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FOR THE RECORD

Cloning, purification, and preliminary characterization by circular dichroism and NMR of a carboxyl-terminal domain of the bacteriophage P22 scaffolding protein

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Abstract: Assembly of double-stranded DNA viruses and bacteriophages involves the polymerization of several hundred molecules of coat protein, directed by an internal scaffolding protein. A 163–amino acid carboxyl-terminal fragment of the 303–amino acid bacteriophage P22 scaffolding protein was cloned, overexpressed, and purified. This fragment is active in procapsid assembly reactions in vitro. The circular dichroism spectrum of the fragment, as well as the 1D-NMR and ^{15}N-^{1}H HSQC spectra of the uniformly-labeled protein, indicate that stable secondary structure elements are present. Determination of the three dimensional packing of these elements into the folded scaffolding protein fragment is underway. Structure-based drug design targeted at structural proteins required for viral assembly may have potential as a therapeutic strategy.

Keywords: NMR; P22; scaffolding protein; virus assembly

P22 is a double-stranded (ds) DNA-containing bacteriophage of *Salmonella typhimurium*. The assembly pathway of P22 shares many common features with herpesviruses (Lee et al., 1988; Sherman & Bachenheimer, 1988) and adenoviruses (D'Halluin et al., 1978) and thus can serve as a model system for studying dsDNA virus assembly (Casjens, 1985; Prevelige & King, 1993). Assembly of the capsid of these icosahedral viruses requires the copolymerization of hundreds of molecules of coat protein, directed by a scaffolding protein, to form an intermediate assemblage called a procapsid. The scaffolding protein then exits the viral shell as or before nucleic acid is packaged (King & Casjens, 1974; Casjens & Hendrix, 1988), followed by expansion of the coat protein shell into the mature capsid form.

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While most current antiviral drugs act by inhibiting enzymes critical to the viral life cycle, computer simulations have suggested that interference with capsid assembly may be a promising new strategy. In theory, the incorporation of a single hexameric coat protein cluster in place of a pentamer (or vice versa) can misdirect assembly into large improper aggregates of coat protein, which are not only useless for virus production but serve as a "sink" for sequestering coat proteins (Berget, 1985; Berger et al., 1994). Teschke et al. (1993) have demonstrated that the small hydrophobic dye 1,1′-bi(4-anilinonaphthalene-5-sulfonic acid) can directly interfere with the subunit–subunit interactions required for bacteriophage P22 capsid assembly, thereby providing proof of concept for small molecule inhibition of virus assembly. Thus, study of the interactions among viral proteins during the assembly process may lead to new methods for interfering with virus multiplication.

In vivo, the assembly of each P22 virion utilizes over 400 molecules of the 47 kDa coat protein and 200–300 molecules of the 34 kDa, 303–amino acid scaffolding protein (King et al., 1973; King & Casjens, 1974; Eppler et al., 1991). Also incorporated in vivo, but not required for assembly, are a dodecamer of portal protein and a small number of several additional proteins. The unassembled scaffolding protein monomer is highly elongated, with an axial ratio of 9–11 to 1 (Fuller & King, 1982; Parker et al., 1997), and appears to exist in an equilibrium among monomers, dimers, and tetramers in solution (Parker et al., 1997). Like the herpesviruses cytomegalovirus (Beaudet-Miller et al., 1996) and herpes simplex type 1 (Hong et al., 1996), the P22 scaffolding protein appears to interact with coat protein subunits via its carboxyl-terminal end (S. Casjens & L. Sampson, unpubl. results).

To date, efforts to crystallize the P22 scaffolding protein have been unsuccessful (P.E. Prevelige et al., unpubl.). This may be related to its rather unusual solution structure. The thermal denaturation profile of scaffolding protein displays little cooperativity, all of the peptide NH groups are accessible to rapid deuterium exchange, and there is an unusually high content of charged amino acids and prolines (Tuma et al., 1996). These findings suggest that

the scaffolding protein is a highly flexible molecule consisting mainly of a series of segmented α-helices and lacking a hydrophobic core.

In order to analyze the functions of different domains of the scaffolding protein, we have cloned and expressed a fragment of the P22 scaffolding protein. One of these domains, amino acid residues 141 through 303 (the C-terminus), is the subject of this report. A DNA fragment was created that carries these scaffolding protein gene codons by polymerase chain reaction amplification using a template derived from a P22 strain (c1-7, 13$^-$amH101), which contains a fortuitous mutation from C to A at position 4668 in the P22 gene sequence. This mutation changes proline 259 in the scaffolding protein to histidine but is phenotypically silent (L. Sampson & S. Casjens, unpubl.). Two oligonucleotide primers, GACGATGCATATGGCTCGCAGCAATGCCGTAGCAG and GTAGAGAGGATCCTTGGAGTGATTGCGGAGATG (*Nde*I and *Bam*HI sites underlined) were used to amplify a fragment that contains codons 141 through 303 as well as 165 bp of P22 sequence 3′ to the scaffolding protein gene translation termination codon. In addition, these primers created an *Nde*I site immediately 5′ to codon 141 (Ala GCT), as well as a *Bam*HI site 3′ to the scaffolding protein gene. This amplified DNA was cleaved with restriction endonucleases *Nde*I and *Bam*HI, ligated into similarly cleaved plasmid pET3a (Studier et al., 1990) and used to transform $CaCl_2$-treated competent *Escherichia coli* strain NF1829 (Schultze et al., 1982). Minilysates of ampicillin-resistant transformants were screened for plasmids with correctly sized DNA inserts. The DNA inserts of several candidates were completely sequenced, and one, pSCAFF(141–303), that contained the correct sequence was used in these studies. This plasmid was then used to transform $CaCl_2$-treated competent *E. coli* BL21 (DE3) carrying the plasmid pLysS (Studier et al., 1990) to ampicillin resistance.

The resulting gene, which encodes amino acids 141 through 303 of the P22 scaffolding protein fused in-frame to an N-terminal Met and the vector's associated transcription and translation initiation signals, expresses high levels of this protein upon induction with isopropyl β-D-thiogalactopyranoside (IPTG). Scaffolding protein modulates its own synthesis *in vivo*, probably at the posttranscriptional level (Casjens & Adams, 1985; Wyckoff & Casjens, 1985). This has no doubt contributed to the fact that it has not been possible to overexpress scaffolding protein unless cellular levels are kept low by concomitant expression of coat protein, which sequesters the scaffolding protein in procapsids as it is formed (S. Casjens, unpubl. results). The amino acid 141–303 fragment described here can be overexpressed in the absence of coat protein. This suggests that the modulatory domain is missing in this fragment and supports the findings of Casjens et al. (1985) that the amino-terminal third of scaffolding protein appears to be responsible for the modulation.

A penultimate Ala, such as this protein has, usually programs N-terminal Met removal in *E. coli* (Flinta et al., 1986). Analysis of the protein using electrospray (Greis et al., 1996) and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy gave a molecular weight of 17,991 (±3) Da, consistent with an expected value of 17,993 for the amino acid 141–303 sequence (with the additional N-terminal methionine removed).

E. coli BL21[DE3]/pLysS/pSCAFF[141-303] were grown at 37 °C in 1 L of either LB broth or, for NMR, M9 minimal media (Miller, 1992) supplemented with $^{15}NH_4Cl$ as the sole nitrogen source. All media contained 30 μg/mL ampicillin and 25 μg/mL chloramphenicol. Cells at mid-log phase were induced with 0.4 mM IPTG and grown for an additional 3 h before centrifugation, decanting, and freezing overnight at −70 °C. Lysis buffer (50 mM tris hydrochloride, 20 mM $MgCl_2$, 1 mM phenylmethanesulfonic acid, pH 7.6; 50 mL) was added to the pellets, which were subjected to two rounds of thawing and freezing in dry ice/ethanol, and then treated with 0.5 mg DNAse I and incubated for 30 min at 37 °C. The suspension was centrifuged for 20 min at 16,000 × *g* and decanted. The pellet was extracted with an additional 50 mL of lysis buffer and shaken for 30 min at 4 °C, followed by centrifugation.

Solid ammonium sulfate was added to the combined supernatants; material that precipitated between 20 and 50% saturation was dissolved in approximately 10 mL of buffer B (50 mM tris hydrochloride, 25 mM NaCl, 2 mM EDTA, pH 7.6) and dialyzed overnight against 2 × 500 mL of this buffer. Insoluble material was removed by centrifugation for 30 min at 12,000 × *g*. The supernatant was loaded onto a 5 ml Hi-Trap SP-Sepharose cation-exchange column in a Pharmacia FPLC system, and eluted with a linear gradient of 25 to 250 mM NaCl in buffer B. The scaffolding protein fragment, which eluted between 125 and 200 mM NaCl, was pooled and dialyzed against 2 × 500 mL of buffer B. The protein was then loaded onto a 5 ml Q-Sepharose (anion-exchange) and a 5 ml SP-Sepharose column connected in series. After thorough washing with buffer B, the Q-Sepharose column was disconnected, and the protein was eluted from the other column by a gradient of 100 to 200 mM NaCl in buffer B. After dialysis, the protein was concentrated using a Millipore Ultrafree-15 5000 molecular weight cutoff centrifugal filter and stored at −20 °C until use. The 280 nm extinction coefficient of the native protein was determined by the method of Gill and von Hippel (1989) to be 0.389 mL/mg. A 1-L preparation in LB medium yields 40–45 mg of >99% pure protein (as determined by SDS-PAGE).

In vitro assembly reactions were conducted at 20 °C as described previously (Prevelige et al., 1988). Scaffolding protein (186 μg/mL, 5.53 μM) or the 163-amino acid fragment (100 μg/mL, 5.56 μM) was added to P22 coat protein (19.9 μM), and the mixtures were incubated for 4–5 h. Aliquots (200 μL) were centrifuged through 5 mL gradients of 5–20% sucrose in buffer B atop 150 μL cushions of 60% CsCl in 20% sucrose for 35 min at 33,000 rpm using an Sw55-Ti rotor (Beckman). The gradients were separated into 15 fractions and analyzed by SDS-PAGE on 13% acrylamide gels.

Procapsid-like particles are expected to sediment approximately halfway to two-thirds through this gradient. In Figure 1A, a peak in the distribution of coat and scaffolding proteins is seen at this position due to the in vitro formation of procapsids (Prevelige et al., 1988). In Figure 1B, a similar distribution occurs when the scaffolding fragment is used in place of the full-length protein. This suggests that the amino acid 141–303 scaffolding fragment can direct the assembly of coat protein into procapsid-sized particles, i.e., that amino acids 141–303 are sufficient for activity. Control experiments with no scaffolding protein or fragment did not give rise to detectable amounts of procapsids (data not shown).

Circular dichroism (CD) of the scaffolding protein fragment at 20.0 mg/mL, pH 6.0 and 0.100 mg/mL, pH 7.6 was measured at 20 °C on an Aviv model 62DS spectropolarimeter, using 0.014 mm and 1.0 mm cells, respectively. Five scans from 250 to 185 nm were averaged, using a 2 nm bandwidth, 1 s signal averaging time, and 0.5 nm intervals, and corrected using buffer as a blank. The spectropolarimeter was calibrated and the pathlength of the shorter cell was measured using *d*-10-camphorsulfonic acid (Yang et al.,

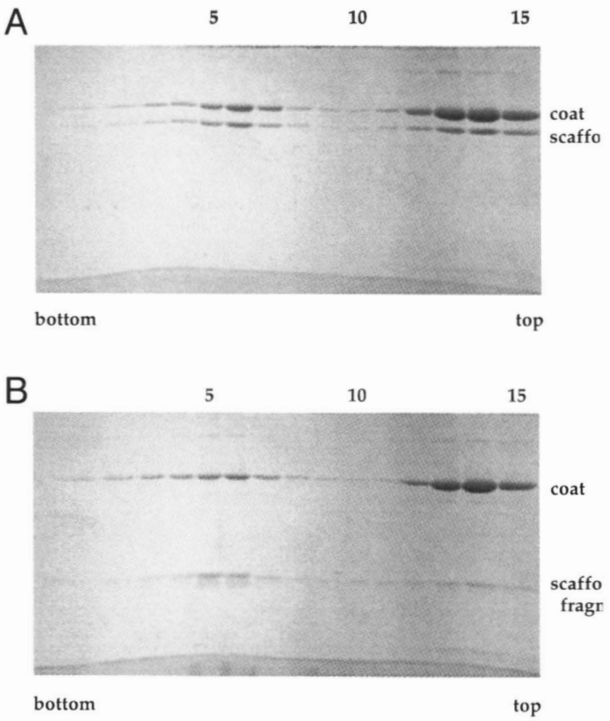

Fig. 1. Formation of procapsids by the full-length scaffolding protein and the amino acid 141–303 fragment. In vitro assembly reactions were carried out at 20 °C for 4–5 h in the presence of coat protein. Aliquots were centrifuged through 5–20% sucrose gradients, fractionated, and analyzed by SDS-PAGE on 13% acrylamide gels. **A:** Full-length scaffolding protein. **B:** Amino acid 141–303 fragment.

1986). CD spectra of the scaffolding protein fragment are shown in Figure 2. Spectra taken under conditions at which the protein is active in assembly (pH 7.6, 100 μg/mL) and at which the NMR spectrum was determined (pH 6.0, 20 mg/ml) were very similar. Analysis of the CD spectra (Yang et al., 1986) gives an estimate of 31% helix, 14% beta sheet, 16% beta turn, and 39% random coil; these are similar to values reported for the full-length scaffolding protein (Teschke et al., 1993; Tuma et al., 1996).

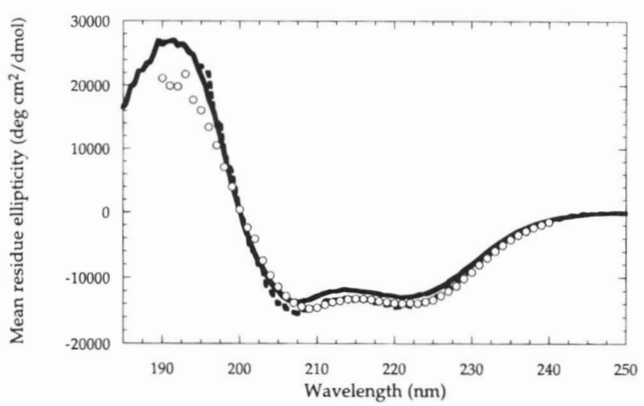

Fig. 2. Circular dichroism spectra of the scaffolding protein amino acid 141–303 fragment. Solid line: 20.0 mg/mL, pH 6.0, dashed line: 0.100 mg/mL, pH 7.6. Circles indicate a fit to 31% alpha helix, 14% beta sheet, 16% beta turn, and 39% random coil (Yang et al., 1986).

^{15}N-labeled protein used for NMR was dialyzed into degassed water. A 10-fold concentrated solution of perdeuterated buffer B (30 μL) was added, and the solution was lyophilized. Samples were reconstituted by adding 225 μL H_2O and 25 μL D_2O and adjusting the pH to 6.0 with 0.1 N HCl. The protein concentration used for NMR was approximately 1.1 mM (20 mg/ml). The sample was heated to 40 °C to remove dissolved air and then transferred to a 5 mm Shegemi microcell. An ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) experiment was performed on a Bruker AM-600 spectrometer at 35 °C. The standard HSQC pulse sequence was modified to include phase cycling of the first 180° ^{15}N pulse and a composite final ^{15}N 180° pulse (Shaw & Stonehouse, 1996). TPPI frequency discrimination in f_1 was accomplished with the use of an external timing accessory, and the ^{15}N decoupling was implemented with an external modulation accessory (both from Tschudin Associates, Kensington, MD). Sixty-four 1K real scans were collected for each of the 512 t_1 increments (52 ms total time). The data were processed with FELIX 950 (Molecular Simulations, Inc., San Diego, California). A 45° shifted sinebell and zero filling to 2K real was applied to both dimensions prior to Fourier transformation. The ^{1}H 1D-NMR spectrum (not shown) is characteristic of a folded protein with well-defined conformation. Figure 3 shows a portion of the ^{15}N-^{1}H HSQC spectrum, displaying well-resolved peaks with distinct ^{15}N chemical shifts, different from random-coil shifts (Wishart et al., 1991).

We have removed 46% (140 amino acids) from the amino-terminal end of the P22 scaffolding protein, and overexpressed and purified the resulting fragment in *E. coli*. The scaffolding protein fragment is assembly-active in vivo (S. Casjens & L. Sampson, unpubl. results) and in vitro (Fig. 1), and has elements of secondary structure (Fig. 2) similar to those of the full-length scaffolding protein (Tuma et al., 1996). The ^{15}N-^{1}H HSQC NMR spectrum (Fig. 3) confirms that stable elements of secondary structure are present.

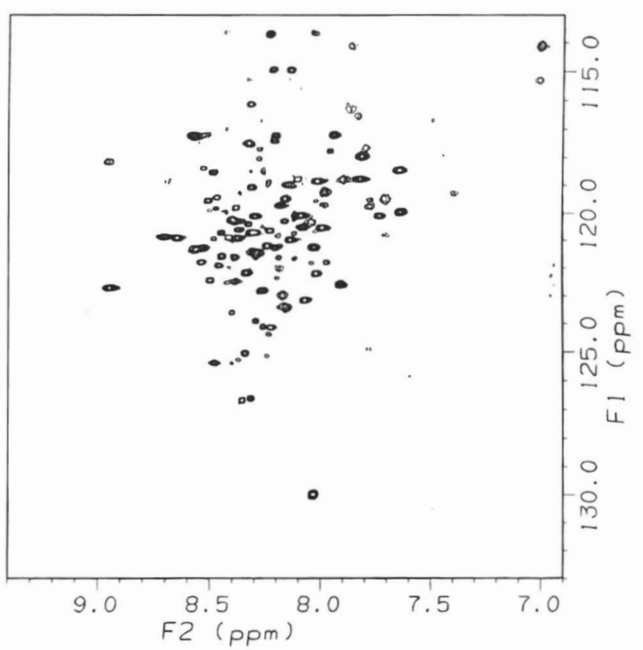

Fig. 3. A portion of the ^{1}H-^{15}N HSQC spectrum of the scaffolding protein fragment at pH 6.0, 1.1 mM (20 mg/mL), 35 °C. F1: ^{15}N. F2: ^{1}H.

No NMR or X-ray crystal structure has yet been reported for a viral scaffolding protein. Determination of the structure of the amino acid 141-303 assembly-active domain of P22 scaffolding protein by NMR is currently underway. This information should yield insights into the subunit interactions required for viral assembly, and allow more sophisticated models for scaffolding action to be developed and tested.

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