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Cooperative Heteroassembly of the Adenoviral L4-22K and IVa2 Proteins onto the Viral Packaging Sequence DNA

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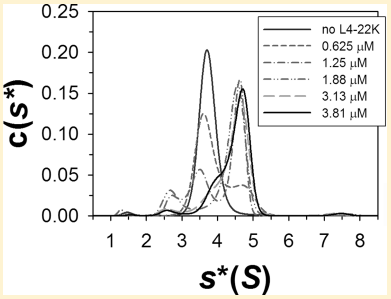
ABSTRACT: Human adenovirus (Ad) is an icosahedral, double-stranded DNA virus. Viral DNA packaging refers to the process whereby the viral genome becomes encapsulated by the viral particle. In Ad, activation of the DNA packaging reaction requires at least three viral components: the IVa2 and L4-22K proteins and a section of DNA within the viral genome, called the packaging sequence. Previous studies have shown that the IVa2 and L4-22K proteins specifically bind to conserved elements within the packaging sequence and that these interactions are absolutely required for the observation of DNA packaging. However, the equilibrium mechanism for assembly of IVa2 and L4-22K onto the packaging sequence has not been determined. Here we characterize the assembly of the IVa2 and L4-22K proteins onto truncated packaging sequence DNA by analytical sedimentation velocity and equilibrium methods. At limiting concentrations of L4-22K, we observe a species with two IVa2 monomers and one L4-22K monomer bound to the DNA. In this species, the L4-22K monomer is promoting positive cooperative interactions between the two bound IVa2 monomers. As L4-22K levels are increased, we observe a species with one IVa2 monomer and three L4-22K monomers bound to the DNA. To explain this result, we propose a model in which L4-22K self-assembly on the DNA competes with IVa2 for positive heterocooperative interactions, destabilizing binding of the second IVa2 monomer. Thus, we propose that L4-22K levels control the extent of cooperativity observed between adjacently bound IVa2 monomers. We have also determined the hydrodynamic properties of all observed stoichiometric species; we observe that species with three L4-22K monomers bound have more extended conformations than species with a single L4-22K bound. We suggest this might reflect a molecular switch that controls insertion of the viral DNA into the capsid.



Human adenovirus (Ad) is an icosahedral, nonenveloped double-stranded DNA virus that causes infections of several tissues.[1,2] Ad infection is potentially lethal in immunocompromised patients, especially organ transplant recipients.[3,4]

Initiation of the viral DNA packaging reaction requires the interaction of at least three viral components: the IVa2 and L4-22K proteins and a conserved region within the genome called the packaging sequence.[5−9] The packaging sequence consists of ∼200 bp and is composed of multiple repeated elements, called A repeats, with a consensus sequence of 5′-TTTG-(N_8)-CGXG-3′.[2,8] The wild-type sequence possesses four exact copies of this sequence, which are numbered AI, AII, AV, and AVI.[2] In addition, the wild-type sequence possesses additional "half-sites" composed of the TTTG or CGXG motif. The exact requirements of these half-sites are not understood, because genetic studies have shown that several other arrangements of A repeats could also support virus assembly and growth.[2,6,8,10−12] In particular, a virus possessing four copies of the consensus sequence, in an AI-AII-AI-AII arrangement, had wild-type levels of viral growth.[11]

Electrophoretic mobility gel shift assays (EMSAs) have shown that the IVa2 protein recognizes the CGXG motif, while the L4-22K protein recognizes the TTTG motif.[5,7,9,11,13] IVa2 binds to CGXG sequences with high affinity and some specificity (∼10-fold stronger affinity for CGXG-containing DNA than for a control DNA molecule without the CGXG sequences[14]). On the other hand, L4-22K requires IVa2 (along with the CGXG motifs) to specifically recognize the TTTG motif.[5,7,9] Thus, true specificity requires the presence of both CGXG and TTTG motifs and both IVa2 and L4-22K proteins.

An understanding of how the assembly of IVa2 and L4-22K on the packaging sequence initiates the virus assembly−DNA packaging process requires the elucidation of the equilibrium binding properties of these proteins bound to packaging sequence DNA. Previous studies from our laboratory showed that at least two IVa2 monomers could bind to a synthetic DNA molecule that contained the Ad A-I-II packaging sequence repeats (buffer H, 25 °C).[14] The first binding event occurs with a strong affinity (K_d ∼ 10−100 nM in buffer H at 25 °C), while the second event occurs with a weak affinity (K_d = 1.4 ± 0.7 μM). Additional studies by our laboratory have characterized the self-assembly properties of the L4-22K protein;[15] we showed that L4-22K reversibly self-associates via an indefinite, isodesmic process. In the work presented here, we have now characterized the stoichiometry of interaction of both the IVa2 and L4-22K proteins with a truncated packaging sequence DNA, called A-I-II, which appears to represent the fundamental unit of function within the packaging sequence. We find that multiple heteroprotein DNA ligation states are populated, beyond the simple prediction that a single IVa2

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loads onto a single CGXG site and a single L4-22K loads onto a single TTTG site. We demonstrate that L4-22K binding promotes cooperative assembly of IVa2 onto the packaging sequence and that saturating levels of L4-22K diminish this cooperativity. We also show that the higher-order L4-22K assemblies do not assemble onto the A-I-II DNA in the presence of the IVa2 protein, implying these large assemblies are not required for activity. We discuss our results in light of current virus assembly hypotheses.

EXPERIMENTAL PROCEDURES

Buffers and Sample Preparation. Buffer H consists of 100 mM NaCl, 40 mM HEPES (pH 7.6) at 25 °C, 10% (v/v) glycerol, 5 mM 2-mercapatoethanol, and 1 mM EDTA. The recombinant L4-22K protein was overexpressed and purified to homogeneity as described previously.[5,15] The IVa2 protein used here is an N-terminal truncation (beginning at amino acid 75) and has been shown previously to possess wild-type viral activity.[16] This protein was overexpressed and purified to homogeneity, and its DNA binding activity was determined as described previously.[14] All DNA molecules were purchased from IDT (Iowa City, IA) and purified by high-performance liquid chromatography. The sequence of the top strand of the double-stranded 48mer (A-I-II-Cy3) is 5′-Cy3-TAG-TAAA**TTTG**GGCGTAAC**CGAG**TAAGA**TTTG**GC-CATTTT**CGCG**GGAA-3′ (A repeat sequences underlined). The DNA concentrations were determined using absorbance spectroscopy as described previously.[14] We annealed the A-I-II-Cy3 double-stranded (ds) DNA molecule by mixing a 1.25-fold excess of the unlabeled, bottom strand DNA with the Cy3-labled top strand in buffer H, boiling the mixture for 5 min, and then cooling it at room temperature for 30 min.[14]

Sedimentation Velocity and Equilibrium Experiments. Analytical ultracentrifugation experiments were performed using a Beckman XLA analytical ultracentrifuge. The protein and DNA samples were first dialyzed into buffer H and then mixed with each other, or the final concentrations were achieved by direct dilution from storage conditions, such that the buffer components were at final concentrations identical to the buffer H concentrations. We have observed no experimental differences between the dialysis or dilution procedures. Samples were loaded into Epon charcoal-filled two-sector centerpieces, and experiments were performed at 25 °C. For sedimentation velocity experiments, 390 μL of sample was loaded into the sample chamber, while 400 μL of buffer was loaded into the reference chamber. For sedimentation equilibrium experiments conducted in the two sector centerpieces, 180 μL of sample was loaded into the sample chamber, and 185 μL of buffer was loaded into the reference chamber. For sedimentation equilibrium experiments conducted in the six-sector centerpieces, 115 μL of sample was loaded into the sample chamber while 120 μL of buffer was loaded into the reference chamber. For sedimentation velocity experiments, the samples were equilibrated for at least 1.5 h prior to initiation of the run in the centrifuge. Data were collected at the indicated wavelength using a radial step size of 0.002 cm with two absorbance replicates in the continuous scan mode. The raw sedimentation velocity data were analyzed using SEDFIT and SEDPHAT,[17] and experimental sedimentation coefficients were corrected to standard conditions (water, 20 °C) using SEDNTERP. For the sedimentation equilibrium experiments, data were collected using a radial step size of 0.001 cm, with 20 replicates. Samples were judged to have reached equilibrium using Winmatch. The data were analyzed using in-house models written for the Nonlinear Least Squares (NLLS) program Scientist (Micromath, St. Louis, MO), according to

$$A_T = \sum_{i=1}^{N} \exp\left(\ln A_i + \frac{M_{b,i}\omega^2}{RT}\frac{r^2 - {r_{ref}}^2}{2}\right) + b \quad (1)$$

where $\ln A_i$ is the natural logarithm of the absorbance of species i at the reference radial position, r_{ref}; r is the radial position from the center of the rotor; ω is the angular velocity of the rotor; R is the gas constant; T is the absolute temperature; b is the baseline offset; and $M_{b,i}$ is the buoyant molecular weight of species i, given by

$$M_{b,i} = M_i(1 - \bar{\upsilon}_i\rho) \quad (2)$$

where M_i is the molecular weight of species i, $\bar{\upsilon}_i$ is the partial specific volume of species i, and ρ is the buffer density. We note here that throughout this work we discuss M_b values for each stoichiometric species, rather than molecular weights, for the following reasons. (1) M_b is what is experimentally measured in the analytical ultracentrifuge. (2) $M_{b,i}$ is given by the sum of each individual M_b for each component. (3) Calculation of the molecular weight from an experimentally determined M_b requires a priori knowledge of the assembly stoichiometry.[18] ρ was calculated for buffer H using SEDNTERP, which yielded a value of 1.033 g/mL. $\bar{\upsilon}_i$ was calculated for each IVa2:L4-22K:DNA species assuming no significant volume changes accompany complex formation, according to

$$\bar{\upsilon}_i = \frac{qM_{IVa2}\bar{\upsilon}_{IVa2} + rM_{L4\text{-}22K}\bar{\upsilon}_{L4\text{-}22K} + sM_{DNA}\bar{\upsilon}_{DNA}}{qM_{IVa2} + rM_{L4\text{-}22K} + sM_{DNA}} \quad (3)$$

where q, r, and s are the stoichiometric coefficients for IVa2, L4-22K, and DNA, respectively. The partial specific volumes for the IVa2 and L4-22K proteins were calculated from their amino acid sequences using SEDNTERP (for 25 °C, yielding values of 0.7406 and 0.7162 mL/g, respectively), while the partial specific volume of the A-I-II-Cy3 DNA was determined experimentally using a sedimentation equilibrium value of 0.5525 ± 0.0083 mL/g.[14]

The frictional coefficient ratio for each macromolecular species was calculated according to[14,19]

$$\left(\frac{f}{f_0}\right)_i = \left[\frac{{M_i}^2(1 - \bar{\upsilon}_i\rho)^3}{162\pi^2(s_{i,20,w})^3\eta^3{N_{av}}^2(\bar{\upsilon}_i + \delta_i\upsilon^0_{H_2O})}\right]^{1/3} \quad (4)$$

where $s_{i,20,w}$ is the sedimentation coefficient of species i corrected to standard conditions (20 °C and water), η is the viscosity of pure water at 20 °C, N_{av} is Avogadro's number, $\bar{\upsilon}_i$ is the partial specific volume of macromolecular species i, ρ is the density of pure water at 20 °C, $\bar{\upsilon}^0_{H_2O}$ is the partial specific volume of pure water at 20 °C, and δ_i is the hydration of macromolecular species i. δ_i was estimated for each species by calculating a weight-average value according to[14]

$$\delta_i = \frac{qM_{IVa2}\delta_{IVa2} + rM_{L4\text{-}22K}\delta_{L4\text{-}22K} + sM_{DNA}\delta_{DNA}}{qM_{IVa2} + rM_{L4\text{-}22K} + sM_{DNA}} \quad (5)$$

where δ_{IVa2} and $\delta_{L4\text{-}22K}$ were calculated from their primary sequences using SEDNTERP, which yielded values of 0.38 and 0.45 g/g, respectively. The value used for δ_{DNA} was 0.74 g/g, as determined previously.[14]

All reported uncertainties correspond to 68.3% confidence intervals and were calculated manually using the F-statistic method.[20]

RESULTS

Sedimentation Velocity Studies of the Assembly of the L4-22K and IVa2 Proteins onto a Truncated Packaging Sequence DNA Containing the A Repeat Pair A-I-II. For these studies, the A-I-II-Cy3 DNA and the IVa2 and L4-22K proteins were mixed with each other at the indicated final concentrations and then studied by sedimentation velocity (buffer H, 25 °C). The wavelength used in these experiments was 522 nm, ensuring that only the Cy3-labled DNA molecule (A-I-II-Cy3) would be detected. Any free (unbound) protein will not be detected directly because it does not absorb at 522 nm. However, if the protein binds to the A-I-II-Cy3 DNA, then this interaction is observed because of the change in the rate of sedimentation of the DNA molecule.

Published results of EMSA experiments showed that purified L4-22K does not bind with appreciable affinity to DNA molecules containing A repeats in the absence of the IVa2 protein,[5,7,9] and we have reproduced this result under our experimental conditions. In the presence of IVa2, L4-22K binds specifically to DNA containing A repeats (midpoint of titration being ~10 nM L4-22K,[5] at 0.1 nM A-I-II DNA and 10 nM IVa2). For the investigation of the stoichiometry of interaction of L4-22K with IVa2 and the A-I-II-Cy3 DNA, by AUC methods, DNA concentrations much higher than those typically used in EMSA experiments are required. In our specific case, ~1 μM A-I-II-Cy3 DNA is required to ensure an adequate absorbance signal from the Cy3 dye. Therefore, all the relevant binding reactions will occur under stoichiometric binding conditions; i.e., the concentration of the bound ligand is expected to be much greater than the concentration of the free ligand. Stoichiometric binding conditions are optimal for measuring ligand:macromolecule stoichiometries.

Figure 1 shows the results of a series of sedimentation velocity experiments investigating the binding of L4-22K to A-I-II-Cy3 DNA (buffer H, 25 °C) in the presence of 1.33 μM IVa2 and 1.25 μM A-I-II-Cy3. The sedimentation velocity data were initially analyzed according to the $c(s)$ approach using SEDFIT.[17] The free A-I-II-Cy3 DNA sediments as a single species with an uncorrected sedimentation coefficient of 2.66 S under our experimental conditions.[14] The 1:1 (IVa2:A-I-II-Cy3) species (i.e., the IVa2:A-I-II-Cy3 species in the absence of added L4-22K) also sediments as a single species, with an uncorrected sedimentation coefficient of 3.7 S,[14] and we have reproduced this result in Figure 1. Upon titration of this mixture with L4-22K, a new peak appears at ~4.6 S, and we observe a concomitant decrease in the area of the 3.7 S peak with an increase in the area of the 4.6 S peak. This indicates the L4-22K protein is binding to the 1:1 IVa2:A-I-II-Cy3 species, increasing the sedimentation coefficient of the ternary complex. However, we also reproducibly observe the emergence of a smaller peak, centered at ~2.7 S, consistent with the known sedimentation coefficient (2.66 S, uncorrected) of the free A-I-II-Cy3 DNA.[14] This result indicates that upon loading of the L4-22K protein onto the IVa2:A-I-II-Cy3 species, some of the A-I-II-Cy3 DNA is released into solution. The only way this can occur is if the L4-22K protein increases the affinity for loading a second (or third etc.) IVa2 monomer onto the A-I-II-Cy3 DNA. In this scenario, in the presence of the L4-22K protein, the equilibrium association constant for loading a second IVa2 onto the A-I-II-Cy3 DNA

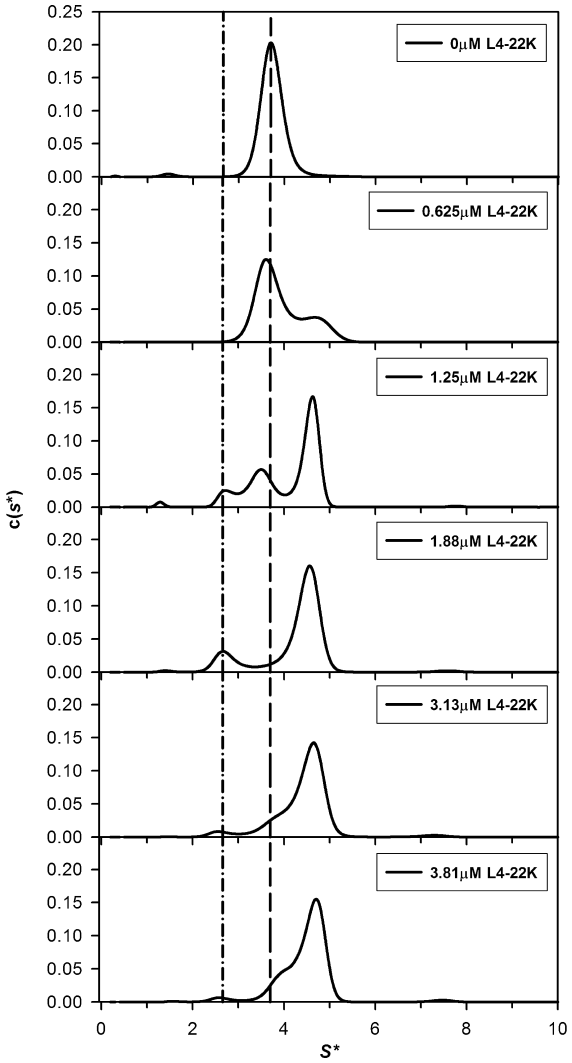

Figure 1. Stoichiometric binding of the L4-22K protein to the 1:1 IVa2:A-I-II-Cy3 complex, studied by sedimentation velocity. IVa2 (1.33 μM) was mixed with 1.25 μM A-I-II-Cy3 in buffer H, along with the indicated total monomer concentration of L4-22K. The samples were sedimented at 50K rpm and 25 °C. The smooth curves are the corresponding $c(s)$ traces obtained from the primary data (not corrected to 20 °C in water). The vertical dashed line corresponds to the previously determined s^* value for the 1:1 IVa2:A-I-II-Cy3 complex of 3.72 ± 0.04 S, while the vertical dashed−dotted line corresponds to the previously determined s^* value for the A-I-II-Cy3 DNA of 2.66 ± 0.02 S. The small peak at ~1.4 S is not consistently observed and cannot correspond to single-stranded DNA, which has an s^* value of 1.87 ± 0.02 S under identical conditions.

increases in affinity such that it can outcompete binding of IVa2 to a single A-I-II-Cy3 DNA; i.e., IVa2 is stripped off A-I-II-Cy3 to be loaded onto complexes that have L4-22K and IVa2 bound. Therefore, it appears that at least one of the roles of the L4-22K protein is to promote positive cooperative binding of IVa2 to the packaging sequence, consistent with the published EMSA data.[5]

While it is clear from the $c(s)$ analysis that some of the ternary complexes (i.e., L4-22K:IVa2:A-I-II-Cy3 complexes) must have at least two IVa2 molecules bound to the A-I-II-Cy3 DNA, it also is clear that additional species must be present. We can deduce this as follows. If the 4.6 S peak is composed of a single species that contains at least two IVa2 monomers bound,

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then each time this complex forms, a IVa2 must have been stripped off another A-I-II-Cy3 DNA, because the only significant source of IVa2 is the 1:1 IVa2:A-I-II-Cy3 complex (the vast majority of the IVa2 is bound to the A-I-II-Cy3 DNA, rather than free in solution). Thus, as L4-22K is titrated, we should see an equivalent increase in the magnitude of the free A-I-II-Cy3 DNA peak with the formation of the 4.6 S peak. This is not what we observe. We see that the area of the 4.6 S peak increases faster than the area of the 2.7 S peak, indicating that multiple ternary complexes, with differing stoichiometries, must compose the 4.6 S peak. In fact, we can be certain that some of these complexes can only have one IVa2 molecule bound, while others must have two or more IVa2 molecules bound, from simple mass conservation considerations.

On the basis of this, the simplest model to account for the sedimentation velocity data is one that includes a total of four sedimenting species: free A-I-II-Cy3 DNA, the 1:1 IVa2:A-I-II-Cy3 species, a ternary complex consisting of IVa2, L4-22K, and A-I-II-Cy3 DNA that includes at least two IVa2 molecules bound to the A-I-II-Cy3 DNA, and a final ternary complex that includes a single IVa2 molecule bound to the A-I-II-Cy3 DNA. We discuss the results of this analysis in a later section.

Finally, we note that even though L4-22K undergoes reversible isodesmic, indefinite self-association at the protein concentrations used in these experiments, these large assemblies do not bind to the IVa2:A-I-II-Cy3 species. The consequences of the coupling of large-scale protein self-association with DNA binding on the determination of ligand stoichiometry have been discussed by us previously[21] and are discussed in a later section of this work.

Sedimentation Equilibrium Studies of the Assembly of the L4-22K and IVa2 Proteins onto the A-I-II-Cy3 DNA. To determine the stoichiometry and size of the ternary species formed on the A-I-II-Cy3 DNA, we used the sedimentation equilibrium technique. Figure 2 shows the results of experiments performed under the same solution conditions used in the sedimentation velocity studies. We mixed 1.25 μM A-I-II-Cy3 DNA with 1.33 μM IVa2 along with either 1.25, 1.88, or 3.13 μM total L4-22K. The mixtures were sedimented to equilibrium at three rotor speeds (14K, 17K, and 21K rpm in buffer H at 25 °C). On the basis of the sedimentation velocity studies, we expect each of these mixtures will contain at least four stoichiometric species: the free A-I-II-Cy3 DNA, the 1:1 IVa2:A-I-II-Cy3 complex, and at least two different ternary complexes, as discussed in the preceding section.

As a first step, we attempted to analyze these data by global, NLLS methods with the four-species model, by fixing the buoyant molecular weights (M_b) of the free A-I-II-Cy3 DNA and the 1:1 IVa2:A-I-II-Cy3 complex to their previously determined values of 12.9 and 22.9 kDa.[14] However, it was not possible to uniquely resolve buoyant molecular weights for two additional ternary complexes. In fact, this analysis suggested that a single additional buoyant molecular weight term was sufficient to describe the sedimentation equilibrium data, because both M_b values, for the two ternary complexes, always tended to float to a single, common value. If we analyzed the data this way, we resolved an M_b value of ~42 kDa (see Table 1 and Figure 2A), which is independent of L4-22K concentration. We further tested the concentration independence of this M_b value by conducting experiments at two additional ratios of IVa2 to A-I-II-Cy3 DNA: 0.5 μM IVa2 with 0.95 μM L4-22K and 0.667 μM IVa2 with 2.5 μM L4-22K. Analysis of these data also yielded an M_b value of ~41 kDa, indicating this value is independent of L4-22K and IVa2 concentration, at least over this range (see Table 1 and Figure 2B). There are two possible explanations for this result. (1) There is only a single additional ternary complex in solution, or (2) there are at least two additional ternary complexes; however, they have very similar buoyant molecular weights such that they cannot be uniquely resolved by sedimentation equilibrium. With regard to the second possibility, because we have varied the IVa2 and L4-22K concentrations, we would expect to vary the relative proportions of the ternary complexes in solution, so if they did indeed have significantly different M_b values, we would expect the single, average M_b value to vary with concentration, reflecting the changing proportions of these multiple species with concentration. In contrast with this, we observe very similar values for the fitted M_b over all concentrations investigated (Table 1). Because the results of our sedimentation velocity studies conclusively rule out option 1, we must conclude that there are at least two ternary complexes with similar buoyant molecular weights. We further conclude, on the basis of our observation of the release of free A-I-II-Cy3 from the IVa2:A-I-II-Cy3 complex, that one of these ternary complexes must have at least two IVa2 molecules bound to the A-I-II-Cy3 DNA while the other must have only one IVa2.

Table 2 lists the calculated buoyant molecular weights for a range of assumed stoichiometries for the L4-22K:IVa2:A-I-II-Cy3 ternary complex. This table shows that there are multiple ways a ternary complex can be formed that have an M_b value close to 41−42 kDa. We were able to uniquely resolve which of these stoichiometries is correct on the basis of direct fitting of the sedimentation velocity data, which we discuss in the following section.

Global, NLLS Analysis of the Primary Sedimentation Velocity Data Incorporating Two Ternary Species with Identical Buoyant Molecular Weights. We have performed a global, NLLS analysis of the sedimentation velocity data shown in Figure 1 using SEDPHAT.[17] In this analysis, we fixed $s_{20,w}$ and M_b for the free A-I-II-Cy3 DNA and the 1:1 IVa2:A-I-II-Cy3 complex at their previously determined values[14] (see Table 1) while allowing the $s_{20,w}$ values for the two ternary complexes to float, along with the fraction of each species in solution at each L4-22K concentration. In addition, we have fixed the buoyant molecular weight of the two ternary complexes to the average of the sedimentation equilibrium experiments (Table 1), which yields an $\langle M_b \rangle$ of 41.7 ± 1.4 kDa. The results of this analysis are shown in Figure 3; it is clear this stoichiometric binding model describes the experimental data well. This analysis resolved $s_{20,w}$ values for the two ternary complexes of 6.13 ± 0.15 and 6.93 ± 0.24 S (see Table 3 for the s^* values measured under the experimental conditions).

We have also analyzed these data, including a single additional ternary species (not shown). F-Statistics were then used to determine if the inclusion of the second additional ternary complex significantly improved the quality of the fit.[20] Inclusion of this species improved the sum of the squared residuals (SSR) from 0.0601 to 0.0581. The critical SSR value, at the 95% confidence interval, is 0.0586. Because the SSR value obtained using the three-species model is larger than the critical SSR value, we are at least 95% confident inclusion of the second additional ternary complex improves the quality of the fit.

Figure 4 shows the fraction of each resolved species, determined from the global NLLS fit, as a function of the loading L4-22K concentration. Figure 4A shows that as the 1:1 IVa2:A-I-II-Cy3 species is titrated with L4-22K, the fraction of free A-I-II-Cy3 DNA increases to a maximum value of ~13%, consistent with the $c(s)$ data shown in Figure 1. Continued addition of L4-22K

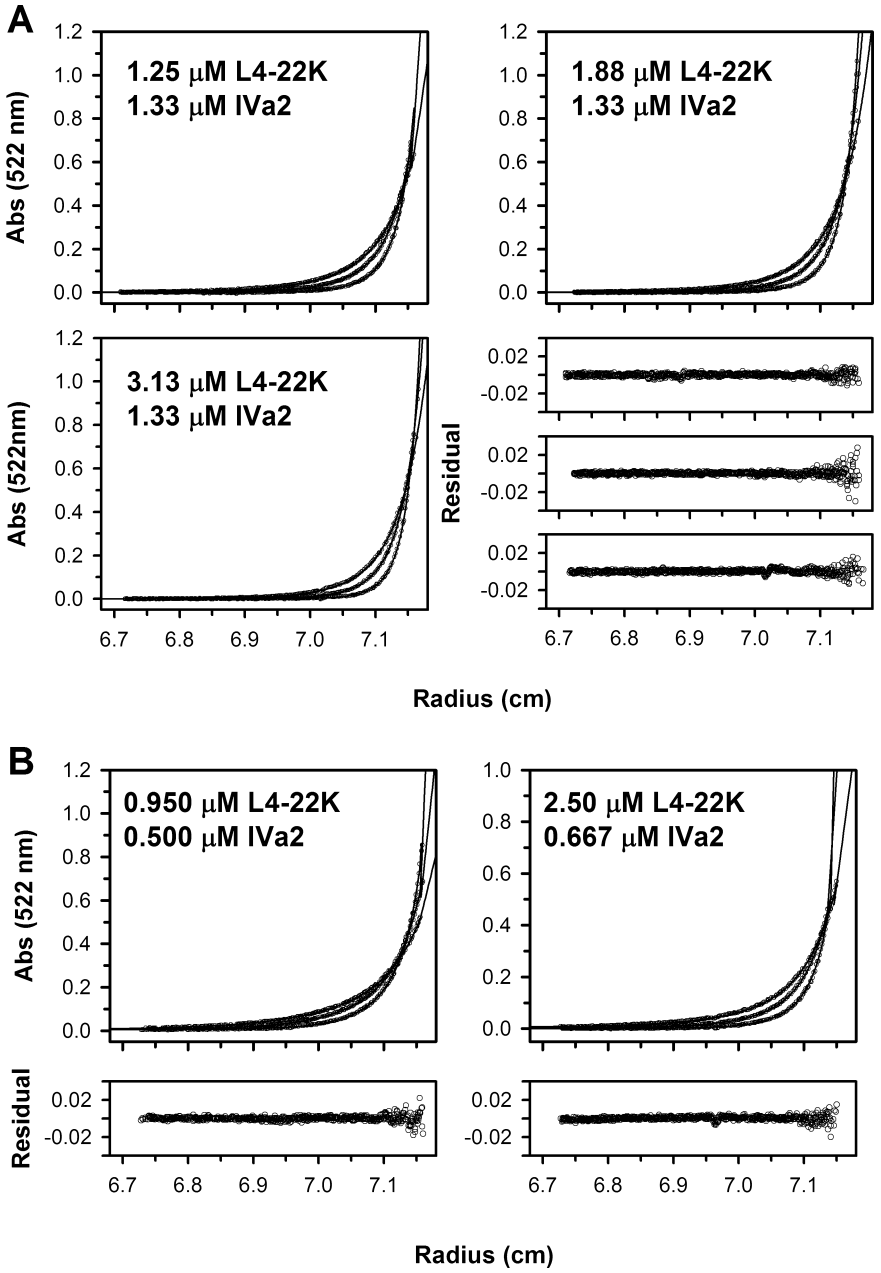

Figure 2. Sedimentation equilibrium experiments to determine the size of the L4-22K:IVa2:A-I-II-Cy3 ternary complexes. (A) IVa2 (1.33 μM) was mixed with 1.25 μM A-I-II-Cy3 in buffer H, along with the indicated total monomer concentration of L4-22K. The samples were loaded into two-sector centerpieces and then sedimented to equilibrium at 14K, 17K, and 21K rpm at 25 °C. The smooth curves are the results of NLLS analyses of the experimental data to an ideal, three-species model, as described in Experimental Procedures. In these analyses, the M_b values of the free A-I-II-Cy3 DNA and the 1:1 IVa2:A-I-II-Cy3 complex were fixed at their experimentally determined values of 12.89 ± 0.26 and 23 ± 1 kDa,[14] respectively, while the M_b value of the third ternary complex was allowed to float for each L4-22K loading concentration. The best fit values for this ternary species are listed in Table 1. (B) Results of the NLLS analyses of the two additional molar ratios of IVa2 and L4-22K.

results in a reduction in the concentration of free A-I-II-Cy3 DNA, which reflects a small amount of weak binding of L4-22K to the free A-I-II-Cy3 DNA at these high protein and A-I-II-Cy3 DNA concentrations.

The fraction of the 5.00 S species (the 1:1 IVa2:A-I-II-Cy3 complex) initially decreases linearly from 100% to a plateau of 20% (Figure 4B). Further addition of L4-22K apparently cannot shift all of the 5.00 S species to the ternary complexes, consistent with the results of the $c(s)$ analysis. Furthermore, we observe an apparent stoichiometric breakpoint (i.e., [L4-22K]/[A-I-II-Cy3]) of ~1.3. While it may appear this indicates a single L4-22K binds to the IVa2:A-I-II-Cy3 complex, the interpretation is actually more complex, for the following reasons.

We have previously shown that the L4-22K protein self-assembles into large oligomeric complexes, according to an indefinite, isodesmic assembly process; i.e., the addition of each L4-22K monomer to the growing oligomer can be described by the same equilibrium constant, L, and the oligomer can continue to assemble indefinitely.[15,22] A consequence of this indefinite self-assembly behavior is that an absolute upper limit is placed on the free L4-22K monomer concentration in solution, which for the isodesmic model is given by $1/L$.[15,21] Under buffer H

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Table 1. Summary of NLLS Analysis of the Sedimentation Equilibrium Data (buffer H, 25 °C)[a]

[IVa2] (μM)	[L4-22K] (μM)	$M_{b,3}$ (kDa)
1.33	1.25	42.6 (38.4, 46.6)
1.33	1.88	40.8 (37.4, 44.5)
1.33	3.13	43.7 (41.0, 45.7)
0.500	0.950	40.8 (32.9, 50.3)
0.667	2.50	40.6 (35.5, 46.3)

[a]The A-I-II-Cy3 DNA concentration was 1.25 μM in each experiment. Each experiment was analyzed using a three-species, stoichiometric binding model (eq 1). The buoyant molecular weight (M_b) values of the first two species were fixed at their previously determined values of 12.9 and 22.9 kDa,[14] while the average M_b value of the additional ternary complexes was allowed to float.

Table 2. Calculation of Predicted Buoyant Molecular Weights (M_b) for Various IVa2 and L4-22K Ligation Stoichiometries[a]

species	M (kDa)	$\bar{v}$ (mL/g)	M_b (kDa)
L4-22K (monomer)	21.85	0.7162	5.685
IVa2 (monomer)	42.77	0.7406	10.05
DNA (A-I-II-Cy3)	30.04	0.5525	12.90
IVa2:DNA	72.81	0.6630	22.94
$(L4\text{-}22K)_1$:$(IVa2)_1$:$(DNA)_1$	94.66	0.6753	28.63
$(L4\text{-}22K)_2$:$(IVa2)_1$:$(DNA)_1$	116.5	0.6830	34.32
$(L4\text{-}22K)_3$:$(IVa2)_1$:$(DNA)_1$	138.4	0.6882	40.00
$(L4\text{-}22K)_4$:$(IVa2)_1$:$(DNA)_1$	160.2	0.6920	45.69
$(L4\text{-}22K)_1$:$(IVa2)_2$:$(DNA)_1$	137.4	0.6956	38.68
$(L4\text{-}22K)_2$:$(IVa2)_2$:$(DNA)_1$	159.3	0.6984	44.36
$(L4\text{-}22K)_3$:$(IVa2)_2$:$(DNA)_1$	181.1	0.7006	50.05
$(L4\text{-}22K)_4$:$(IVa2)_2$:$(DNA)_1$	203.0	0.7023	55.74
$(L4\text{-}22K)_1$:$(IVa2)_3$:$(DNA)_1$	180.2	0.7063	48.73
$(L4\text{-}22K)_2$:$(IVa2)_3$:$(DNA)_1$	202.0	0.7074	54.41
$(L4\text{-}22K)_3$:$(IVa2)_3$:$(DNA)_1$	223.9	0.7082	60.10
$(L4\text{-}22K)_4$:$(IVa2)_3$:$(DNA)_1$	245.8	0.7089	65.78

[a]These calculations assume no significant volume change accompanies complex formation. The values for the L4-22K monomer, the IVa2 monomer, the A-I-II-Cy3 DNA, and the 1:1 IVa2:DNA compex were taken from our previous publications.[14,15] M_b was calculated using eq 2 with a ρ of 1.033 g/mL (buffer H, 25 °C).

conditions, using the identical approach described in ref 15, we measure an L of 74 (71, 77) μM^{-1}. Because all binding processes are fundamentally driven by the chemical potential of the free ligand concentration,[23] this indicates that for a ligand that participates in an indefinite, self-assembly process there will be an upper limit for the fractional saturation of the ligand with its binding site, which will be <100%.[21] The linkage between indefinite ligand assembly and receptor binding is discussed in detail in a recent publication from our laboratory.[21] This explains why we are not able to drive complete conversion of the IVa2:A-I-II-Cy3 complex into the ternary complexes; continued addition of L4-22K will not guarantee a continued increase in the free L4-22K monomer concentration. For our specific case, at ~20% IVa2:A-I-II-Cy3 complex, the free L4-22K monomer concentration in solution is 1/74 μM^{-1} (13.5 nM). Furthermore, because it is not possible to drive complete conversion of the IVa2:A-I-II-Cy3 into ternary complexes, this also indicates that any apparent stoichiometric breakpoint must occur at a lower total L4-22K concentration.[21]

Figure 4C shows that the fraction of the 6.13 S ternary complex increases up to an apparent breakpoint of ~1.5, after which it reaches a plateau at 50% saturation. Figure 4D shows that the fraction of the 6.93 S ternary complex increases to an average value of ~18%. Adding the fractions of the two ternary complexes together results in an apparent stoichiometric titration, with an apparent breakpoint of ~1.3 (Figure 4E).

These fractions have allowed us to determine the precise binding stoichiometries associated with the 6.13 and 6.93 S species, by calculating the total concentration of either the IVa2 or the L4-22K proteins on the A-I-II-Cy3 DNA for any assumed stoichiometry. The resulting calculations must obey mass conservation; they must be consistent with the observation that the 6.13 and 6.93 S species must both have M_b values close to 41.7 kDa, and one of them must contain at least two IVa2 molecules, to explain the release of free A-I-II-Cy3 DNA from the 1:1 IVa2:A-I-II-Cy3 complex observed upon titration with L4-22K. For example, if we assume the 6.13 S species corresponds to a 1:2:1 L4-22K:IVa2:A-I-II-Cy3 stoichiometry [M_b = 38.7 kDa (Table 2)], and the 6.93 S species corresponds to a 3:1:1 stoichiometry [M_b = 40.0 kDa (Table 2)], then we can calculate the total concentration of IVa2 and L4-22K that must compose each stoichiometric species, for any added L4-22K concentration. Next, we can compare the total IVa2 and L4-22K concentrations with the known total concentrations to account for all added protein. For the 6.13 S species, the plateau value for the species fraction is 50% (Figure 4C). For the 6.93 S species, we use a plateau value of ~18% (Figure 4D) and ignore the small increase at the two highest L4-22K concentrations, because this is likely due to nonspecific binding of L4-22K to the A-I-II-Cy3 DNA. We already know the 5.0 S species corresponds to a 1:1 IVa2:A-I-II-Cy3 species.[14] For this species, we use a plateau value of 20% (Figure 4B). Using these fractions and assuming the stoichiometries mentioned above, we calculate that the total IVa2 and L4-22K concentrations must be (1.25 μM) × (2 × 0.5 + 0.2 + 0.18) = 1.73 μM IVa2 and (1.25 μM) × (0.5 + 3 × 0.18) = 1.30 μM L4-22K. Considering that it takes ~2 μM L4-22K to reach saturation of the 6.13 S species fraction, it is clear the calculated total L4-22K concentration of 1.3 μM underestimates the known total concentration required to saturate the species fraction (i.e., 1.3 μM $\ll$ 2 μM) and that the calculated IVa2 concentration overestimates the total concentration added (i.e., 1.73 μM $\gg$ 1.33 μM). On the other hand, if we assume the 6.13 S species corresponds to the 3:1:1 stoichiometry while the 6.93 S species corresponds to the 1:2:1 species, then we calculate total IVa2 and L4-22K plateau concentrations of 1.33 and 2.1 μM, respectively. These calculations correspond very closely to the known total amounts added to solution at saturation and thus indicate these are very likely the correct species stoichiometries. We point out here that even though these calculations strongly suggest our assignment of these stoichiometries is correct, the M_b values of 40.0 and 38.7 kDa fall just outside of the experimentally determined M_b value of 41.7 ± 1.4 kDa (Table 1 and Figure 2). This might reflect small errors in calcuating the M_b for each stoichiometry assuming additivity of the individual components. This could arise if there are significant changes in volume upon complex formation.

Additional evidence of the validity of the 3:1:1 stoichiometry comes from consideration of the indefinite assembly of the L4-22K ligand. As discussed above, the maximal concentration of the free L4-22K monomer is 13.5 nM, which in this specific case ensures that 100% saturation of the A-I-II-Cy3 DNA can never be achieved by increasing only the total L4-22K

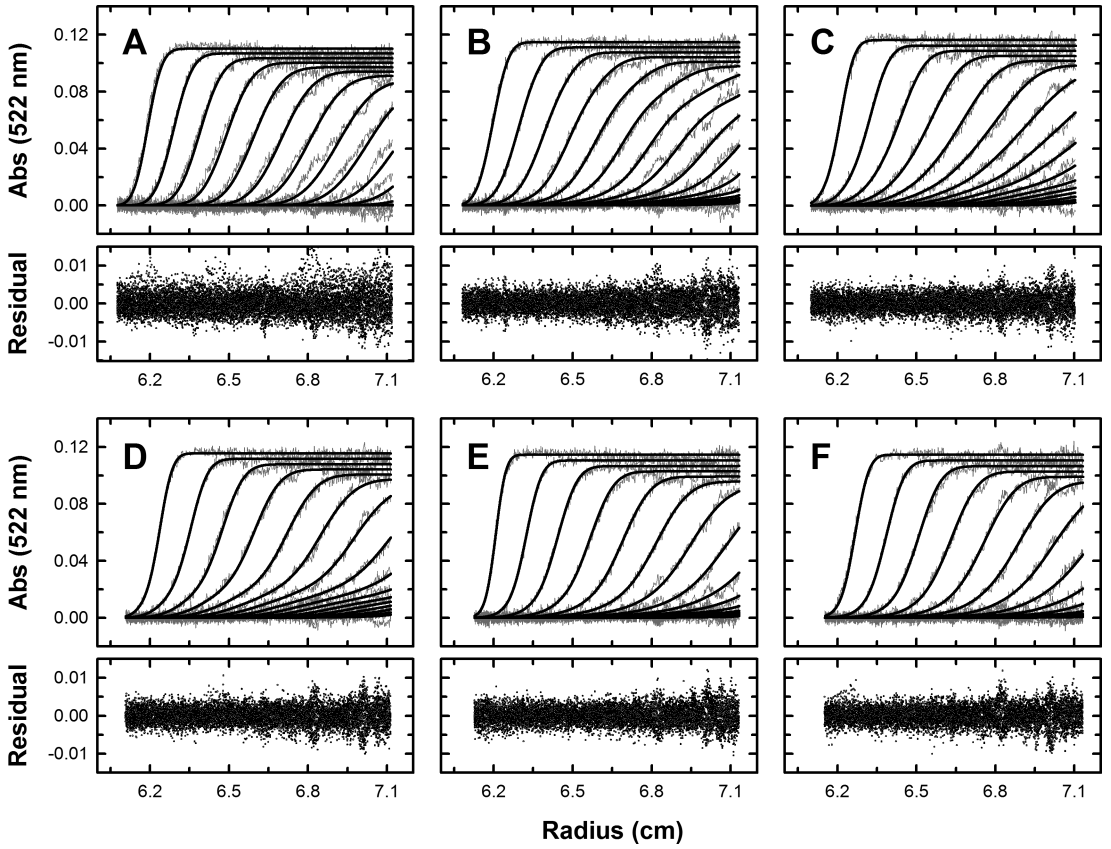

Figure 3. Global, NLLS analysis of the primary sedimentation velocity data via fitting to a stoichiometric, four-species model. Panels A–F show results of experiments performed at total L4-22K monomer concentrations of 0, 0.625, 1.25, 1.88, 3.13, and 3.81 μM, respectively. The smooth curves are simulations generated using the NLLS best fit parameters from the SEDPHAT analysis of the primary data. In this global NLLS analysis, the $s_{20,w}$ values of the free A-I-II-Cy3 DNA and the 1:1 IVa2:A-I-II-Cy3 species were fixed at their previously determined values of 3.48 ± 0.03 and 5.00 ± 0.05 S, respectively. The buoyant molecular weights of the free A-I-II-Cy3 DNA and the 1:1 IVa2:A-I-II-Cy3 species were fixed at their previously determined values of 12.89 ± 0.26 and 23 ± 1 kDa, respectively. The M_b value of the two additional species was assumed to be the same, as described in the text, and was fixed at 41.7 ± 1.4 kDa.

Table 3. Summary of the Hydrodynamic Properties of Each Detectable Component Present in the Ternary Assembly Reaction[a]

species	M (kDa)	$\bar{v}$ (mL/g)	M_b (kDa)	$s_{20,w}$ (S)	s^* (S)	f/f_0
L4-22K (monomer)	21.92	0.716	5.71	2.16 ± 0.04	1.58 ± 0.03	1.18 ± 0.02
IVa2 (monomer)	42.77	0.741	10.1	2.88 ± 0.02	2.07 ± 0.01	1.29 ± 0.01
DNA (A-I-II-Cy3)	30.04	0.553	12.9	3.48 ± 0.03	2.66 ± 0.02	1.37 ± 0.01
IVa2:DNA	72.81	0.663	22.9	5.00 ± 0.05	3.72 ± 0.04	1.33 ± 0.01
$(L4\text{-}22K)_3$:$(IVa2)_1$:$(DNA)_1$	138.6	0.688	40.1	6.13 ± 0.15	4.52 ± 0.11	1.55 ± 0.04
$(L4\text{-}22K)_1$:$(IVa2)_2$:$(DNA)_1$	137.5	0.696	38.7	6.93 ± 0.24	5.10 ± 0.18	1.34 ± 0.04
$(L4\text{-}22K)_3$:$(IVa2)_2$:$(DNA)_1$	181.3	0.701	50.1	7.11 ± 0.02	5.22 ± 0.01	1.54 ± 0.01

[a]The values reported for the L4-22K monomer, the IVa2 monomer, the A-I-II-Cy3 DNA, and the 1:1 IVa2:DNA species were taken from our previous publications.[14,15] The $s_{20,w}$ value for the IVa2:DNA complex is slightly different from that reported in ref 14 (which was 4.92 ± 0.09 S) because we have averaged in several recent measurements; the uncertainty is the standard deviation of six independent measurements.

concentration. To test if the 3:1:1 stoichiometry is correct for the 6.13 S species, we need to extrapolate the stoichiometric species fraction plots to 100% saturation[21] and then determine if the total amount of added L4-22K (by reading off the L4-22K concentration at 100% saturation) yields an L4-22K:A-I-II-Cy3 ratio of 3:1. This extrapolation yields an L4-22K concentration of 3.9 μM, which corresponds to an [L4-22K]/[A-I-II-Cy3] ratio of 3.9:1.25 (3.1). Thus, this extrapolation also supports our assignment of the 3:1:1 stoichiometry to the 6.13 S species.

Determination of the Maximal IVa2:L4-22K Stoichiometry on the A-I-II-Cy3 DNA. The experiments we described in the previous section were performed close to a 1:1 IVa2:A-I-II-Cy3 loading concentration ratio. As such, these experiments were sensitive to intermediate stoichiometries, identifying the 1:2:1 and 3:1:1 L4-22K:IVa2:A-I-II-Cy3 stoichiometries. However, it is not clear from these experiments what the maximal IVa2 and L4-22K binding stoichiometries are for loading onto the A-I-II DNA. To determine this, we have titrated the A-I-II-Cy3 DNA with both IVa2 and L4-22K in an attempt to populate a single, saturated complex.

Figure 5 shows a series of sedimentation velocity experiments performed at 2.5 μM IVa2 and 1.25 μM A-I-II-Cy3, which is enough IVa2 to completely occupy both IVa2 binding sites within the A-I-II-Cy3 DNA (both CGXG sites) if both binding

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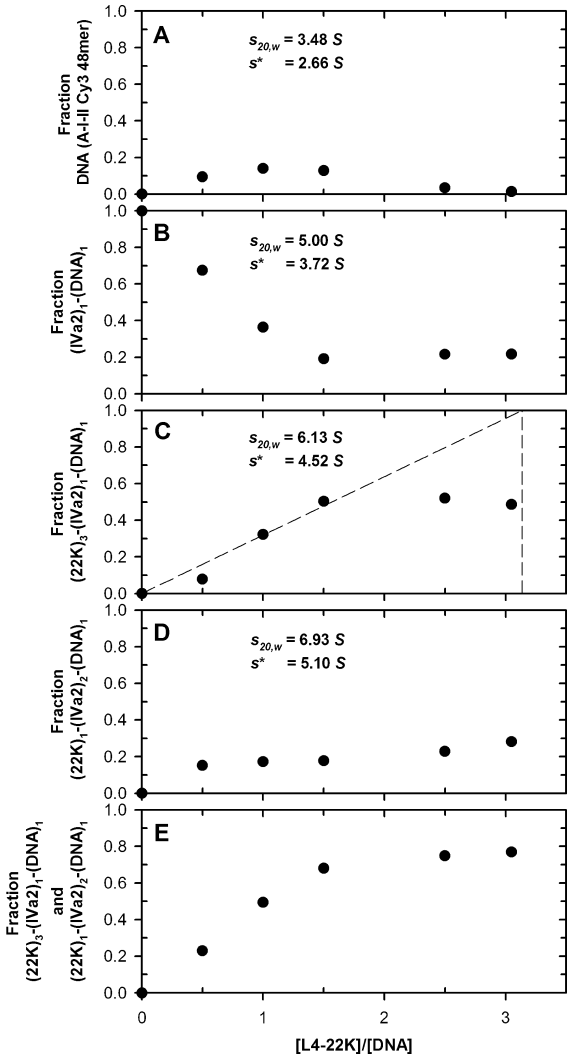

Figure 4. Species fractions determined from the global, NLLS analysis of the sedimentation velocity data shown in Figure 3. Panels A–D show the best fit values for the species fractions plotted as a function of [L4-22K]/[DNA], where [L4-22k] is the total monomer concentration. Consistent with the $c(s)$ analysis of these same data, this global analysis also shows that upon titration of the 1:1 IVa2:A-I-II-Cy3 species with L4-22K, a significant fraction of the A-I-II-Cy3 DNA is released from the IVa2:A-I-II-Cy3 species. Continued titration with L4-22K results in a decrease in this fraction, because of weak binding of L4-22K to the DNA at these high protein and DNA concentrations. The dashed line in panel C is the result of a linear least-squares fit of the linear part of the species fraction data to allow an extrapolation to a species fraction of one. This extrapolation allows one to determine the number of L4-22K monomers that are bound to the A-I-II-Cy3 DNA, as described in the text and ref 21. Panel E plots the sum of the fractions from panels C and D.

reactions are conducted under stoichiometric conditions. In the absence of added L4-22K, we see a heterogeneous $c(s)$ distribution that we have already shown reflects a mixture of at least 1:1 and 2:1 IVa2:A-I-II-Cy3 complexes; the K_d for adding the first IVa2 to the A-I-II-Cy3 DNA is ~10–100 nM, while the K_d for binding of the second IVa2 is 1.4 ± 0.7 μM.[14] Addition of 2.5 μM L4-22K results in a shift of essentially 100% of the distribution toward a 5.1 S species. Addition of 5 and 10 μM L4-22K results in a further small shift toward an apparently homogeneous, 5.2 S species. This implies that we have formed

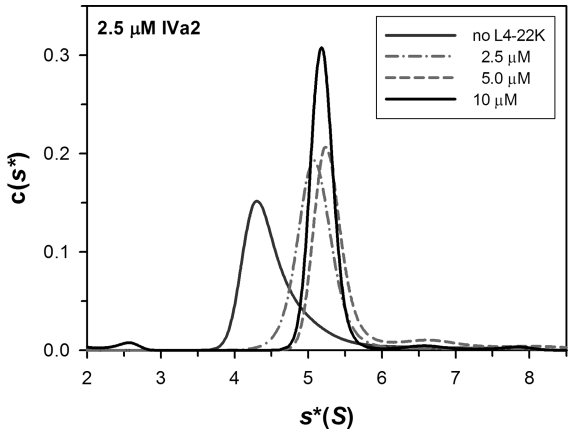

Figure 5. Sedimentation velocity experiments to determine the maximal binding stoichiometry of the IVa2 and L4-22K proteins on the A-I-II-Cy3 DNA. IVa2 (2.5 μM) was mixed with 1.25 μM A-I-II-Cy3 DNA in buffer H, along with the indicated concentrations of L4-22K. The samples were sedimented at 50K rpm and 25 °C. The smooth curves are the corresponding $c(s)$ traces obtained from the primary data (not corrected to 20 °C in water). At 10 μM L4-22K, a single, symmetric peak is observed, implying a single, saturated species is in solution. This idea was tested by sedimentation equilibrium experiments presented in Figure 6.

a saturated complex, with the maximal numbers of IVa2 and L4-22K molecules bound to the A-I-II-Cy3 DNA.

To determine the size of this 5.2 S species, we conducted a series of sedimentation equilibrium experiments as a function of IVa2 and L4-22K concentration (Figure 6). First, we mixed 5 μM L4-22K with 1 μM A-I-II-Cy3, along with either 2.1 or 4 μM IVa2. The resulting equilibrium distributions were described well by a single-ideal species model, returning M_b values for the single, saturated complex of 48.2 (46.5, 49.9) kDa and 50.4 (48.5, 52.3) kDa for 2.1 and 4 μM IVa2, respectively. The fact that the data are well described by a single-ideal species model and the fact that we measure identical M_b values for both IVa2 concentrations indicate we have completely saturated all available IVa2 binding sites on the A-I-II-Cy3 DNA. To further test this interpretation, we performed an additional experiment at 4 μM IVa2 along with 6 μM L4-22K. These data were also described well by a single-ideal species model, returning an M_b of 51.7 (50.0, 53.5) kDa. The fact that this M_b is identical within error to the previously measured values at the lower L4-22K concentration indicates we have also saturated all the L4-22K binding sites on the A-I-II-Cy3 DNA. Thus, we conclude the 5.2 S species identified by sedimentation velocity has an average M_b value of 50.1 ± 1.8 kDa, and that this species represents the saturated A-I-II-Cy3 DNA species; i.e., all of the available L4-22K and IVa2 sites are occupied with ligand. Inspection of Table 2 indicates this complex most likely has a 3:2:1 L4-22K:IVa2:A-I-II-Cy3 (M_b = 50.0 kDa) stoichiometry.

L4-22K and IVa2 Do Not Interact with Each Other in the Absence of the A-I-II-Cy3 DNA. We tested if IVa2 and L4-22K heteroassociate with each other in the absence of the A-I-II-Cy3 DNA substrate by sedimentation velocity. These experiments were conducted in buffer H at 280 nm, and the results are shown in Figure 7. First, the two proteins were analyzed by $c(s)$ separately (at 1.75 μM L4-22K and 1 μM IVa2). Consistent with our previous results,[14,15] IVa2 sediments as a single monomeric species, while the L4-22K protein shows

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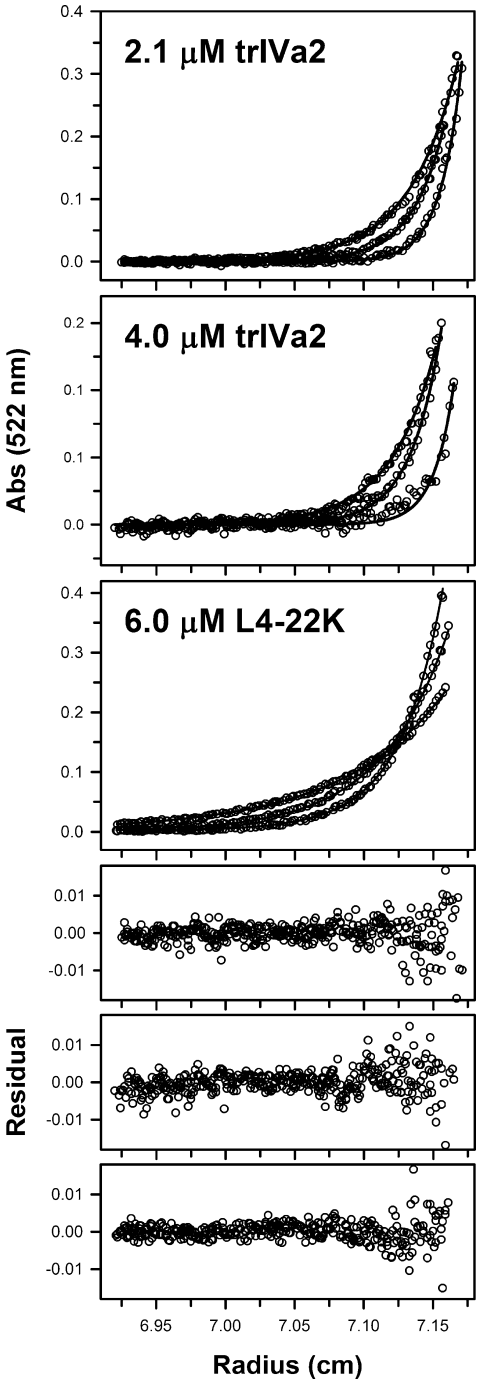

Figure 6. Sedimentation equilibrium experiments to determine the maximal binding stoichiometry of the IVa2 and L4-22K proteins with A-I-II-Cy3 DNA. The top two panels correspond to experiments in which either 2.1 or 4 μM IVa2 was mixed with 1 μM A-I-II-Cy3 DNA along with 5 μM L4-22K (total monomer). The mixtures were loaded into a six-sector centerpiece and then sedimented to equilibrium at 14K, 17K, and 21K rpm, in buffer H at 25 °C. The data were analyzed by NLLS according to a single-ideal species model, returning M_b values of 48.2 (46.5, 49.9) kDa and 50.4 (48.5, 52.3) kDa for 2.1 and 4 μM IVa2, respectively. The panel labeled 6 μM L4-22K corresponds to an experiment in which 4 μM IVa2, 6 μM L4-22K, and 1 μM A-I-II-Cy3 DNA were mixed, loaded into a six-sector centerpiece, and then sedimented to equilibrium at 9K, 11K, and 14K rpm, in buffer H at 25 °C. The NLLS analysis of this data returned an M_b of 51.7 (50.0, 53.5) kDa. The top residual plot corresponds to the data labeled 2.1 μM IVa2; the middle residual plot corresponds to the data labeled 4 μM IVa2, while the bottom residual plot corresponds to the data labeled 6 μM L4-22K.

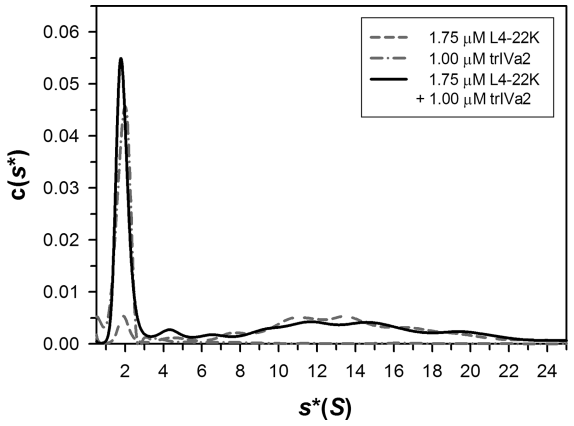

Figure 7. L4-22K and IVa2 do not interact with each other in the absence of the A-I-II-Cy3 DNA. The dashed line shows 1.75 μM L4-22K (buffer H, 25 °C) was subjected to a sedimentation velocity experiment (λ = 280 nm, 40K rpm), and the data were analyzed by $c(s)$ using SEDFIT. As observed previously,[15] the L4-22K protein exists as a heterogeneous mixture of aggregation states. The dotted–dashed line shows 1 μM IVa2 (buffer H, 25 °C) was subjected to a sedimentation velocity experiment (λ = 280 nm, 40K rpm), and the data were analyzed by $c(s)$ using SEDFIT. As observed previously,[14] the IVa2 protein sedimented as a single monomeric species. The solid line shows 1.75 μM L4-22K was mixed with 1 μM IVa2 (buffer H, 25 °C). The mixture was then subjected to a sedimentation velocity experiment (λ = 280 nm, 40K rpm), and the data were analyzed by $c(s)$ using SEDFIT. This experiment provides no evidence of an interaction between the IVa2 and L4-22K proteins; no new peaks are observed, which might indicate a complex has been formed.

the presence of a wide range of species, with s^* values between ~5 and ~25 S. We have already shown that the L4-22K protein self-associates into large aggregates.[15] We have shown this self-association behavior is reversible and is described well by an indefinite, isodesmic self-association model.[15] Next, the two proteins were mixed together at the identical final concentrations and analyzed by $c(s)$. Inspection of the $c(s)$ trace for the mixture indicates no evidence of interaction of these two proteins in the absence of the A-I-II-Cy3 DNA; the $c(s)$ trace is described well by the sum of the two $c(s)$ traces for the individual components. This result indicates that these two proteins do not interact with each other significantly at these concentrations, putting a lower limit on an apparent K_d of ~10 μM.

Does IVa2 Load L4-22K onto Nonspecific DNA? It is known that the conserved CGxG and TTTG motifs present within the A repeats are required for the observation of IVa2-dependent, L4-22K DNA binding, as analyzed by EMSAs.[5,7,9] To assess whether loading of L4-22K onto the A-I-II-Cy3 DNA also requires these motifs, we investigated the assembly of L4-22K and IVa2, by sedimentation velocity, onto a mutant A-I-II-Cy3 DNA, called A-I-II-mut-Cy3, where both the TTTG and CGXG motifs have been mutated to ACAC and ATXC, respectively (generating a nonspecific, relative to IVa2 and L4-22K binding, DNA molecule). We have already characterized the loading of the IVa2 protein onto this construct.[14] We found that IVa2 can still bind nonspecifically to this construct; however, it does so with a relatively high affinity (K_d < ~100 nM).

First, we mixed 0.52 μM IVa2 with 1 μM A-I-II-mut-Cy3 DNA, in buffer H at 25 °C, and subjected the sample to a sedimentation velocity experiment. As we have found previously, we observe a 3.7 S peak corresponding to a 1:1

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IVa2:A-I-II-mut-Cy3 complex (Figure 8). Next, we repeated this experiment including 1 μM L4-22K. Contrary to what we observe when we perform this experiment using the A-I-II-Cy3 DNA, we do not observe a significant shift of the 3.7 S peak. This result indicates that the TTTG and CGXG motifs are required to assemble the IVa2:L4-22K complex onto the DNA, consistent with the published data.[5,7,9]

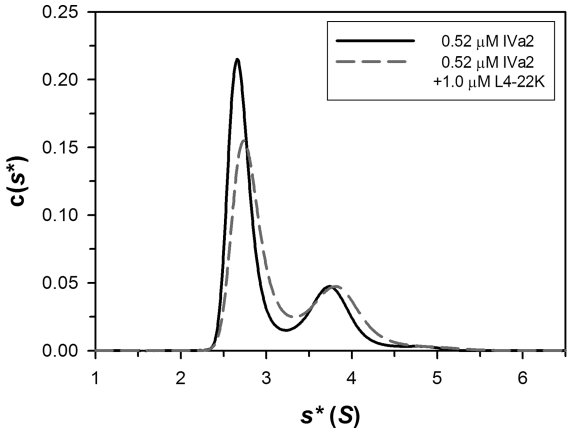

Figure 8. IVa2 cannot load L4-22K onto nonspecific DNA. The solid line shows 0.52 μM IVa2 was mixed with 1.25 μM A-I-II-mut-Cy3 DNA and subjected to a sedimentation velocity experiment at 50K rpm, and the data were analyzed by $c(s)$ using SEDFIT. As observed previously, IVa2 binds nonspecifically to this DNA, generating a 1:1 IVa2:A-I-II-mut-Cy3 species (3.7 S). Next, the same experiment was performed including 1 μM L4-22K. The shape of the $c(s)$ distribution is not significantly altered, indicating IVa2 is not loading L4-22K onto the A-I-II-mut-Cy3 nonspecific DNA.

DISCUSSION

Previous work has shown that the viral DNA packaging reaction in human Ad absolutely requires at least three viral components: the viral DNA packaging sequence, the L4-22K protein, and the IVa2 protein.[5−9] Both IVa2 and L4-22K specifically interact with conserved elements in the packaging sequence, with IVa2 binding to a conserved CGXG motif and L4-22K binding to a conserved TTTG motif.[5,7,9,11,13] For human Ad type 5, the wild-type sequence is complicated and contains multiple copies of the CGXG and TTTG motifs.[2] However, wild-type viral replication does not require the full packaging sequence. Elegant genetic studies have shown that several different combinations of the so-called A repeats can also support wild-type or near wild-type levels of viral replication.[2,6,8,10−12] Of particular interest is a simple packaging sequence construct that possesses two adjacent copies of the A-I-II sequences, which is termed the A-I-II dimer, and supports wild-type viral replication levels.[11] A single copy of the A-I-II segment is not sufficient to support viral replication, implying that cooperative interactions between the two adjacent A-I-II segments within the A-I-II dimer are required for signaling the viral DNA packaging reaction.[11] The arrangement of the conserved IVa2 and L4-22K binding motifs within this construct is shown in Figure 9.

To begin to understand how the IVa2 and L4-22K proteins assemble on this minimal packaging sequence, we have determined the stoichiometries of interaction of the IVa2 and L4-22K proteins onto a DNA containing a single A-I-II segment; i.e., if we think of the A-I-II segment as the fundamental unit of function within the A-I-II dimer, we have determined the stoichiometries of

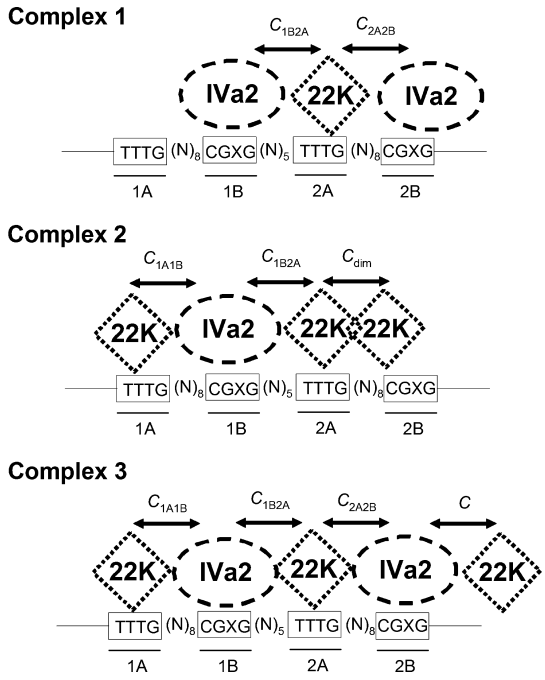

Figure 9. Model for assembly of L4-22K and IVa2 onto the A-I-II-Cy3 DNA. The 6.93, 6.13, and 7.11 S species are proposed to assemble onto the A-I-II-Cy3 DNA as shown in complexes 1−3, respectively. At limiting concentrations of L4-22K relative to the DNA, we observe a species that contains two IVa2 monomers and one L4-22K monomer bound to the A-I-II-Cy3 DNA. To explain how a single bound L4-22K monomer can strip a IVa2 monomer off a 1:1 IVa2:A-I-II-Cy3 complex for construction of complex 1, we propose that heterocooperative interactions exist between adjacently bound IVa2 monomers, indicated as C_{1B2A} and C_{2A2B} in complex 1. Continued titration of the 1:1 IVa2:A-I-II-Cy3 mixture with L4-22K, such that L4-22K is no longer limiting, yields a species that contains three L4-22K monomers, along with a single IVa2 monomer, bound to the A-I-II-Cy3 DNA. To explain why this species is favored energetically as opposed to other possible intermediate ligation states, we propose that L4-22K can self-assemble with itself on the bound A-I-II-Cy3 DNA, and this reaction can compete sterically with binding of IVa2 to the 2B site. Furthermore, at the higher L4-22K concentrations, site 1A can now become occupied with an additional L4-22K monomer. To form complex 3, which corresponds to the maximal 3:2:1 L4-22K:IVa2:A-I-II-Cy3 binding stoichiometry we observed, we can imagine titrating complex 2 with IVa2; as IVa2 levels increase, IVa2 binds to site 2B and disrupts the L4-22K dimer. IVa2 bound to site 2B then serves as a site for loading of an additional L4-22K monomer through similar heterocooperative interactions (labeled C) proposed in complex 1 (labeled C_{2A2B}). This model can explain our observed DNA ligation species and our inability to observe intermediate ligation states, through cooperative interactions between adjacently bound ligands.

interaction of the IVa2 and L4-22K proteins with this unit and can use these results when building physical chemical models to understand the energetics of assembly of these proteins onto the full A-I-II dimer. The ultimate goal of this work is to develop a predictive, physical chemical model for how assembly of these proteins onto the packaging sequence controls the onset of viral assembly, and the work presented here represents the first steps that occur in the virus assembly process.

Model for Cooperative Assembly of IVa2 and L4-22K onto A-I-II-Cy3 DNA. A simple prediction for the saturating stoichiometry for the A-I-II-Cy3 DNA is that a single L4-22K monomer will bind to each TTTG sequence while a single IVa2 monomer will bind to each CGXG sequence; in this case, we

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would predict a saturating L4-22K:IVa2:A-I-II-Cy3 stoichiometry of 2:2:1. However, at saturation (when all binding sites are occupied with the protein ligands), we see that while two IVa2 molecules do indeed bind the A-I-II-Cy3 DNA, three L4-22K monomers are assembled. Furthermore, we are able to detect only a limited number of possible intermediate ligation states. For example, we do not detect significant concentrations of a 1:1:1 L4-22K:IVa2:A-I-II-Cy3 species. These observations suggest heterocooperative interactions that suppress some of the intermediate stoichiometries are present. To interpret these results, we have proposed the model shown in Figure 9.

Our previous work showed that while the binding of the first IVa2 to the A-I-II-Cy3 DNA occurred with a K_d of ~10−100 nM, binding of a second IVa2 occurred with a much weaker K_d of 1.4 ± 0.7 μM.[14] However, the picture changes in the presence of L4-22K. We have shown that under conditions where the L4-22K protein is limiting, we are able to form a 1:2:1 L4-22K:IVa2:-A-I-II-Cy3 complex with high affinity. In other words, when a single L4-22K monomer binds to the A-I-II-Cy3 DNA, the affinity for placing a second IVa2 onto the A-I-II-Cy3 DNA must become much stronger; for the concentrations of IVa2 and A-I-II-Cy3 DNA used in our experiments, simple mass conservation considerations indicate the second IVa2 is being stripped off other A-I-II-Cy3 DNA molecules and subsequently loaded onto the A-I-II-Cy3 DNA to form the 1:2:1 L4-22K:IVa2:A-I-II-Cy3 complex. Thus, one function of L4-22K is to modulate cooperativity between adjacently bound IVa2 proteins. Investigation of the primary structure of the A-I-II DNA suggests this complex corresponds to a single L4-22K bound to the central TTTG site (labeled 2A in Figure 9). We propose that cooperative interactions between this bound L4-22K and the 5′- and 3′-bound IVa2 (to CGXG motifs 1B and 2B, respectively) stabilize this complex. Next, continued titration with L4-22K (while maintaining a 1:1 IVa2:A-I-II-Cy3 DNA ratio) results in the binding of two additional L4-22K monomers to the complex but also results in the release of a IVa2 monomer. We propose that one of the L4-22K monomers binds to the 5′-TTTG site (labeled 1A in Figure 9), while the other dimerizes with the L4-22K monomer on site 2A. We further propose that this dimerization reaction competes with heterocooperative, protein−protein interactions between IVa2, bound to site 2B, and L4-22K, bound to site 2A. The fact that L4-22K assembles with itself[15] supports this possibility and suggests this reaction may also serve a functional role as a competitor with L4-22K−IVa2 heteroprotein interactions on the viral DNA. Finally, upon titration of the 3:1:1 complex with IVa2, enough IVa2 finally becomes available to drive binding to both CGXG sites; binding of IVa2 to site 2B disrupts the L4-22K dimer bound to site 2A. To explain the presence of three L4-22K monomers bound to this saturated complex, we propose that sites 1A and 2A each are bound by an L4-22K monomer, while the third L4-22K monomer binds to the end of the IVa2 bound to site 2B, through the same proposed heterocooperative interaction that exists between sites 1B and 2A.

Our data show that even though the L4-22K protein polymerizes into large oligomeric complexes[15] at the concentrations utilized in these experiments, these large structures do not remain stably bound to the IVa2:A-I-II-Cy3 DNA complexes. From a thermodynamic perspective, this means that either these structures cannot bind with appreciable affinity to the IVa2:A-I-II-Cy3 DNA complex or if they can bind, they are not stable; in other words, if large L4-22K oligomers bind to the IVa2:A-I-II-Cy3 DNA complexes, they dissociate, leaving either monomers or dimers bound to the IVa2:A-I-II-Cy3 DNA complex. It is not possible to determine which of these paths (or if a combination of both) occurs from equilibrium experiments alone. Therefore, it is possible these large structures do not play a direct mechanistic role in the specific assembly on packaging sequence DNA; alternatively, the fundamental role of L4-22K polymerization may be simply placing an upper limit on the free L4-22K monomer concentration in the cell.[21] This possibility is currently under investigation in our laboratory.

Hydrodynamic Properties of the L4-22K:IVa2:A-I-II-Cy3 Complexes. Because we were able to determine the L4-22K and IVa2 ligation stoichiometries for binding of the A-I-II-Cy3 DNA, we were able to calculate the frictional coefficient ratios (f/f_0) for each L4-22K:IVa2:A-I-II-Cy3 complex. The f/f_0 value represents the experimentally determined frictional coefficient of the complex divided by the predicted value for a hydrated sphere of identical partial specific volume and molecular weight and has a lower limit of 1. Values close to 1 indicate the complex adopts essentially a spherical shape in solution; values significantly greater than 1 indicate the complex adopts a more extended (i.e., nonspherical) shape. Table 3 shows the calculated frictional coefficient ratios for each individual component separately, as well as each measured A-I-II-Cy3 DNA ligation state.

Comparison of these values shows that both complexes that contain three bound L4-22K monomers have significantly more extended structures in solution ($f/f_0 \sim 1.55$) than the 1:1 IVa2:A-I-II-Cy3 and 1:2:1 L4-22K:IVa2:A-I-II-Cy3 complexes ($f/f_0 \sim 1.35$). While the mechanistic roles that IVa2 and L4-22K play in viral assembly are not understood, it is clear both proteins and their interactions with the packaging sequence DNA are required to package the viral DNA.[7,24,25] Furthermore, recent immunogold labeling experiments have shown the presence of six to eight copies of IVa2 at a unique vertex of the assembled viral particle,[26] which suggests IVa2 may play a role as a terminase and/or portal protein by catalyzing the insertion of the Ad genome into the interior of a preassembled capsid, although other explanations are also possible.[26] On the basis of this, it is tempting to speculate that L4-22K levels may play a role in switching on virus assembly and DNA packaging; at low L4-22K concentrations (more compact packaging sequence; $f/f_0 = 1.35$), the IVa2:packaging sequence DNA complex cannot initiate capsid assembly and DNA packaging, while at saturating L4-22K levels (more extended packaging sequence; $f/f_0 = 1.55$), the IVa2:packaging sequence DNA complex undergoes a conformational change (f/f_0 increases from 1.35 to 1.55), which activates DNA packaging. One possibility is that this conformational change might reflect the opening of a portal:terminase complex to allow entry of the linear DNA genome into the capsid interior, although there are no direct data to support this idea.

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Notes

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