Interactions at the Nucleic Acid Binding Site of the Avian Retroviral Nucleocapsid Protein: Studies Utilizing the Fluorescent Probe 4,4′-Bis(phenylamino)(1,1′-binaphthalene)-5,5′-disulfonic Acid†

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ABSTRACT: The structural and functional properties of the nucleocapsid (NC) protein of the avian myeloblastosis virus were examined by steady-state fluorescence and fluorescence anisotropy measurements of the complex between the NC and the extrinsic fluorophore 4,4′-bis(phenylamino)(1,1′-binaphthalene)-5,5′-disulfonic acid (bis-ANS). The intrinsic fluorescence of bis-ANS is enhanced many fold upon forming a complex with the NC. Between 2 and 10 molecules of bis-ANS bind strongly to the NC, with an overall K_d of less than 10^{-6} M. The emission of bis-ANS in the complex can also be induced by excitation at 298 nm, indicating that energy is transferred from Trp 80, the sole tryptophan in the NC protein, to bis-ANS. The energy transferred between the Trp 80 and bis-ANS was analyzed to yield a calculated distance of separation between these fluorophores of 28 ± 3 Å; thus, Trp 80 is well removed from the nearest bound bis-ANS. The fluorescence emission of bis-ANS in the NC·bis-ANS complex is efficiently quenched by added salts and by poly(A), suggesting that salt (presumably anions), nucleic acid, and bis-ANS bind to the same, positively charged region on the NC protein. A site size of six nucleotides was determined for nucleic acid binding to the NC protein, with an estimated K_d of less than 10^{-6} M. Salt (anion) binding is strong, but nonspecific, with a K_{app} of 4 mM, raising the possibility that anion binding *to the NC protein* might regulate the interaction of the NC with viral RNA inside the host cell.

The avian myeloblastosis virus (AMV) nucleocapsid protein (NC) is an 88 amino acid phosphoprotein that is synthesized as part of the *gag* precursor polypeptide, $Pr76^{gag}$ (McGinnis et al., 1978; Kerr et al., 1976). The NC protein has been shown to be an essential component of replication competent retroviruses in part because it plays a role in the specific recognition of genomic viral RNA during the early stages of viral assembly (Jentoft et al., 1988; Gorelick et al., 1988; Meric & Spahr, 1986). The NC protein has also been suggested to act as a histone-like protein in packaging viral RNA within the retroviral particle, based on the fact that about 2000 copies of this RNA binding protein are associated with the single-stranded genomic RNA within viral particles (Jentoft et al., 1988; Fleissner, 1971; Davis & Ruecker, 1972).

Retroviral nucleocapsid proteins are characteristically small, hydrophilic, and highly basic (Katz & Jentoft, 1989). Circular dichroism studies of the AMV NC protein (Jentoft et al., 1988) and of the Molony murine leukemia virus NC protein (Roberts et al., 1988) were interpreted to suggest that both proteins contained about one-third β sheet, no α helix, and two-thirds turns and "random" structure. All NC proteins have at least one characteristic sequence motif, called the cys-his region (Cys-X-X-Cys-X-X-X-Gly-His-X-X-X-X-Cys) (Copeland et al., 1983; Sanchez-Pescador et al., 1985). Although this motif is reminiscent of the "zinc finger" proteins of transcriptional activation factors (Berg, 1986), the isolated AMV NC protein does not contain stoichiometric amounts of zinc nor is the RNA binding or the structure of the NC protein altered by added zinc(II) (Jentoft et al., 1988). Most significantly, the zinc(II) content of the AMV virus particle is insignificant relative to the level of nucleocapsid protein within the particle (Jentoft et al., 1988), an observation confirmed for the Molony murine leukemia virus (Gorelick et al., 1988). The structural and/or functional role played by this region is not yet known, but it is known from mutation studies that it is residues within these cys-his regions of the NC protein that are essential for the production of replication-competent virus particles (Jentoft et al., 1988; Méric et al., 1988; Méric & Goff, 1989).

Although some biological roles have been assigned to the NC protein, its biochemical structure–function relationships are still poorly defined. In this report, we extend the characterization of the solution properties of the NC protein from AMV by means of fluorescence spectroscopy. Since the fluorescence of Trp 80, the single tryptophan, of the NC protein was not sensitive to nucleic acid binding or to changes in solution conditions (Fu et al., 1985), an extrinsic fluorophore was sought that would bind to the NC protein and that could then be used to monitor changes in the NC protein when salts were added or nucleic acid was bound. The extrinsic fluorophore 4,4′-bis(phenylamino)(1,1′-binaphthalene)-5,5′-disulfonic acid (bis-ANS) is shown in this report to bind noncovalently to the NC protein with an enhancement of its intrinsic fluorescence. The NC·bis-ANS complex is characterized and evidence is presented that bis-ANS, salt, and nucleic acid bind to overlapping sites on the NC protein.

Materials and Methods

Materials. The extrinsic fluorescent probes bis-ANS, 2-(*p*-toluidinyl)naphthalene-6-sulfonyl chloride (2,6-TNS), 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS), and 2-(*N*-methylanilino)naphthalene-6-sulfonic acid (2,6-MANS) were obtained from Molecular Probes, Inc., Eugene, OR. Other chemicals were of the highest commercial quality.

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Isolation of NC. The NC protein was isolated from pelleted avian myeloblastosis virus as described previously (Johnson et al., 1983; Jentoft et al., 1988). Protein was stored at −70 °C as the lyophilized powder. NC protein was homogeneous as judged by SDS–PAGE and Western blot. Concentrations of the NC protein were measured spectrophotometrically, using an $A^{1\%}$ of 0.84 at 280 nm (Katz et al., 1986).

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. In general, measurements were obtained with an excitation slit width of 3 nm and an emission slit width of 10 nm. A 0.5 × 0.5 cm quartz cuvette with a sample size of ≥250 μL was used for all experiments. The sample temperature was regulated to 27 ± 0.2 °C. Inner filter corrections were applied where appropriate (Hêlêne et al., 1969). Fluorescence was stable with time under all conditions. Fluorescence intensity varied somewhat among preparations of the NC protein; except for the data in Table I, all of the studies reported here utilized a single preparation of NC from pelleted virus. A 1 μM concentration of NC was used for most studies since this provided a reasonable balance between signal intensity and conservation of protein. Titrations were performed without dilution of NC or bis-ANS by including identical concentrations of NC and bis-ANS in both the initial and stock solutions.

The spectra shown in Figure 1 have been corrected for the buffer baseline, which has a peak at ca. 375 nm for excitation at 330 nm and at 330 nm for excitation at 298 nm, due to the Raman band from water. The emission spectra used for determination of quantum yield and for determination of the relative emission intensity (see below) were also corrected for instrument-dependent distortions.

Fluorescence Polarization. A Perkin-Elmer manual polarizer accessory was installed in the excitation and emission path to measure fluorescence polarization (P) (Cantor & Schimmel, 1980). The steady-state polarization was calculated from

$$P = [(I_{\parallel})_V - G(I_{|})_V]/[(I_{\parallel})_V + G(I_{|})_V] \quad (1)$$

where

$$G = (I_{\parallel})_H/(I_{|})_H \quad (2)$$

where the subscripts refer to the vertical (V) and horizontal (H) intensities of the excitation and emission polarizers, respectively, | and ∥ are the perpendicular and parallel components, and G is a wavelength-dependent factor that is used to correct for the effects of the polarization response of the instrument. The excitation and emission wavelengths used for polarization measurements (see footnotes to Table II) are well removed from the 0,0 absorption at 435 nm. Measurements were made in the region of the emission spectrum where there was little wavelength dependence to the polarization data.

Distance Calculation. In the Förster theory of dipole–dipole energy transfer (Lakowicz, 1983; Stryer, 1978), the distance between a fluorescent donor and absorbing acceptor can be estimated from the efficiency of energy transfer. The "Förster distance" R_0 is defined as the distance (Å) at which 50% energy transfer occurs:

$$R_0 = 9.79 \times 10^3(J\kappa^2 Q_d \eta^{-4})^{1/6} \quad (3)$$

where κ^2 is the orientation factor describing the relative orientation of the transition dipoles of the donor and acceptor, Q_d is the quantum yield of the donor, and η is the refractive index of the medium. While κ^2 theoretically ranges from 0 to 4, an average value of $\kappa^2 = {}^2/_3$ accounts for random orientations between the emission and absorption dipoles (Lakowicz, 1983). The refractive index, η, is usually equal to 1.33 for protein media. The spectral overlap integral, J, between the emission spectrum of the donor and the absorption spectrum of the acceptor is described by the following expression:

$$J\ (\mathrm{m^{-1}\ cm^3}) = \int F_d(\lambda)E_a(\lambda)\lambda^4\ d\lambda / \int F_d(\lambda)\ d\lambda \quad (4)$$

where $F_d(\lambda)$ and $E_a(\lambda)$ are the corrected relative emission intensity (%) of the donor and the molar extinction coefficient (M^{-1} cm^{-1}) of the acceptor at each wavelength, respectively, and λ is the wavelength in nanometers.

The actual distance between the donor and acceptor (r) is then calculated as

$$r = [R_0^6(1 - E)/E]^{1/6} \quad (5)$$

where $E = (1 - I/I_0)$ is the efficiency of energy transfer and I and I_0 are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively. Uncertainties in the value of r arise primarily from uncertainties in the value of κ^2. Haas et al. (1976) assumed a value of 2/3 for κ^2 and related the resulting probable uncertainty in r to the rotational motion of the donor and acceptor, estimated from their respective polarization in the complex. The tables of Haas et al. (1976) were used to set the ranges of r in Table II.

Data Analysis. The data from the salt titration of the NC·bis-ANS complex were fit to a hyperbola by using an inverse weighting of the data and a Marquardt gradient–analytical search procedure in iterating to the best parameter fits by least-squares analysis (program BASICFIT, written by K. Neet, personal communication).

Table I: Comparison of the Effect of the NC Protein upon the Intrinsic Fluorescence of Naphthalenesulfonic Acid Derivatives

extrinsic fluorescent probe in presence of NC	fluorescence max		fold enhancement
	excitation (nm)	emission (nm)	
bis-ANS[a]	330	496	5.8
2,6-TNS[a]	330	476	1.7
2,6-ANS[a]	330	470	1.2
2,6-MANS[a]	330	515	1.2
1,8-ANS[b]	330	520	1.2

[a] 20 μM probe. [b] 40 μM probe.

Results

Since the intrinsic fluorescence of the NC protein is insensitive to changes in solution conditions or binding events (Fu et al., 1985), an extrinsic fluorophore was sought that would bind to the NC protein with a resulting enhancement of its fluorescence and, in the complex, serve as a probe for the response of the NC protein to external events.

Identification of bis-ANS as a Probe. A variety of naphthalenesulfonic acid derivatives that have been used as fluorescent probes for studying other proteins were chosen as candidate probes for this study. These included bis-ANS, 2-(*p*-toluidinyl)naphthalene-6-sulfonyl chloride (2,6-TNS, 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS), and 2-(*N*-methylanilino)naphthalene-6-sulfonic acid (2,6-MANS). The effect of added NC protein (to a final concentration of ca. 1 μM) upon the fluorescence emission spectra of a 20 or 40 μM solution of each fluorophore was measured and the results summarized in Table I. As shown in Table I, a 6-fold enhancement was observed for the emission spectrum of the bis-ANS in the presence of added NC protein, while less than

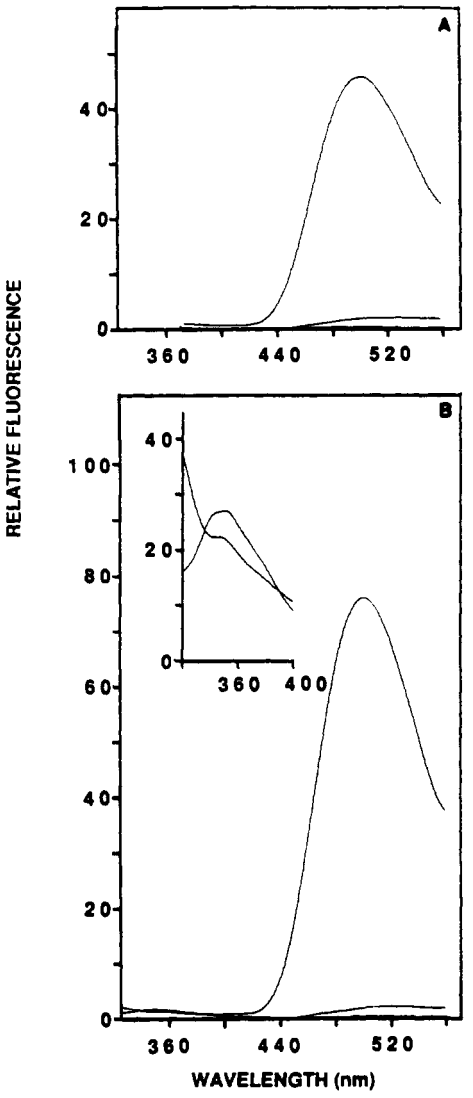

FIGURE 1: Emission spectra of bis-ANS, the NC protein, and the NC·bis-ANS complex in 40 mM MES at pH 5.5. All spectra are corrected for the buffer baseline. Panel A: Emission spectra obtained from excitation of the intrinsic bis-ANS fluorescence at 330 nm (scale factor of 1). The lower spectrum shows the intrinsic fluorescence spectrum of a solution containing 3 μM bis-ANS. The upper spectrum, obtained from a solution 3 μM in bis-ANS and 1 μM in NC protein, shows the large enhancement and blue shift in the emission maximum induced by the addition of NC. The emission spectrum of a 1 μM solution of the NC protein excited at 330 nm is indistinguishable from the baseline. Panel B: Emission spectra obtained from excitation at 298 nm of Trp 80 in NC (scale factor of 1). The emission of a 1 μM solution of the NC protein (scale factor of 1) shows a maximum at 360 nm and is barely distinguishable from baseline above 440 nm. The inset shows the intrinsic fluoescence of Trp 80 in the NC protein for a 1 μM solution at a scale factor of 10 (symmetrical peak centered at 360 nm). The emission of a solution containing 1 μM NC and 3 μM bis-ANS shows a decreased intensity at 360 nm (inset) and the existence of a second, intense emission at 496 nm, indicating that fluorescence energy has been transferred from the NC to bis-ANS. The emission of 3 μM bis-ANS excited at 298 nm gives rise to the lower emission spectrum with a broad maximum near 510 nm. All measurements were made by using standard conditions as defined in Materials and Methods.

2-fold enhancement was observed under similar conditions for the other naphthalenesulfonic acid derivatives. As a result of these observations, further investigations were limited to the interaction between bis-ANS and the NC protein, at reduced concentrations of fluorophore.

Fluorescence Spectra of the NC·bis-ANS Complex. The intrinsic fluorescence emission (λ_{ex} of 330 nm) of a 3 μM solution of bis-ANS in aqueous solution is of very low intensity (Figure 1A, lower spectrum). At higher concentrations it can be seen that the emission maximum is centered at 512 nm (data not shown). Upon making the solution 1 μM in NC protein, a 30-fold increase in the emission intensity and a blue shift in the emission maximum were seen (Figure 1A, upper spectrum). This increase in intensity and shift in emission maximum to lower wavelength suggest that bis-ANS interacts with the NC protein and that it binds to a region of reduced polarity relative to the solvent (40 mM MES).

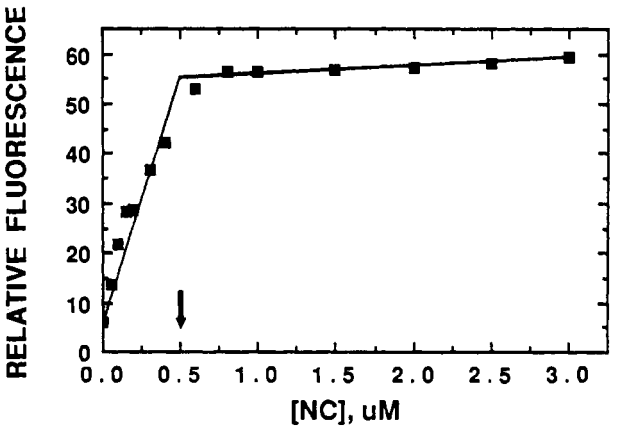

FIGURE 2: Change in relative fluorescence intensity of the intrinsic fluorescence (excitation at 385 nm, emission at 496 nm) of 5 μM bis-ANS as a function of added NC protein. The buffer was 40 mM MES at pH 5.5.

The NC protein contains only three aromatic amino acids, two tyrosines and a single tryptophan, Trp 80 (Jentoft et al., 1988). In the present study an excitation wavelength of 298 nm was used to maximize the contribution to the emission from Trp 80. The fluorescence emission spectrum of a mixture of 1 μM NC and 3 μM bis-ANS is shown in Figure 1B. The major feature is an intense emission band with a maximum at 496 nm, which arises from energy transferred from Trp 80 to the bound bis-ANS. The much less intense emission maximum from Trp 80, at 360 nm, is shown at a 10-fold higher scale factor in the inset to Figure 1B (asymmetrical spectrum). In contrast, the intrinsic fluorescence spectrum for 1 μM NC has no emission above background at 498 nm but, as indicated in the insert to Figure 1B, has a relatively low intensity, symmetrical emission centered at 360 nm. The spectrum for 3 μM bis-ANS alone has as its sole feature a weak emission around 500 nm (relative fluorescence below 5 in Figure 1B). Reducing the emission slit width had no effect on the width of emission band centered at 496 nm (data not shown).

NC Binding to bis-ANS. The change in the relative fluorescence emission of bis-ANS was used to monitor the interaction between bis-ANS and the NC protein. As shown in Figure 2, the relative fluorescence of a solution at pH 5.5 containing 5 μM bis-ANS increases as a function of added NC protein. Since the data show a biphasic rather than hyperbolic dependence on NC, the NC concentration is presumed to be larger than the K_d. The number of bis-ANS molecules bound per molecule of NC can be estimated from the equivalence point, which occurs at a concentration of 0.5 μM NC, as indicated in Figure 2. Thus, as many as 10 bis-ANS molecules bind to each NC.

The stoichiometry of the NC·bis-ANS complex was also investigated by the method of continuous variation. In this experrment, the independent variable was the relative fluorescence emission at 496 nm excited at 298 nm, while the

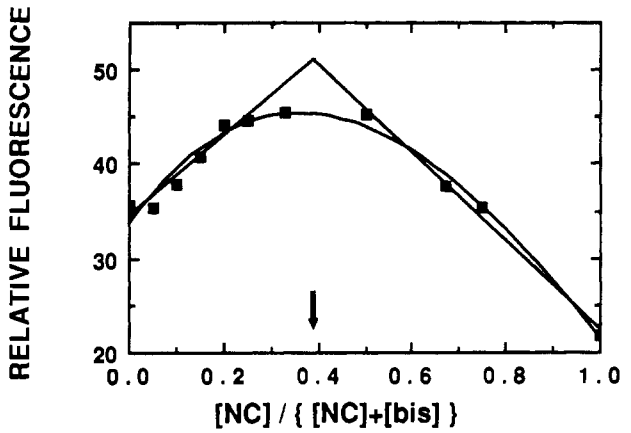

FIGURE 3: Job's plot of continuous variation for the NC protein and bis-ANS. Fluorescence emission at 496 nm as a result of excitation at 298 nm was monitored in 40 mM MES, pH 5.5, as a function of the changing mole fraction of the NC protein, at a constant total molar concentration of the two components of 2 μM. The extensions of the sloping portions of the curve (derived from a linear least-squares fit) intersect at a mole fraction of NC of 0.38, corresponding to a NC-(bis-ANS)$_2$ complex.

dependent variable was the mole fraction of NC at a constant (2 μM) total concentration of NC plus bis-ANS. The results are shown in a Job's plot (Job, 1928) in Figure 3. The maximum fluorescence intensity occurs over a broad range, between a mole fraction of 0.2 and 0.5, with the extrapolated maximum occurring at 0.38, as shown. The maximum corresponds to a ratio of NC to bis-ANS of 1:2, and the range covers ratios of 1:4 to 1:1.

Taken together the data in Figures 2 and 3 indicate that the NC protein has multiple sites for bis-ANS, with the various methods indicating that between 2 and 10 bis-ANS molecules can bind to the NC protein.

Distance between Trp 80 and bis-ANS. The emission band of Trp 80 in the NC protein overlaps the excitation band of the bis-ANS, which is centered at 330 nm (see Figure 1). As a result of this spectral overlap, energy is transferred from Trp 80 to bound bis-ANS. The relative efficiency of this energy transfer is dependent upon the distance of separation between the two fluorophores (eq 5) (Stryer, 1978). The parameters in eqs 3–5 were utilized to calculate the distance between Trp 80 and the closest bound bis-ANS for three different molar ratios of NC and bis-ANS (1:1, 1:3, and 1:10). The experimental values of the parameters and the resulting distances are given in Table II. The distances calculated for the three solution conditions were the same, viz., 28 Å, and the extreme range of the distances estimated by the treatment of Haas et al. (1976) is ±10% or ±3 Å.

Modulation of the Interaction. To see if the NC-induced enhanced fluorescence of the bis-ANS could be used as a probe for studying the conformation of, and ligand binding to, the NC protein, the intensity of the fluorescence emission at 496 nm from the NC·bis-ANS complex was monitored as a function of addition of salt and addition of nucleic acid, respectively.

Salt Effect. NaCl proved to be an unusually effective quencher of the fluorescence emission of the NC·bis-ANS complex, as shown in Figure 4. Emission at 496 nm is quenched completely at concentrations of NaCl above 20 mM. The K_{app} for NaCl is about 4 mM, indicating an uncharacteristically high affinity for ions by the NC protein (Record et al., 1978).

The ability of NaCl to efficiently quench the bis-ANS fluorescence in the NC·bis-ANS complex prompted a further

Table II: Parameters Determined for the Calculation of the Distance of Separation between Trp 80 and bis-ANS

parameter	NC·bis-ANS complex[a] 1:1	1:3	1:10
quantum yield of donor[b]	0.015	0.015	0.015
I/I_0[c]	0.926	0.911	0.909
J factor, M^{-1} cm^3	1.95×10^{-14}	1.95×10^{-14}	1.95×10^{-14}
R_0, Å	18.8	18.8	18.8
efficiency of transfer	0.074	0.089	0.091
r, Å	28.7	27.7	27.6
polarization of donor[d]	0.086	0.086	0.086
polarization of acceptor[e]	0.045	0.043	0.042
probable error in r, %	2	2	2
extreme limits of r, %	10	10	10

[a] All energy transfer data were obtained at an excitation wavelength of 298 nm in solutions containing 1 μM NC and 40 mM MES, pH 5.5, at 27 °C. [b] The quantum yield was obtained by integrating the corrected emission spectrum of the NC from 310 to 460 nm and comparing it to the integration from 295 to 460 nm of the corrected emission spectrum of *N*-acetyl-L-tryptophanamide (excitation wavelength 280 nm) at pH 7 in 50 mM Hepes. The quantum yield was determined from the concentration-normalized ratio of integrated intensities, using the known quantum yield of *N*-acetyl-L-tryptophanamide of 0.12 (Börresen, 1967). [c] The ratio of corrected fluorescence intensities was measured at 340 nm for NC in the presence and absence of bis-ANS. [d] Polarization was measured for a 3 μM solution of NC in 40 mM MES, pH 5.5, at 27 °C, using an excitation wavelength of 295 nm and an emission wavelength of 360 nm. [e] Polarization was measured for solutions containing 1 μM NC and 1, 3, and 10 μM bis-ANS, respectively, in 40 mM MES, pH 5.5, at 27 °C, using an excitation wavelength of 395 nm and an emission wavelength of 510 nm.

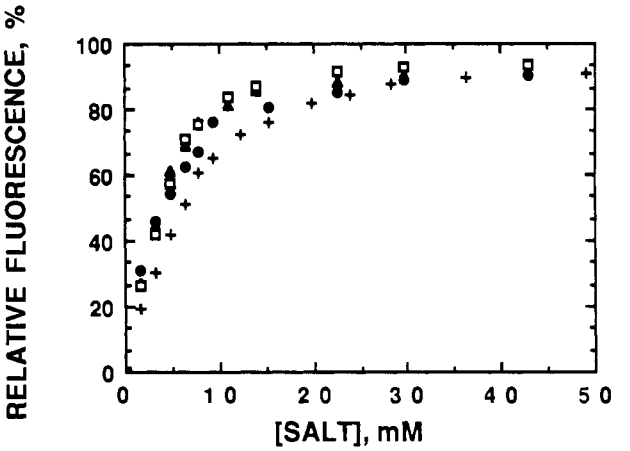

FIGURE 4: Quenching of bis-ANS fluorescence (excited at 298 nm) of the NC·bis-ANS complex by salts. Solutions containing 1 μM NC and 2 μM bis-ANS in 40 mM MES, pH 5.5, were titrated separately with a 400 mM stock solution of the respective salt: (▲) NaCl, (□) Na_2HPO_4, (●) KCl, and (+) potassium glutamate. The K_{app} for NaCl, Na_2HPO_4, and KCl is 4 mM, and that for potassium glutamate is 6 mM.

investigation. To determine if the quenching observed with NaCl was due to a cation- or anion-specific interaction with the NC protein, the K_{app} was determined for the sodium salts of SO_4^{2-}, HPO_4^{2-}, acetate, Cl^-, NO_3^-, and I^- (arranged from most efficient to least efficient in salting-out efficiency). All K_{app} values were the same (4 mM) within the estimated error of the measurement (±1 mM). The relative ability of cations (as the Cl^- salt) to displace bis-ANS from the NC was also examined for Mg^{2+}, NH_4^+, Na^+, and K^+. Again, no dependence on cation was detected, and the K_{app} for all salts was 4 mM. The K_{app} for potassium glutamate was also determined since it has been suggested to be the predominant intracellular salt (Richey et al., 1987), and its K_{app} was found to be 6 mM, a value only slightly larger than that of the other salts. Representative titration data are shown in Figure 4. Note that

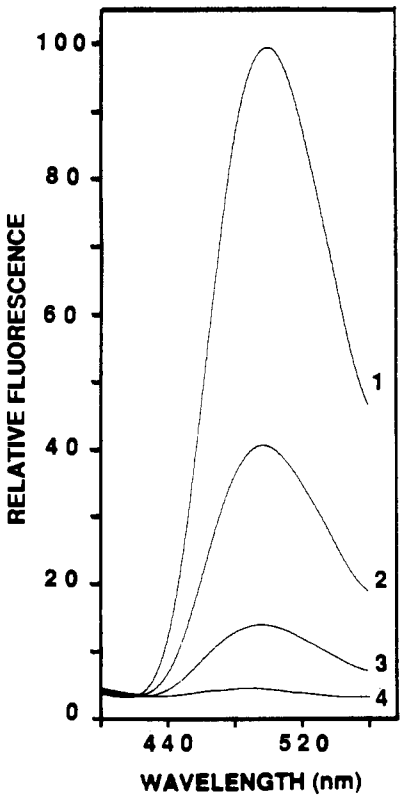

FIGURE 5: Quenching of the bis-ANS emission (excited at 298 nm) of the NC·bis-ANS complex by the addition of poly(A) in 40 mM MES, pH 5.5. The fluorescence emission at 496 nm arising from energy transferred from Trp 80 to bis-ANS is quenched. Concentrations of NC (1 μM) and bis-ANS (2 μM) were kept constant, and poly(A) additions were made from a stock solution containing 200 μM poly(A) bases. Scans 1–4 represent the following poly(A) concentrations (μM): 0, 1.6, 3.2, and 27.6.

all measurements were made in the presence of 40 mM MES buffer; decreasing the buffer concentration to 20 mM had no effect on the K_{app} for NaCl, while increasing the MES concentration to 80 mM increased the K_{app} from 4 to 7 mM.

Nucleic Acid Binding. The effect of added poly(A) upon the fluorescence intensity of the energy transfer band of the NC·bis-ANS complex is shown in Figure 5 and the dependence of quenching upon the concentration of poly(A) bases is plotted in Figure 6. These figures show that the emission band centered at 496 nm is quenched progressively upon successive additions of poly(A) until the molar ratio of NC to poly(A) bases is 6, at which point the fluorescence is completely quenched. These results suggest that the decrease in the bis-ANS emission can be used as an indirect probe to monitor nucleic acid binding. The plot in Figure 6 clearly shows that poly(A) binds tightly to the NC at the micromolar concentrations of protein and poly(A) used in these studies, indicating that the apparent dissociation constant for the interaction is $<10^{-6}$ M. This equivalence point provides an estimate of the site size of the NC protein. The site size of six is consistent with the value of 5 ± 1 determined in previous studies by using the nucleic acid probe poly(ethenoadenylic acid) (Jentoft et al., 1988; Karpel et al., 1987).

Discussion

In this study, we have demonstrated that bis-ANS can be used as an extrinsic fluorescence probe for monitoring a variety of interactions of the NC protein.

The NC protein was shown to have between 2 and 10 binding sites for bis-ANS. Multiple binding sites for bis-ANS have been reported for other proteins such as RNA polymerase

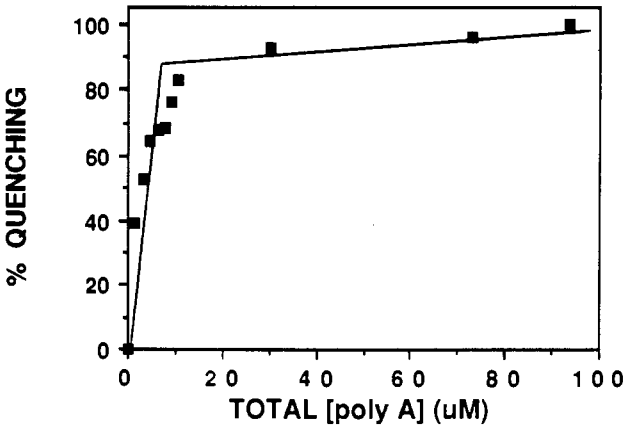

FIGURE 6: Plot of the quenching of the bis-ANS fluorescence in the NC·bis-ANS complex by poly(A) (representative data shown in Figure 5).

(Wu & Wu, 1978), *E. coli* lac repressor (Worah et al., 1978), and tubulin (Prasad et al., 1986).

The efficiency of energy transfer from the NC Trp 80 to the bis-ANS is reflected in the intensity of the emission at 496 following excitation at 298 nm. Consequently, events that give rise to changes in this energy-transfer emission are those that perturb Trp 80 or bis-ANS to alter their relative orientation and/or separation. In contrast, events that affect only bis-ANS are observed at 496 nm subsequent to excitation at 330 nm. Either measurement will provide a means of determining if the NC·bis-ANS complex changes with alterations in solution conditions, but insight into the nature of the change can be obtained by comparing the results obtained for excitation at both wavelengths. Thus, e.g., a conformational change that perturbed either Trp 80 or bis-ANS would give rise to differential changes in the intensity of the emitted fluorescence after excitation at 298 nm versus that at 330 nm. On the other hand, changes in the amount of bis-ANS bound to the NC protein would give rise to parallel decreases in fluorescence intensity after excitation at the two wavelengths. We found that either added salt or added poly(A) resulted in equivalent and, ultimately, complete quenching of bis-ANS fluorescence for both excitation wavelengths. This indicates that quenching is not caused by, e.g., movement of the NC tryptophan relative to the bound bis-ANS, but is more likely the result of the displacement of bis-ANS from the NC protein.

The detailed characterization of the interaction of the NC protein with RNA is of great interest since the NC protein is associated with viral RNA within retroviral particles. The advantages of monitoring the interaction by a protein-based probe include the opportunity to study an unrestricted variety of nucleic acids and the potential to detect binding-induced changes in the protein.

The data presented in Figures 5 and 6 suggest that bis-ANS and nucleic acid bind to the same region of the NC protein. This is supported by reports that bis-ANS has a particular affinity for proteins that have nucleotide binding sites (Worah et al., 1978; Wu & Wu, 1978; Prasad et al., 1986; Lin et al., 1983; Holler et al., 1971). The tight binding between the NC protein and poly(A), as measured by the displacement of bis-ANS, is consistent with previous observations, as is the site size of 6 (Jentoft et al., 1988). These results suggest that the fluorescence of bis-ANS in the NC·bis-ANS complex can be used to monitor the NC–nucleic acid interaction, although the strong interaction of anions with the NC protein may limit the investigation of salt dependence of nucleic acid binding. Since the intrinsic fluorescence of the NC protein is insensitive

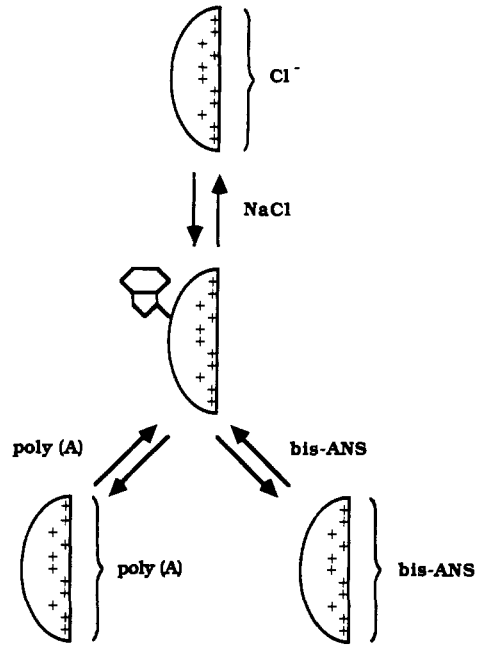

FIGURE 7: Model summarizing the interactions of bis-ANS, salt, and poly(A) with the NC protein. The half ovoid represents the NC protein and the flat, positively charged side represents its binding region. Trp 80 is shown on unliganded NC opposite the binding region, consistent with the determination of a separation >20 Å between the tryptophan and the bis-ANS binding region. The binding region is shown to interact with anions, bis-ANS, and poly(A), consistent with the observation that both salt and poly(A) competitively displace bis-ANS from the NC protein.

to nucleic acid binding, the NC·bis-ANS complex provides a protein-based probe for monitoring nucleic acid binding to the AMV NC protein.

The effect of added salt was not expected to modulate the fluorescence emission of the NC·bis-ANS complex; rather this experiment was viewed initially as a control for the subsequent study of salt effects on the NC–nucleic acid interaction. Unexpectedly, a variety of salts were shown to compete for the bis-ANS binding sites on the NC, without any evidence of specificity. (Since salt binds at concentrations at least 10-fold lower than that of the MES buffer, the 2-(*N*-morpholino)ethanesulfonate anion is presumed to be a relatively noninteracting anion.) Since NC has a calculated pI >12, it is reasonable to assume that the anions interact with the protein. The high affinity of the anions for the NC is unusual, although within the range of reported K_d values for anion–protein interactions (Record et al., 1978). The results of these binding experiments suggest that the strongly basic NC protein contains nonspecific, high-affinity anion binding sites that overlap bis-ANS and nucleic acid binding sites. It should be noted that bis-ANS contains two anionic sulfonic acid groups; the tendency for bis-ANS to bind to proteins that interact with nucleic acid binding proteins could be due in part to its anionic nature.

Further insight into the strong binding of anions by the NC protein is provided by examination of the anion binding properties of ribonuclease (Saroff & Carrol, 1962; Loeb & Saroff, 1964). In ribonuclease clusters of three or more basic residues were found to form effective binding sites for anions. These clusters occurred only near the active site in ribonuclease (Richards & Wyckoff, 1971). Interestingly, the NC protein contains a cluster of four and a cluster of three basic residues along its primary sequence (Lys_{36}-Lys-Arg-Lys, Arg_{61}-Lys-Arg), and it is assumed that the nucleic acid binding site, which can accomodate six nucleic acids (Jentoft et al., 1988), is lined with many of the 5 lysines and 11 arginines, including those in these clusters (Jentoft et al., 1988; Fu et al., 1985). The lack of anion specificity (but not the lack of charge dependence) noted for the NC protein may result from the binding sites being formed by flexible lysine and arginine side chains.

The high affinity of the NC protein for anions has biological implications, since intracellular salt concentrations are at higher levels than that required to saturate the sites on free NC. This raises the possibility that, after fusion of a retrovirus particle with the target cell membrane, dissociation of the NC–RNA complex found in the core may be facilitated or regulated by the interaction of cellular anions with the NC protein.

The intrinsic fluorescence of the NC protein, which would be the fluorescence probe of choice, has been shown to be relatively insensitive to quenchers or to added nucleic acids in the AMV NC protein (Fu et al., 1985). The quantum yield of 0.015 that was determined for Trp 80 in the NC protein is less than 10% of the maximal value quoted for tryptophans in proteins (Cantor & Schimmel, 1980). A low quantum yield might be expected if Trp 80 had a high degree of solvent exposure; in fact, the low value for the polarization of Trp 80 (Table II), as well as NMR chemical shifts (data not shown) and fluorescent quenching experiments (Craig Gelfand, private communication), are all consistent with Trp 80 being primarily solvent exposed. Since bis-ANS and nucleic acid bind to the same region on the NC protein, we can now provide a structural explanation for the insensitivity of the Trp 80 fluorescence to nucleic acid binding, specifically, that the Trp 80 is more than 20 Å from the nucleic acid binding site.

These interactions of the NC protein with bis-ANS, salts, and nucleic acids are summarized by the model shown in Figure 7. The model is supported by (1) the basic nature of the NC protein, (2) the anionic nature of salt, bis-ANS, and nucleic acid, and (3) the ability of both salt and poly(A) to completely displace bis-ANS from the NC. As the model indicates, the same extended basic region on the NC protein is proposed to interact with bis-ANS, anions, and nucleic acids, although the precise limits of the site may vary from ligand to ligand. On the basis of the studies to date, this site is large enough to accomodate five or six nucleic acids, up to 10 dianionic bis-ANS molecules, and an unknown number of anions. Note that Trp 80 is placed far away from the binding site, consistent with its >25-Å separation from the closest bis-ANS.

This study, in placing Trp 80 more than 20 Å from the nucleic acid binding site, provides the first spatial information for the NC protein.

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An Inverse Face-Centered Cubic Phase Formed by Diacylglycerol–Phosphatidylcholine Mixtures†

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ABSTRACT: Fully hydrated unsaturated diacylglycerol–phosphatidylcholine mixtures are found to adopt an inverse face-centered cubic phase, of crystallographic cubic aspect 15. The same behavior is observed for either the 1,2- or 1,3-isomer of the diacylglycerol. This Q_{15} cubic phase, of probable space group *Fd3m* (Q^{227}), occurs between an inverse hexagonal (H_{II}) phase and an inverse micellar (L_2) solution, with increasing diacylglycerol concentration, which implies that the mean curvature of the interface is more negative than that of the H_{II} phase. This behavior is quite different from that of the more usual bicontinuous inverse cubic phases *Pn3m* (Q^{224}), *Im3m* (Q^{229}), and *Ia3d* (Q^{230}), which normally occur between the lamellar L_α and the H_{II} phases. One possible structure for the *Fd3m* cubic phase has recently been proposed (Mariani, P., Luzzati, V., & Delacroix, H. (1988) *J. Mol. Biol. 204*, 165–189), consisting of tetrahedrally arranged clusters of inverse micelles surrounded by a continuous cage of tetrahedrally connected water/lipid (inverse) channels.

The effect of diacylglycerols (DG)[1] on membrane structure is currently a topic of great interest because of their role in signal transduction in cells. In response to stimulation of various membrane receptors, diacylglycerols are formed from phosphatidylinositols, and they then activate the membrane-bound enzyme protein kinase C (Berridge, 1984; Berridge & Irvine, 1984; Nishizuka, 1983, 1986). In addition, diacylglycerols enhance the hydrolysis by phospholipases C and A_2 of phospholipids such as PC (Dawson et al., 1983). This was found to be correlated with the appearance of an isotropic component in the ^{31}P NMR spectrum, which appeared when the amount of incorporated DG exceeded 15 mol % (Dawson et al., 1984). Further addition of DG caused the appearance of a hexagonal-type line shape, and by 50 mol % DG the

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[1] Abbreviations: PC, phosphatidylcholine; egg PC, phosphatidylcholine from egg yolk; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DG, 1,2- or 1,3-diacylglycerol; egg DG, 1,2-diacylglycerol derived from egg PC; 1,2-DOG, 1,2-dioleoylglycerol; 1,3-DOG, 1,3-dioleoylglycerol; c_{DOG}, weight fraction of dioleoylglycerol; L_1, normal micellar solution; L_2, inverse micellar solution; L_α, fluid lamellar (bilayer) phase; H_{II}, inverse hexagonal phase; *Fd3m* (Q^{227}), cubic phase of crystallographic space group number 227.

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