Inhibition of Viral Capsid Assembly by 1,1′-Bi(4-anilinonaphthalene-5-sulfonic Acid)†

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Received May 7, 1993; Revised Manuscript Received July 21, 1993®

ABSTRACT: The precursor shells of dsDNA bacteriophages are assembled by the polymerization of competent states of coat and scaffolding subunits. The fluorescent dye 1,1′-bi(4-anilinonaphthalene-5-sulfonic acid) (bisANS) binds to both the coat and scaffolding proteins from the *Salmonella typhimurium* bacteriophage P22. It displays little affinity for the polymerized forms of the proteins. The subunits with bound bisANS are incapable of assembling into procapsids. The binding constants of bisANS for both coat and scaffolding protein monomers have been measured and are 7 and 6 μM, respectively. Binding of bisANS to coat protein has little effect on the conformation as determined by circular dichroism and susceptibility to proteolysis. Binding of bisANS to scaffolding protein induces a change in the secondary structure consistent with a loss of α-helix, and an altered susceptibility to proteolysis. We suggest that the bisANS is probably binding at sites responsible for intersubunit interactions and thereby inhibiting capsid assembly.

The formation of the capsids of all known viruses involves the polymerization of hundreds of protein subunits into structures large with respect to the subunit dimensions. The subunit interactions required for successful assembly are very specific and rarely use host proteins or structures as the basis for the viral capsid structure. Where multiple protein species are involved, these must be precisely located for the formation of closed biologically active shells (Kellenberger, 1969; Casjens & King, 1975).

In the dsDNA-containing bacteriophage, as well as the herpesviridae, a proteinaceous precursor shell is formed into which the nucleic acid is subsequently packaged [reviewed in Casjens and Hendrix (1987)]. In this case, the direct product of protein subunit polymerization is a procapsid shell in which an inner core of scaffolding protein is surrounded by the outer shell of coat protein. During assembly, a single vertex is differentiated from the other 11 by virtue of the addition of an oligomer of a portal protein. The viral DNA is packaged into the procapsid through this portal vertex. Removal of the scaffolding protein, either by proteolysis or by direct exit, precedes DNA packaging.

A rational target for the design of antiviral agents would be the capsid subunits prior to polymerization into capsids. The specificity of subunit/subunit interactions required for viral assembly suggests that agents which inhibit these processes would be quite specific for the viral proteins and not interfere with cellular processes. However, in part because of the limited characterization of the *in vivo* polymerization processes in animal viruses, this avenue has not been systematically explored. In the dsDNA phages, procapsids can be assembled *in vitro* from purified precursor subunits. Such *in vitro* capsid assembly reactions have been particularly well characterized for the dsDNA bacteriophage P22. In an effort to find polymerization inhibitors, we examined the effect of small hydrophobic dyes on the polymerization of capsid subunits *in vitro*.

Salmonella bacteriophage P22 is a double-stranded DNA-containing bacteriophage whose capsid is composed of 420 identical 47-kDa coat protein molecules arranged in a $T = 7$ lattice (Casjens, 1979; Prasad *et al.*, 1993). P22 assembly proceeds through a mechanism requiring the copolymerization of the coat protein with approximately 300 molecules of a 33-kDa scaffolding protein (Figure 1) (Prevelige *et al.*,1989; Fuller & King, 1980; King & Casjens, 1974; King *et al.*, 1973). Unlike many other viruses, there is no proteolytic processing or covalent modification during the assembly process. The scaffolding protein is contained internally with protein density extending from a radius of 50 Å to nearly the inner edge of the coat protein lattice (215 Å) (Earnshaw *et al.*,1976). The portal vertex is marked by the presence of 12 molecules of the 90-kDa gene 1 encoded portal protein (Bazinet *et al.*,1988).

The viral DNA is replicated as a concatamer. Specific pac sites on the DNA are recognized by the protein products of the phage-encoded genes 2 and 3, and the DNA is packaged by a headful mechanism through the portal vertex. Coupled to the DNA packaging is the exit of scaffolding protein without proteolytic cleavage, an expansion of the capsid lattice resulting in a substantial increase in internal volume, and the closing of holes in the lattice (King & Casjens, 1974; Prasad *et al.*,1993). Following DNA packaging, the portal vertex is closed by the addition of the protein products of genes 4, 10, and 26 (Strauss & King, 1984). Binding of the tailspike protein to the portal vertex renders the phage infectious.

The critical role of the scaffolding protein in directing the assembly process has been demonstrated *in vivo* through the use of mutants unable to synthesize functional scaffolding protein (King *et al.*,1973; Lenk *et al.*,1975; Earnshaw & King, 1978). In these mutants, the coat protein remains unassembled until a high intracellular concentration has been reached. At that point, assembly proceeds, but the product is aberrant polymeric forms such as spirals and small shells (Earnshaw & King, 1978). A similar process occurs *in vitro*, and Raman studies have demonstrated that the coat protein subunits in these polymers have a largely native conformation (Prevelige *et al.*,1990).

† Supported by NIH Grants GM17980 (J.K.) and GM47980 (P.E.P.).
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® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

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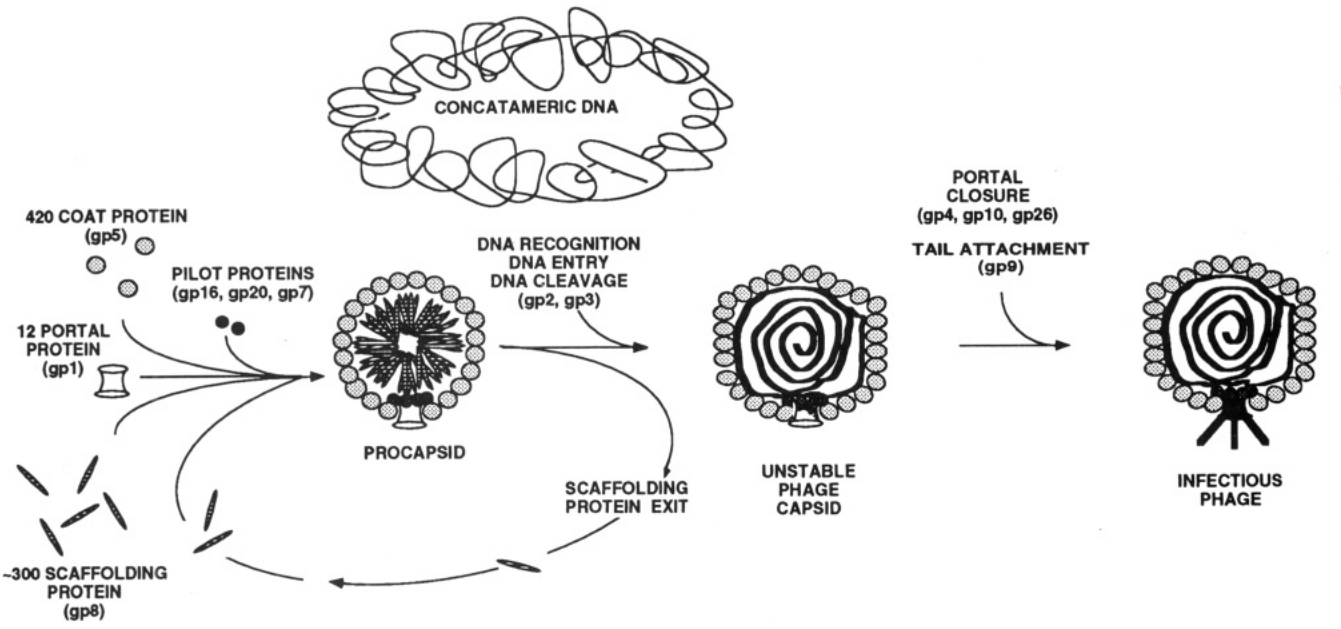

FIGURE 1: Assembly pathway of bacteriophage P22 *in vivo*. The assembly of bacteriophage P22 *in vivo* requires the copolymerization of 420 molecules of the gene 5 encoded coat protein (47 kDa), ~300 molecules of the gene 8 encoded scaffolding protein (33 kDa), 12 molecules of the gene 1 encoded portal protein (90 kDa), and between 12 and 20 molecules each of the products of genes 16 and 20. The $T = 7$ procapsid has a diameter of 580 Å. The DNA is replicated as a concatamer and packaged in a processive headful mechanism through the actions of the products of genes 2 and 3. As the DNA is packaged through the portal channel, the scaffolding protein exits the shell to participate in further rounds of assembly, and the capsid lattice increases in diameter to 630 Å. The portal channel is closed by the binding of the products of genes 4, 10, and 26. Binding of up to six trimers of the gene 9 encoded tailspike protein renders the phage infectious.

The coat and scaffolding proteins can be purified from the procapsid of the *Salmonella* bacteriophage P22 in a biologically active form (Fuller & King, 1980, 1981; Prevelige *et al.*,1988). When mixed together, these subunits rapidly and efficiently assemble into procapsid-like particles (Prevelige *et al.*,1988). Kinetic analysis of the assembly reaction *in vitro* has demonstrated that it displays the behavior of a nucleation-limited polymerization, with a pentamer of coat protein a likely component of the nucleus (Prevelige *et al.*,1993). The procapsid is stabilized by intersubunit hydrophobic interactions (Fuller & King, 1980; Prevelige and Silva, manuscript in preparation). In this paper, we report on the inhibition of procapsid assembly by the hydrophobic dye 1,1′-bi(4-anilinonaphthalene-5-sulfonic acid) (bisANS).

MATERIALS AND METHODS

Chemicals and Supplies. BisANS was purchased from Molecular Probes, guanidine hydrochloride from Fisher Scientific, and acrylamide from Bio-Rad. Centricon 30 concentrators were from Amicon. Chymotrypsin–agarose was purchased from Sigma. All other chemicals were reagent grade.

Buffers. The buffer used in all experiments with the exception of the circular dichroism (CD)[1] experiments was 50 mM Tris, 25 mM NaCl, and 2 mM EDTA, pH 7.6. The buffer used in the CD experiments was 20 mM sodium phosphate, pH 7.6. The phosphate buffer was calculated to have approximately the same ionic strength as the Tris buffer.

Preparation of Procapsids, Shells, and Coat and Scaffolding Proteins. Procapsids were prepared as described previously (Prevelige *et al.*,1988). Briefly, *Salmonella typhimurium* cells were infected with bacteriophage P22 mutants which could not package DNA. The infected cells accumulate procapsids intracellularly. Cells were harvested by centrifugation and lysed by repeated cycles of freezing and thawing, and the procapsids were purified by differential centrifugation. The scaffolding protein and portal protein were extracted by repeated treatment with 0.5 M GuHCl and further purified by chromatography on a DEAE column. The shells of coat were purified by size-exclusion chromatography in 0.5 M GuHCl.

Monomeric coat protein was prepared by dissociation of shells and denaturation of the coat protein in 3 M GuHCl. The protein was purified by size-exclusion chromatography in 2 M GuHCl, and was refolded by dialysis from GuHCl at 4 °C.

Fluorescence Methods. All fluorescence measurements were performed with a Hitachi F4500 spectrofluorometer thermostated at 25 °C and interfaced with a personal computer. The excitation slit was set to 2.5 nm and the emission slit to 5 nm. The PMT voltage was set at 700 V. Light scattering was determined by setting both the excitation and emission wavelengths to 500 nm, the slit widths to 5 nm, and the PMT voltage to 400 V.

The binding of bisANS to scaffolding protein was measured as described in Teschke and King (1993). Briefly, a concentrated solution of bisANS was made in buffer and the concentration determined by the absorbance at 385 nm using an extinction coefficient of 16 790 L mol^{-1} cm^{-1} (Farris *et al.*, 1978). Scaffolding protein (25 μg/mL, 750 nM) was incubated in increasing concentrations of bisANS at room temperature for ~1 h. The emission intensity of bisANS was measured using $\lambda_{excit} = 400$ nm and $\lambda_{emit} = 490$ nm. The quenching of the tryptophan by nonradiative energy transfer was monitored at 340 nm when the solution was excited at 280 nm. In all cases the fluorescence was corrected for the inner filter effect by the formula $F_{corrected} = F_{observed}$ antilog-$[(OD_{ex} + OD_{em})/2]$ where OD_{ex} and OD_{em} are the optical densities at the excitation and emission wavelengths, respectively (Lakowicz, 1983). The data were analyzed by the method of Webb (1963) as described by Bagshaw and Harris for direct fluorescence enhancement or by the modified Stern–Volmer equation for tryptophan quenching (Bagshaw &

[1] Abbreviations: CD, circular dichroism; GuHCl, guanidine hydrochloride.

Harris, 1987; Eftink & Ghiron, 1981; Prasad *et al.*, 1986). The maximum fluorescence enhancement was determined by a plot of $1/F$ against $1/[\text{bisANS}]$ where the y intercept is the maximum fluorescence. The analysis of binding according to Webb (1963) does not require that the bisANS be present in great excess over the number of binding sites. All linear data were analyzed using linear least-squares analysis.

The binding of bisANS to procapsids, shells, and coat protein subunits was monitored as follows. Procapsids and shells (10 μg/mL in coat protein, 210 nM) or coat protein subunits (10 μg/mL, 210 nM) were added to 2 μM bisANS and incubated at room temperature for 30 min. Fluorescence emission spectra upon binding of bisANS to the particles were measured at an excitation wavelength of 400 nm.

Extraction of Scaffolding Protein from Procapsids with BisANS. Procapsids ($\sim$1 mg/mL, 30 nM) were incubated in the presence or absence of 60 μM bisANS for 2 h at room temperature; 200 μL of each sample was layered on top of a 5–20% (w/v) sucrose gradient. The gradient to which the sample incubated in the presence of bisANS was applied contained 60 μM bisANS throughout the gradient. The gradients were centrifuged at 35 000 rpm in an SW50.1 rotor at 20 °C for 35 min. Following centrifugation, they were fractionated and analyzed on 10% SDS–polyacrylamide gels.

The kinetics of extraction were monitored by the increase in the fluorescence of bisANS at 495 nm and by the decrease in light scattering at 500 nm as described above. Enhancement of bisANS fluorescence monitored the accessibility of binding sites, and the decrease in light scattering monitored, the extraction of scaffolding from procapsids. The relaxation time of these processes was determined with a computer using a first-order rate equation with two exponentials.

Assembly of Procapsids in Vitro. The assembly of procapsids *in vitro* was done as described in Prevelige *et al.*, (1988, 1993). Assembly reactions were monitored by turbidity in a Gilford Response spectrophotometer thermostated at 20 °C. Coat protein at $\sim$1 mg/mL (21 μM) was incubated in spectrophotometer cuvettes with increasing concentrations of bisANS, prepared as described under *Fluorescence Methods.* Approximately one part in six of a stock solution of scaffolding protein was added and manually mixed so that the final concentration of scaffolding protein was 1 mg/mL (30 μM). The turbidity at 315 nm was monitored with time. Tangents to the initial slope were used to measure the initial rate of the reaction. For each concentration of added bisANS, the fraction of the maximal rate (obtained in the absence of added bisANS) was plotted.

Uncontrolled Polymerization of Coat Subunits. Uncontrolled polymerization of coat protein subunits was performed as described in Prevelige *et al.* (1990) with the following modifications. Coat protein was mixed with bisANS such that the final concentration was 1 mg/mL (21 μM) protein and 300 μM bisANS. A sample in which buffer was substituted for bisANS was prepared in parallel. The solutions were concentrated from 2 mL to $\sim$200–300 μL using a Centricon 30 concentrator. Samples were taken from each to observe by negative-stain electron microscopy. The solutions were centrifuged in a Beckman TL 100 centrifuge using a TLA-100 rotor at 100 000 K rpm. The supernatants were removed and the pellets suspended in an equal volume of buffer. Equal volume samples were applied to an SDS–polyacrylamide gel.

Circular Dichroism Spectroscopy. CD spectra were recorded on an Aviv Model 60DS spectropolarimeter. Temperature control of the samples was maintained by employing the temperature control accessory. Spectra were recorded in either 20 mM sodium phosphate, pH 7.6, or 25 mM NaCl, 50 mM Tris, pH 7.6. The protein concentration was determined spectrophotometrically. The extinction coefficients for denatured coat protein and for denatured scaffolding protein were obtained computationally using the method of Johnson (1988) for protein unfolded in GuHCl. In order to determine the extinction coefficients of the folded protein, the spectrum of the protein was recorded in buffer, and then a careful dilution into GuHCl was made and the spectra compared. The extinction coefficients obtained in this manner were 1.61×10^4 L mol^{-1} cm^{-1} for scaffolding protein and 4.45×10^4 L mol^{-1} cm^{-1} for coat protein, in reasonable agreement with those obtained by the Lowry assay (Fuller & King, 1981). All CD spectra were recorded in a 0.1-cm cuvette, and base lines of buffer and buffer plus bisANS were recorded for each run. In order to minimize the potential for errors in protein concentration between samples to be compared, the samples were prepared by mixing a 500 μL protein with either 7 μL of buffer or bisANS stock solution. BisANS absorbs strongly in the far-UV region, and this absorbance can result in spectral artifacts. Therefore, a practical maximal upper bisANS concentration was determined by recording the CD spectrum of serial dilutions of the sample containing bisANS until there was no change in the normalized spectrum upon dilution. The spectral bandwidth was 1.5 nm, the step size was 1 nm, the averaging time was 5 s, and five scans were taken and averaged for each sample. Instrument calibration was verified using *d*-10-camphorsulfonic acid as described by Johnson (1988).

Limited Proteolysis with Chymotrypsin. Coat protein subunits and scaffolding protein (0.5 mg/mL, 10.5 and 15 μM, respectively) were treated for increasing times with chymotrypsin–agarose at room temperature. A 1:1 slurry (by volume) of chymotryspin–agarose was diluted 1:10 into coat and scaffolding protein and mixed on a Nutator platform rocker. At times after addition, the samples were centrifuged for 10 s in an Eppendorf centrifuge. The supernatants were removed and added directly into tubes containing SDS sample buffer and boiled. Equal volumes of protein were loaded onto an SDS–polyacrylamide gel.

RESULTS

Binding of BisANS by Procapsid Proteins. BisANS, a dimer of ANS, is a fluorescent probe that has been shown to bind to hydrophobic areas on proteins (Brand & Gohlke, 1972). BisANS has the useful property of being nonfluorescent in polar solvents but highly fluorescent when in a hydrophobic environment (Farris *et al.*, 1978). It has previously been reported that each soluble coat protein subunit binds one molecule of bisANS with a K_d of 7.2 μM, as determined by direct bisANS fluorescence enhancement and by nonradiative energy transfer (Teschke & King, 1993). To determine if bisANS was also bound by scaffolding protein, the binding of bisANS to scaffolding protein was followed by the enhancement of bisANS emission upon binding. The maximum fluorescence enhancement after correction for the inner filter effect was ascertained graphically to be 630 units. The binding of bisANS to scaffolding protein indicated 12–16 binding sites with a K_d of 6 μM (Figure 2).

In order to determine if a bisANS binding site was in the proximity of the single tryptophan residue at position 134 of the 303 amino acids in scaffolding protein (Eppler *et al.*, 1991), the quenching of tryptophan fluorescence by nonradiative energy transfer to bound bisANS was monitored. This requires physical proximity between the two groups on the order of

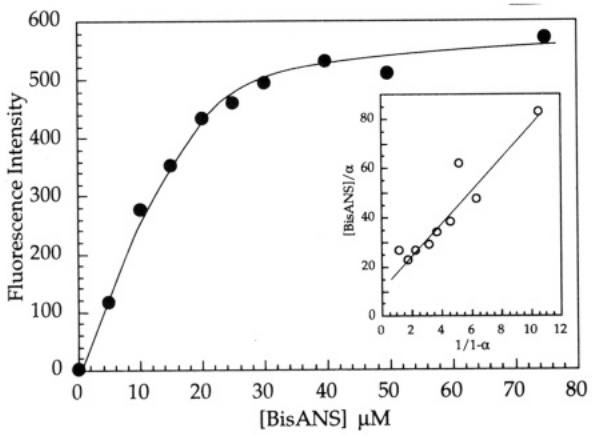

FIGURE 2: Binding of bisANS to scaffolding protein. Scaffolding protein (25 μg/mL, 750 nM) was added to increasing concentrations of bisANS. The fluorescence enhancement at 490 nm when excited at 400 nm due to the binding of bisANS was monitored. The inset shows the linearized data as described under Materials and Methods. α is F/F_{max}. The slope gives the K_d and the y intercept the total concentration of binding sites.

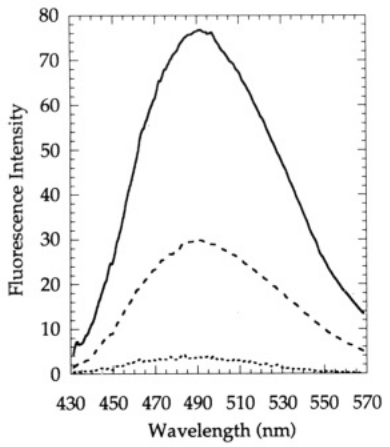

FIGURE 3: Fluorescence emission spectra of bisANS bound to coat protein, procapsids, and shells. The emission spectra of samples of coat protein monomer (solid line), procapsids (dashed line), and shells (dotted line) containing 2 μM bisANS were recorded. The excitation wavelength was 400 nm. The coat protein concentration was the same for all three samples and was 10 μg/mL (200 nM).

20–50 Å (Lakowicz, 1983). Using this methodology a single binding site for bisANS was detected with a K_d of 5 μM, suggesting that only 1 of the 12–16 molecules of bisANS bound to scaffolding protein was near tryptophan-134. Hydrodynamic data suggest that the scaffolding protein is a highly elongated rod (Fuller & King, 1982), and therefore it is reasonable that only a single binding site is in the proximity of tryptophan-134.

In order to determine whether the binding sites on coat and scaffolding protein remain available in the polymerized forms, the binding of bisANS to coat protein shells and procapsids was compared to coat protein subunits. In contrast to coat protein monomers, coat protein shells showed little fluorescence enhancement by bisANS. In addition, binding of bisANS to the empty shells did not appreciably quench the tryptophan fluorescence (data not shown). The decreased affinity of the shells for bisANS coupled with the lack of tryptophan quenching suggests that the sites of binding in the polymerized form are different than in the unpolymerized coat protein. The procapsids displayed an increase in bisANS fluorescence relative to the shells (Figure 3).

To determine if the increased binding of bisANS to procapsids was due to extraction of the scaffolding protein,

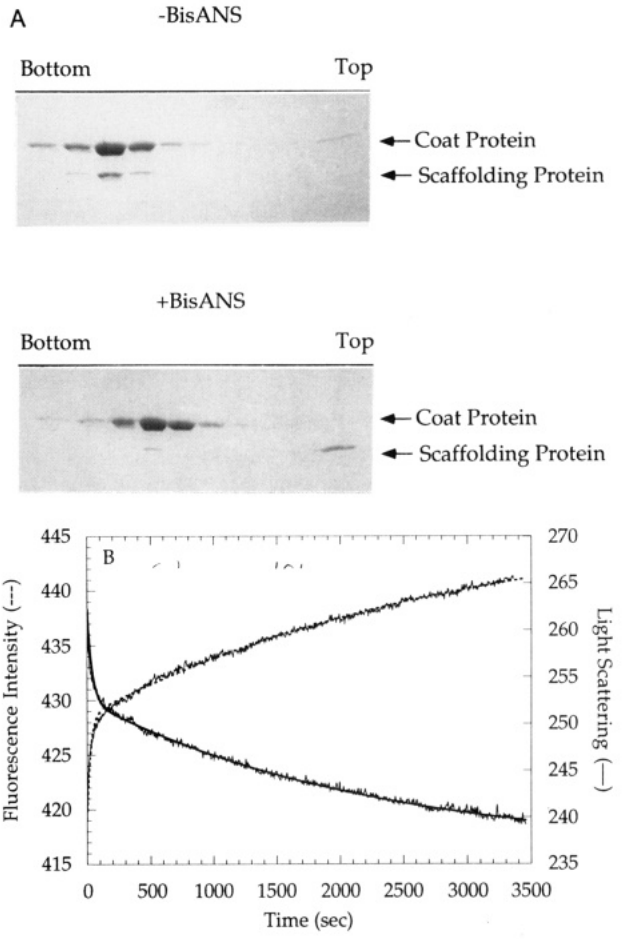

FIGURE 4: Extraction of scaffolding protein by bisANS. (Panel A) Procapsids (~1 mg/mL, 30 nM) were incubated in the absence (top) or presence (bottom) of 60 μM bisANS at 20 °C for 2 h and centrifuged through a 5–20% sucrose gradient. Each fraction was analyzed for protein content by SDS–PAGE. Soluble proteins remain at the top of the gradient, whereas procapsids and shells centrifuge three-fourths of the way down the gradient. (Panel B) The kinetics of bisANS binding to scaffolding protein and scaffolding release were followed by the enhancement of fluorescence intensity at 495 nm and the decrease in light scattering at 500 nm, respectively. The lines drawn through the data represent the theoretical curves calculated using the parameters obtained by fitting the data to a double-exponential decay.

purified procapsids were incubated 2 h at room temperature in the presence of bisANS. The particles were centrifuged through a sucrose gradient and the fractions analyzed by SDS–PAGE. The scaffolding protein remained at the top of the gradient. In turn, the procapsids were depleted of scaffolding protein (Figure 4A). These results suggest that the bisANS binds to procapsids and extracts the scaffolding protein.

In order to determine if the binding of bisANS to procapsids induced scaffolding exit, we measured both the kinetics of bisANS binding using fluorescence enhancement and the kinetics of scaffolding release using loss of light scattering (Figure 4B). Neither progress curve was well fit by a single exponential, but both were well described by two exponentials. The rate constants obtained by fitting either the fluorescence data or the light-scattering data were essentially the same, and consisted of a fast process with a relaxation time of ~30 s that accounted for one third of the change and a slow phase with a relaxation time of ~2500 s that accounted for the remaining two-thirds. There was no light-scattering change in empty shells upon bisANS binding (data not shown). The fact that there were two phases detected by turbidity with the

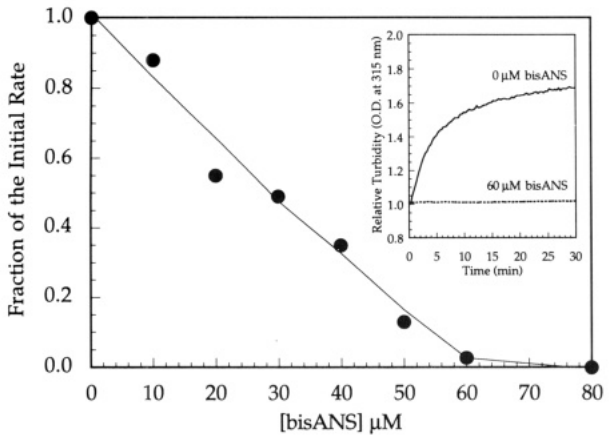

FIGURE 5: Inhibition of procapsid assembly by bisANS. Coat and scaffolding proteins were mixed at a final concentration of ~1 mg/mL (20 and 30 μM, respectively) of each in the presence of increasing concentrations of bisANS at 20 °C. Assembly was monitored by following the increase in turbidity at 315 nm. The initial rate of assembly was determined by drawing tangents to the progress curves and plotted versus the concentration of added bisANS. Inset: Progress curve obtained in the absence and presence of 60 μM bisANS.

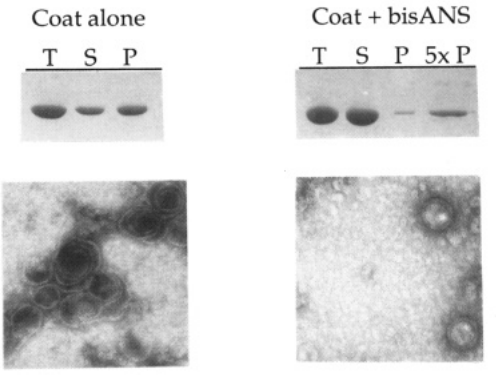

FIGURE 6: Uncontrolled polymerization in the presence and absence of bisANS. Coat protein monomers at 1 mg/mL (20 μM) were concentrated in a Centricon 30 concentrator in the absence or presence of 300 μM bisANS. The samples were concentrated 10-fold. SDS–polyacrylamide gels (upper panels) of equal volumes of the total (T) sample centrifuged, the supernatant (S), and the pellet (P) after centrifugation in the absence and presence of bisANS are shown. Negative-stain electron micrographs of each concentrated sample are shown in the lower panels.

procapsids suggests that there are two classes of scaffolding protein in the sample. Since we have not observed free scaffolding protein in solutions of procapsids, it is likely that these two classes are both internal to the procapsid. Similar suggestions of two classes of scaffolding protein have been made from analysis of *in vitro* assembly experiments (Prevelige *et al.*,1988; B. Greene, personal communication).

Inhibition of Assembly by BisANS. Procapsids can be assembled *in vitro* by mixing purified coat and scaffolding protein (Prevelige *et al.*, 1988, 1993; Thomas & Prevelige, 1991). In order to determine the effect of bisANS on procapsid assembly, monomeric coat protein was preincubated with various concentrations of bisANS, and scaffolding protein was then added. The rate of assembly was monitored by following the increase in turbidity at 315 nm. The initial rate was determined by drawing tangents to the progress curves as previously described (Prevelige *et al.*, 1993). The rate of assembly decreased linearly with increasing concentration of bisANS until it was completely inhibited at a concentration of approximately 60 μM (Figure 5 and inset).

The assembly of procapsids from subunits involves a nucleation step followed by repeated growth steps (Prevelige *et al.*,1993). The presence of bound bisANS could interfere with either or both of these steps and thereby result in the accumulation of assembly intermediates or aberrant polymerization products. We examined the products of the assembly reactions by both sucrose gradient analysis and electron microscopy at both intermediate and inhibitory concentrations of bisANS. At intermediate concentrations, a decreased yield of morphologically normal, fully assembled, procapsid-like particles but no partially formed shells was observed. At high concentrations, we were unable to observe any completed procapsids or partially assembled species.

This result can be explained either by bisANS blocking nucleation only or by bisANS blocking both nucleation and growth; it is not consistent with blocked growth only. If bisANS blocked growth only, nucleation would still occur, and we would expect to accumulate nucleation complexes, and perhaps partially assembled procapsids. There was no evidence of any polymerized form other than procapsids in either the sucrose gradients or the electron micrographs. If only nucleation were inhibited and growth could still occur, a decreased number of initiation events would occur, resulting in both a decreased rate of assembly and a decreased yield, but particles once started would proceed to completion. If bisANS blocked both the nucleation and growth steps, it could be possible for partially assembled procapsids to complete their assembly with unliganded protein.

In an attempt to directly determine if inhibition of assembly was due to bisANS binding one or the other of coat or the scaffolding protein, we preincubated the proteins individually with a saturating amount of bisANS and then tried to remove the unbound bisANS by using desalting columns. The bisANS was released too rapidly from both proteins for us to isolate protein with bisANS bound. A second attempt to answer this question involved premixing bisANS with either the coat or the scaffolding protein and then adding the other component of the reaction while looking for a burst of assembly. Such a burst of assembly might be detected if the binding of bisANS to the added subunit was slow or comparable to the rate of subunit polymerization. As we were unable to detect such a burst of assembly, either the presence of bisANS bound to either of the subunits can block assembly, or the binding of bisANS to the added subunit is fast relative to subunit polymerization.

Since the binding of bisANS to the protein subunits was so strong as to inhibit assembly, we also examined the binding of ANS, which has been reported to bind less tightly to other proteins. We saw little fluorescence enhancement of ANS at a concentration to 150 μM, indicating that little ANS was bound to either the coat or the scaffolding proteins, and no effect of ANS on the rate or extent of assembly was observed.

Inhibition of Uncontrolled Polymerization. In order to discern whether the inhibition of assembly by bisANS involved the coat or the scaffolding protein, we took advantage of the ability of coat protein to polymerize in the absence of scaffolding protein. Coat protein has been shown to polymerize into an ensemble of morphological forms *in vitro* at high concentrations (Prevelige *et al.*, 1990). These forms are the product of uncontrolled polymerization, but are not due to nonspecific aggregation.

Purified coat protein monomer was prepared at 1 mg/mL (21 μM) and then concentrated at 20 °C by filtration in a Centricon 30 concentrator in either the presence or the absence of 300 μM bisANS. Following concentration, the samples were centrifuged in a Beckman TL-100 centrifuge under conditions calculated to pellet any species greater than 28 S, and the pellet and supernatant fractions were analyzed by SDS–PAGE (Figure 6).

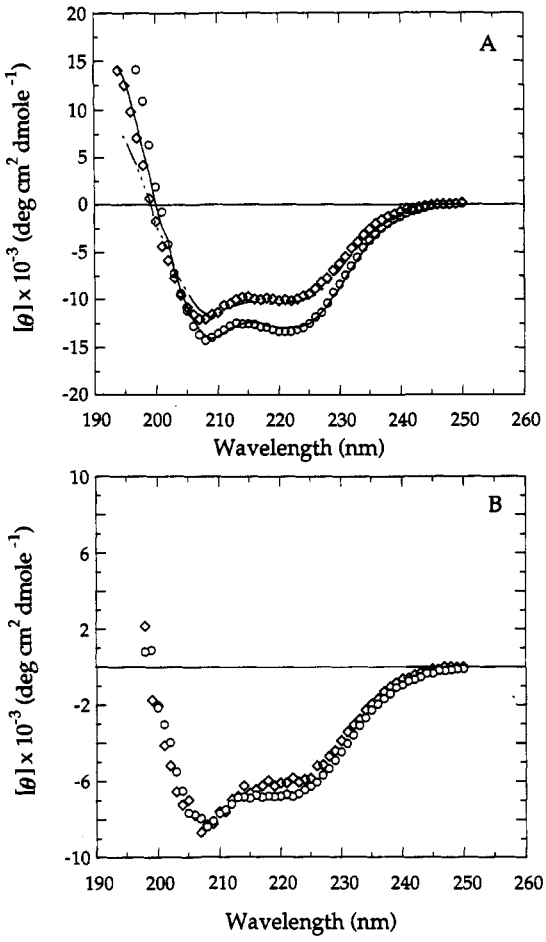

FIGURE 7: CD spectra of coat and scaffolding proteins in the presence and absence of bisANS. CD spectra of scaffolding protein (panel A) and coat protein (panel B) at concentrations of 8.8 and 6.0 μM, respectively, in an AVIV Model 60DS spectropolarimeter. All spectra were recorded in a 0.1-cm path-length cell in 20 mM sodium phosphate, pH 7.6 at 10 °C. In the case of the spectra with bisANS added (diamonds), the concentration of bisANS was 60 μM. For the scaffolding protein, the line indicates the calculated spectrum for a protein containing the fractional amounts of the elements of secondary structure indicated in the text.

These results suggest that binding of bisANS may be sufficient to inhibit coat protein polymerization, but do not exclude the possibility that binding of bisANS to the scaffolding protein is also sufficient to inhibit procapsid assembly.

Effect of BisANS on the Conformation of Capsid Proteins. The inhibition of bisANS could be due to either (a) blocking a hydrophobic site necessary for polymerization or (b) binding of bisANS inducing a more global conformational change. In order to detect any conformational change induced in the subunits by the binding of bisANS, we recorded both near- and far-UV CD spectra and performed limited proteolysis.

The CD spectrum of the 8.8 μM scaffolding protein was recorded in 20 mM phosphate, pH 7.6, 10 °C, in the absence and presence of 60 μM bisANS (Figure 7A). The CD spectra were done in phosphate buffer because the Tris buffer has a high absorbance in the far ultraviolet region. CD spectra of scaffolding protein in Tris buffer in the absence of bisANS were superimposable with those obtained in the phosphate buffer described here. The added absorbance due to bisANS precluded obtaining spectra of scaffolding protein liganded to bisANS in Tris buffer.

The CD spectrum of scaffolding protein in both the presence and absence of bisANS displays prominent negative peaks at 208 and 222 nm typical of a protein containing α-helical regions. Using the Chang, Wu, and Yang standards (Chang *et al.*,1978), we estimate the composition of the unliganded scaffolding protein to be 30% α-helix, 10% β-sheet, 20% turn, and 40% random coil. The observation that scaffolding protein is highly helical is in accord with that made by Raman spectroscopy (Thomas *et al.*,1982).

In the presence of bisANS, there was a decrease in the negative ellipticity at 208 and 222 nm. This decrease is consistent with a loss in helical content. Analysis of the spectrum using the Chang, Wu, and Yang standards (Chang *et al.*,1978) yielded 23% α-helix, 17% β-sheet, 20% turn, and 40% random coil. Although these values suggest a conversion of secondary structure from α-helix to β-sheet upon bisANS binding, they must be interpreted cautiously as the possibility of contribution from bisANS bound in a chiral fashion cannot be ruled out. Furthermore, it must be noted that under these conditions the scaffolding protein was not saturated with bisANS, but had on average half of the sites liganded. Therefore, the actual change may be greater than that observed in this experiment.

The CD spectrum of coat protein was recorded at 6 μM under the conditions described for the scaffolding protein in both the presence and absence of 60 μM bisANS (Figure 7B). As was the case with the scaffolding protein, we saw no effect of buffer on the conformation of the unliganded protein as determined by CD. The coat protein CD spectrum was essentially unchanged by the addition of bisANS. We were unable to obtain a good fit to the CD data using the Chang, Wu, and Yang reference standards. A fit with 20% α-helix, 25% β-sheet, and 55% irregular as suggested by Raman spectroscopy (Thomas *et al.*,1982) described the data, as well as fits with significantly more β-sheet structure.

Digestion of the Coat and Scaffolding Proteins with Chymotrypsin. The CD changes suggested that there was a change in the secondary structure in the scaffolding protein upon binding of bisANS with no detectable change in the tertiary structure by fluorescence. In order to determine if there were perceivable changes in the tertiary structure, samples of both coat and scaffolding protein with or without bound bisANS were subjected to limited proteolytic digestion with chymotrypsin (Figure 8).

The coat protein was rapidly digested by chymotrypsin to a series of bands of ~30 and ~15 kDa (Figure 8). The same pattern of bands was obtained whether or not bisANS was present during the reaction. Scaffolding protein showed a different proteolytic pattern in the presence or absence of bisANS. In the absence of bisANS, the initial digestion gave a single band of ~30 kDa and a number of bands of ~13 kDa. In the presence of bisANS, the initial bands were a doublet of ~25 kDa and quartet of ~18 kDa as well as the same series of bands at ~13 kDa. Increasing the time of incubation with the chymotrypsin did not change the pattern of the bands in the scaffolding digestion. The rate of digestion of both coat and scaffolding proteins appeared to be slowed in the presence of bisANS. Chymotrypsin was shown to bind bisANS and could account for difference in rate (data not shown). However, digestion of the coat protein showed that the specificity of the chymotrypsin was unchanged by the bisANS.

CONCLUSIONS

The presence of bisANS in an assembly reaction containing coat and scaffolding protein subunits effectively inhibited the polymerization of those subunits into procapsids. Procapsid formation requires the presence of both coat and scaffolding protein, and since both proteins bind bisANS, it is difficult

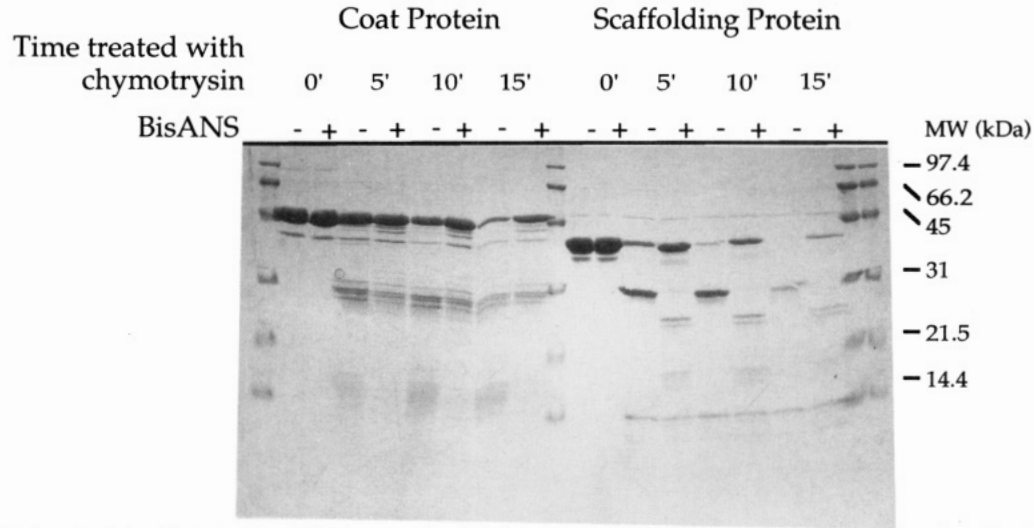

FIGURE 8: Limited proteolysis of coat and scaffolding protein with chymotrypsin. Coat (0.5 mg/mL, 10.5 μM) and scaffolding (0.5 mg/mL, 15 μM) proteins in the absence or presence of 125 μM bisANS were incubated with chymotrypsin–agarose as described under Materials and Methods. At the times indicated, the reactions were stopped. The samples were loaded onto a 15% SDS–polyacrylamide gel. The molecular masses of marker proteins are indicated on the right of the gel.

to assign the inhibition of assembly to either one. However, the concentration-induced uncontrolled polymerization of coat protein is also blocked by the presence of bound bisANS suggesting that bisANS, bound to coat protein is sufficient to inhibit polymerization.

Using a K_d of 7.2 μM for the binding of bisANS to coat protein, we estimate the ΔG of binding to be –7 kcal/mol. If the binding energy of coat protein subunits for each other was larger than this value, the coat protein subunits would be expected to displace the bisANS and polymerize. The fact that bisANS inhibits polymerization of the coat protein suggests that the intersubunit binding energy is less than this value.

Although this calculation is strictly only valid for the uncontrolled polymerization, kinetic analysis has suggested that procapsid assembly proceeds from a pentamer of coat protein. It is reasonable to suppose that the uncontrolled polymerization initiates in a similar fashion, and therefore that this estimate of binding energy can be applied to procapsid formation. Procapsid formation is a nucleation-limited reaction which displays an apparent critical concentration of 0.3 mg/mL coat protein (Prevelige *et al.*, 1993). One characteristic of a nucleation-limited reaction is that the equilibrium constant for the formation of the nucleus is unfavorable relative to that of the growth steps. In the case of procapsid assembly, this unfavorable step would correspond to the formation of a pentamer of coat protein. The estimated value of –7 kcal/mol for inhibition may then apply only to the nucleation steps, not to the growth steps. An explanation of this sort would then reconcile the observation that the coat protein shell of the assembled procapsid is stable in the presence of bisANS with the observation that bisANS inhibits assembly.

The fact that polymerized forms of coat protein cannot bind bisANS suggests that the bisANS binding sites are inaccessible in the polymer. It is conceivable that the binding sites are simply on the innersurface of both the procapsid and shell and are therefore inaccessible. This would not explain the observed inhibition of assembly, and it has been shown in the case of T4 and T7 that internal bisANS binding sites are accessible (Griess *et al.*, 1991; Khan *et al.*, 1992). The empty shells of P22 have been shown to have holes of approximately 25 Å, presumably large enough for bisANS to pass through, and it has been shown that ethidium bromide can intercalate into the DNA contained within the capsid (Prasad *et al.*, 1993; Griess *et al.*, 1986). Therefore, we suggest that the reason that the bisANS cannot bind to the assembled forms is coupled to the mechanism of inhibition of assembly. There are two mechanisms by which binding of bisANS could inhibit assembly: direct steric effects in which the bisANS binding site overlaps with an intersubunit bonding site, and indirect effects on the conformation of the protein subunit.

In the case of rhinovirus, infection requires binding of the virus to the cell-surface receptor ICAM-1 at a region on the viral capsid termed the canyon. This leads to endocytosis, and subsequent viral uncoating is triggered by the acidic pH of the endosome. A class of antiviral compounds called the WIN compounds have been developed that appear to inhibit infection by two mechanisms [reviewed in McKinlay *et al.*(1992)]. Binding of the hydrophobic WIN compounds underneath the canyon floor results in a 5-Å upheaval of the canyon floor, thereby blocking binding to ICAM-1. Additionally, the presence of the bound WIN compounds prevents viral uncoating. Although the mechanism of inhibition of uncoating has not been fully elucidated, the WIN compounds bind in a pocket formed beneath the floor of the canyon surrounding each 5-fold symmetry axis. Normally, the WIN pocket is occluded by a strand of the VP1 capsid subunit. One hypothesis is that the empty pocket is required to allow the subunit conformational changes that accompany uncoating.

In the case of the $T = 7$ procapsid of bacteriophage P22, the coat protein subunits must form seven distinct types of intersubunit contacts. In all the viruses whose crystal structure has been determined, this multibonding potential is realized through conformational changes within the subunits. It is possible that the presence of bisANS bound to the coat protein subunits prevents them from adopting one or more of the conformations necessary for assembly. In fact, in the presence of bisANS, coat protein subunits seem to form small oligomers on the order of dimers or trimers, but are blocked from further polymerization (Prevelige, unpublished observations).

It has been shown that binding of bisANS to tubulin monomers blocks polymerization. Tubulin can be induced to polymerize through a variety of mechanisms including the addition of microtubule-associated proteins, taxol, DMSO, and glutamate. BisANS inhibits the MAP-induced polymerization, but not polymerization induced by the other agents (Mazumdar *et al.*, 1992). Tubulin in the presence of bisANS

remains able to bind the MAP2 subunits, but cannot undergo assembly. This suggests that subunits remain in the conformation required for assembly, but not necessarily the conformation required for initiation of assembly. It has been shown that bisANS binds to a flexible region of the tubulin, and it has been suggested that a conformational change in this region is required for assembly (Prasad *et al.*, 1986).

Hydrophobic dyes have been shown to bind to the molten globule states of proteins (Kim & Baldwin, 1990). The molten globule has secondary structure and fluctuating tertiary structure (Kuwajima, 1989). In the case of the coat protein, the binding of the single molecule of bisANS does not seem to induce a change in either the secondary structure based on CD or the tertiary structure as assayed by chymotrypsin digestion. For the scaffolding protein, binding 12–16 molecules of bisANS alters the secondary structure and generates new sites that can be recognized by chymotrypsin. This suggests that the binding of bisANS induces a more global unfolding that might be consistent with the formation of a molten globule.

The hydrophobic dye bisANS binds to both the scaffolding and coat proteins in their monomeric forms, but binds with substantially less affinity to polymeric forms, procapsids and empty shells. BisANS appears to bind to procapsids better than to coat protein shells. The fact that the addition of bisANS to procapsids results in the stripping of the scaffolding protein and that the kinetics of binding and scaffolding removal are the same suggest that the reason for the enhanced fluorescence of procapsids over shells is due to the binding of bisANS to a monomeric form of scaffolding protein. Whether the bisANS binds to scaffolding protein associated with the procapsid, resulting in its rapid release, or traps transiently released scaffolding molecules remains an open question. We have not been able to detect free scaffolding protein in solution in procapsid samples either by sucrose gradient centrifugation or by HPLC analysis. This suggests that if the scaffolding protein is transiently released, it does not exit the shell. Kinetic analysis of the removal of scaffolding protein by bisANS demonstrated that there were two phases. This result implies that there are two classes of scaffolding protein within the procapsid. A similar suggestion has been made on the basis of analysis of *in vitro* assembly data (Prevelige *et al.*,1988) and a kinetic analysis of scaffolding extraction from procapsids by GuHCl (B. Greene, personal communication).

This study was designed to test the hypothesis that binding of a hydrophobic compound to the monomeric form of a viral coat protein subunit could block assembly of the virus. The results obtained in this study validate this approach and suggest that binding of agents with only moderate affinity can indeed inhibit assembly of viral capsids that once assembled are very stable, probably due to the highly cooperative nature of viral capsid assembly (Prevelige *et al.*,1993). The intersubunit interfaces responsible for virus assembly are likely to be highly conserved. Development of agents which inhibit assembly that are targeted at these interfaces could prove to be a valuable therapeutic avenue.

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