adsorbed cations from long nucleic acid polymers (Lohman, 1986). Although there are exceptions [including *Escherichia coli*

ribosomal protein S1 (Draper & von Hippel, 1978) and the dnaB protein of *E. coli* (Biswas et al., 1986)], many other nucleic acid binding proteins including recA (Cotterill et al., 1982), gene 5 protein (Coleman et al., 1976; Alma et al., 1982), and SSB protein (Kraus et al., 1981), have K_d values for mononucleotides more than 100-fold weaker than that reported here for NC.

The ribonucleotide monophosphates all bind with similar affinity to NC, indicating that NC binds to all the bases equally well. This is consistent with its histone-like role in packaging RNA within the virion. This lack of discrimination fails to provide support for sequence-specific binding by NC, although it is possible that NC binds with enhanced affinity to a specific tertiary structure or a precise oligonucleotide sequence.

When the affinities for adenosine and deoxyadenosine are compared, it can be seen that deoxyadenosine is 5 times better than adenosine in displacing bis-ANS from NC, suggesting that NC may prefer to bind deoxynucleotides. This finding was unexpected since NC is bound to RNA within virus particles.

The large increase in binding affinity for ribose 5-phosphate compared to that for inorganic phosphate leads to the unexpected conclusion that the ribose moiety contributes substantially to the binding interaction between the NC and mononucleotides. On the other hand, the less than 10-fold differences in K_d for nucleotide bases and nucleosides suggest that the contribution of ribose and/or base to the interaction is more complicated than simply filling a subsite within the NC binding region, since the binding affinities for ribose and for base are not additive. A molecular explanation for this observation has not yet been formulated. The usual invocation of an increase in affinity caused by a conformational change at the binding site induced by the presence of either a base or a ribose is an initially attractive possibility. However, since the displacement reactions occur at three independent sites on NC, the explanation based on a conformational change actually requires three independent, but equal, conformational changes, one for each NC site. Such changes are possible but unlikely, especially in a 9.5-kDa protein. Further, at this time, we know of no spectroscopic evidence supporting the existence of multiple conformations of active NC.

The conclusion that NC has 3 sites for bis-ANS and for nucleotide-type ligands is significant, since it is a fundamental functional characteristic of the protein. It is reasonable to predict that RNAs and DNAs will directly interact with NC at these same three subsites. The number of subsites is smaller than the occluded site size of 5 or 6 polynucleotides (Jentoft et al., 1987; Secnik et al., 1990) as expected, since the occluded site size includes nucleic acids that interact with protein sites and those that are physically shielded from binding interactions by steric factors.

The relative affinities of nucleotides and bis-ANS for NC were quantified, as shown in Table II. bis-ANS binds to NC no more than 6 times more tightly than mononucleotide phosphates, corresponding to $\leq$1.1 kcal/mol of additional binding energy. Thus, contacts between NC and bis-ANS or nucleotides differ by no more than one or two van der Waal's contacts or one weak hydrogen bond (Fersht, 1985). Since bis-ANS binding is stoichiometric under the conditions of the experiments, K_d for bis-ANS is less than 10^{-7} M. Accordingly, an upper limit for the K_d of adenosine, the weakest nucleotide ligand, is 10^{-6} M.

The internal variation in affinities for NC between nucleotide ligands is very small, certainly accounting for no more than one or two contact interactions. Therefore, NC shows very little ability to differentiate between types of nucleotides or to differentiate between phosphorylated and unphosphorylated nucleotide ligands. Thus, any in vivo ability of NC to discriminate between RNA and DNA must depend upon the three-dimensional structure of the polymeric nucleic acids. On the other hand, the affinity of NC for nucleotides and anions may be biologically relevant since these compounds could interact with NC during those parts of the retroviral replication cycle that occur within the cytosol.

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Registry No. Phosphate, 14265-44-2; ribose 5-phosphate, 4300-28-1; deoxyadenosine, 958-09-8; adenine, 73-24-5; adenosine, 58-61-7; deoxyribose, 533-67-5.

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Role of Magnesium Ion in the Interaction between Chromomycin A_3 and DNA: Binding of Chromomycin A_3–Mg^{2+} Complexes with DNA

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ABSTRACT: Chromomycin A_3 is an antitumor antibiotic which blocks macromolecular synthesis via reversible interaction with DNA template only in the presence of divalent metal ions such as Mg^{2+}. The role of Mg^{2+} in this antibiotic–DNA interaction is not well understood. We approached the problem in two steps via studies on the interaction of (i) chromomycin A_3 and Mg^{2+} and (ii) chromomycin A_3–Mg^{2+} complex(es) and DNA. Spectroscopic techniques such as absorption, fluorescence, and CD were employed for this purpose. The results could be summed up in two parts. Absorption, fluorescence, and CD spectra of the antibiotic change upon addition of Mg^{2+} due to complex formation between them. Analysis of the quantitative dependence of change in absorbance of chromomycin A_3 (at 440 nm) upon input concentration of Mg^{2+} indicates formation of two types of complexes with different stoichiometries and formation constants. Trends in change of fluorescence and CD spectroscopic features of the antibiotic in the presence of Mg^{2+} at different concentrations further corroborate this result. The two complexes are referred to as complex I (with 1:1 stoichiometry in terms of chromomycin A_3:Mg^{2+}) and complex II (with 2:1 stoichiometry in terms of chromomycin A_3:Mg^{2+}), respectively, in future discussions. The interactions of these complexes with calf thymus DNA were examined to check whether they bind differently to the same DNA. Evaluation of binding parameters, intrinsic binding constants, and binding stoichiometry, by means of spectrophotometric and fluorescence titrations, shows that they are different. Distinctive spectroscopic features of complexes I and II, when they are bound to DNA, also support that they bind differently to the above DNA. Measurement of thermodynamic parameters characterizing their interactions with calf thymus DNA shows that complex I–DNA interaction is exothermic, in contrast to complex II–DNA interaction, which is endothermic. This feature implies a difference in the molecular nature of the interactions between the complexes and calf thymus DNA. These observations are novel and significant to understand the antitumor property of the antibiotic. They are also discussed to provide explanations for the earlier reports that in some cases appeared to be contradictory.

Chromomycin A_3 ($CHRA_3$,[1] structure shown in Figure 1; Thiem & Meyer, 1979; Van Dyke & Dervan, 1983) is an antitumor antibiotic produced from *Streptomyces griseus* (Gause, 1975). This antibiotic, along with structurally related antibiotics like mithramycin and olivomycin, belongs to the aureolic acid group (Bakhara et al., 1968; Thiem & Meyer, 1979; Calabresi & Parks, 1985). They bind to double-stranded DNA, thereby blocking its function as a template for DNA and RNA polymerases (Wakisaka et al., 1963; Ward et al., 1965; Calabresi & Parks, 1985). The reversible interaction with DNA leading to the inhibitory effects on macromolecular synthesis requires the presence of a divalent metal ion, Mg^{2+} (Kersten et al., 1966; Goldberg & Friedmann, 1971). Optical and NMR spectroscopy (Ward et al., 1965; Behr et al., 1969; Hayasaka & Inoue, 1969; Nayak et al., 1973, 1975; Shashiprabha, 1979; Berman et al., 1985; Keniry et al., 1987; Kam et al., 1988; Gao & Patel, 1989, 1990; Banville et al., 1990a,b), enzymatic and chemical footprinting (Van Dyke & Dervan, 1983; Fox & Howarth, 1985; Fox & Waring, 1986; Cons & Fox, 1989), and hydrodynamic studies (Behr & Hartmann, 1965; Kersten et al., 1966; Waring, 1970; Cobreros et al., 1982) were carried out to understand the molecular basis of $CHRA_3$–DNA interaction in the presence of Mg^{2+}. Spectrophotometric methods indicated a GC base specificity of the antibiotic; the origin of the base specificity was ascribed to hydrogen bonding between a potential site in the antibiotic and an amino group of the guanine base in GC residues (Goldberg & Friedmann, 1971). Footprinting studies using

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[1] Abbreviations: CD, circular dichroism; $CHRA_3$, chromomycin A_3; FPA, fluorescence polarization anisotropy; MTR, mithramycin; poly(dG-dC), double-stranded alternating copolymer, poly(dG-dC)·poly(dG-dC); poly(dG-me^{5}dC), double-stranded alternating copolymer, poly(dG-me^{5}dC)·poly(dG-me^{5}dC).

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