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Minireview

Are weak protein–protein interactions the general rule in capsid assembly?

Adam Zlotnick*

University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

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How do spherical viruses assemble?

Assembly of spherical viruses, which may involve hundreds of components, is not well understood on a biological or a physical basis. Recently, tremendous progress has been made with alphaviruses and retroviruses by efforts to capture intermediates in their respective assembly reactions. Using different strategies, investigators showed that when association was too weak to support assembly of monomers, assembly could be induced by dimerizing the capsid protein or by adsorbing capsid protein to a nucleic acid scaffold. In this review we will summarize those results and compare them to assembly of experimentally more tractable hepatitis B virus (HBV) and to mathematical models of assembly. These fundamental models show that in vitro dimerization of subunits is expected to favor capsid assembly, independent of the role of dimers in vivo. This analysis suggests that weak association energy may be a mechanism for in vivo regulation of assembly by, for example, dimerization factors and/or scaffold.

In vitro assembly of an alphavirus core

Alphaviruses (e.g., Sindbis virus, SINV) are enveloped viruses that assemble a $T = 4$ icosahedral core in the cytoplasm (Fig. 1A). For SINV, this core consists of 240 copies of the 264 residue capsid protein (CP) and viral RNA. The N-terminal 72 residues of CP are particularly basic and likely provide most of the interaction with nucleic acid. Recently, an efficient in vitro assembly system has been developed using SINV CP expressed in *Escherichia coli* (Tellinghuisen et al., 1999). It was found that CP is a monomer in solution and requires nucleic acid (NA), either RNA or single-stranded DNA, for assembly. A nearly full-length CP (residues 19–264) was competent to assemble into capsid-like particles (CLPs) using viral RNA or DNA oligonucleotides as short as 14 bases (Fig. 1B). The CLPs were very similar to native particles (Mukhopadhyay et al., 2002). Efficient assembly with the DNA oligos was observed using a 48-mer and a stoichiometry of two CPs per oligo. Short oligonucleotides were not bound as efficiently. Surprisingly, excess CP inhibited assembly of CLPs, suggesting free protein could interact with NA-bound protein.

An N-terminal truncation of CP, CP81–264, was discovered that was unable to assemble into CLPs on its own (in the presence of NA), but could participate with full-length CP in NA-driven assembly of CLPs (Tellinghuisen et al., 1999). Though CP81–264 lacked most of the basic N-terminal sequence, it was still able to bind NA and, clearly, still able to interact with full-length CP.

CP81-264 also proved useful for identifying contacts between CP molecules in the context of a capsid. When CP81-264 was bound to a DNA 48-mer, a pair of lysines (K250) from adjacent proteins was susceptible to chemical crosslinking (Tellinghuisen and Kuhn, 2000). Unlike monomeric CP81-264, the purified dimeric protein was assembly-competent in the presence of NA (Fig. 1C)—dimerization rescued the assembly activity of this truncation mutant (Tellinghuisen et al., 2001). CLPs were also assembled from CP81-264 that was crosslinked with a labile reagent; when the crosslink was broken, the CLPs dissociated into disordered aggregate. Correct assembly of SINV CLPs depended on both protein–NA and protein–protein interactions. The normal protein–protein interactions could be replaced by crosslinking. Though CP–CP interactions were insufficient for assembly in the absence of NA, some interactions were required for capsid stability.

Originally, it was suggested that the crosslinked dimer

* OUHSC, Biochemistry and Molecular Biology, P.O. Box 26901, BRC464, Oklahoma City, OK 73190. Fax: +1-405-271-3910.

E-mail address: adam-zlotnick@ouhsc.edu

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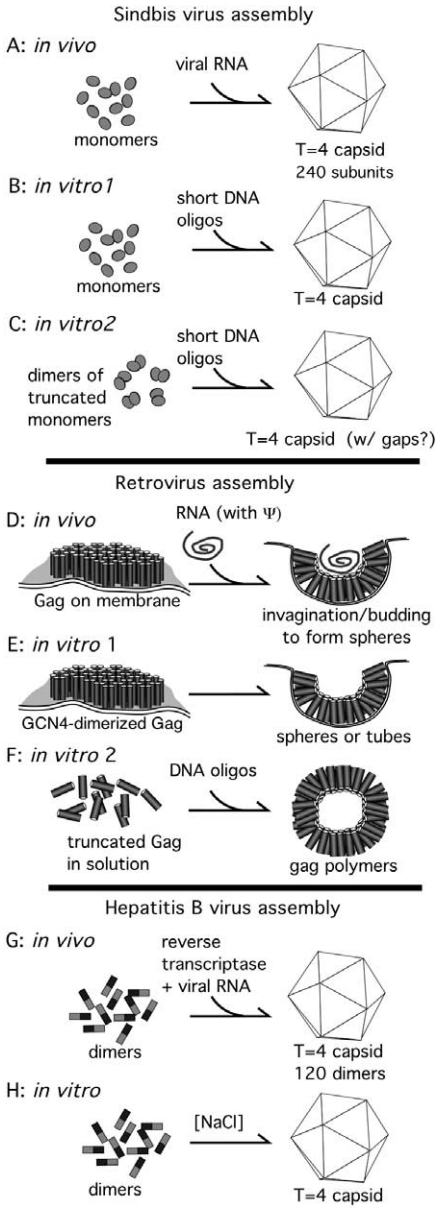


Fig. 1. Schematic models of virus assembly. (A) In vivo, 240 copies of SINV CP condense around viral RNA in the cytoplasm to form the $T = 4$ icosahedral nucleocapsid. (B) Purified CP assembles in response to DNA oligonucleotides to form CLPs (Tellinghuisen et al., 1999). (C) Chemically dimerized truncated CP, though it lacks much of the nucleic acid binding domain, also assembles into $T = 4$ CLPs in response to DNA oligos (Tellinghuisen et al., 2001). (D) In vivo, the Gag protein of type C retroviruses associate with the plasma membrane, bind RNA carrying the ψ sequence, and bud from the membrane. In the absence of viral RNA, host RNA lacking ψ is encapsidated (Muriaux et al., 2001). (E) Assembly of membrane-bound Gag can be induced in the absence of RNA by replacing the RNA-binding NC domain with a GCN4 leucine zipper to dimerize the protein (Johnson et al., 2002). (F) Soluble truncations of Gag, lacking the MA domain, assemble into virus-like particles around DNA oligomers (Feng et al., 2002; Ma and Vogt, 2002; Yu et al., 2001). (G) In vivo assembly of HBV cores is cytoplasmic: 120 CP dimers assemble around a complex of viral RNA and reverse transcriptase. (H) The kinetics (Zlotnick et al., 1999) and thermodynamics (Ceres and Zlotnick, 2002) have been characterized for NaCl-induced assembly of empty HBV capsids.

could reflect a natural intermediate in the assembly process. However, the current model of SINV, obtained by fitting the crystal structure of monomeric CP into cryo-EM density, indicated that the crosslink connected proteins that are adjacent around icosahedral fivefold and quasi-sixfold vertices (Mukhopadhyay et al., 2002). Since a fivefold vertex cannot be built exclusively of such dimers, it is likely that the CLPs assembled from dimeric CP81-264 have gaps, though they were stable enough to survive native agarose gel electrophoresis and negative stain EM.

Retrovirus assembly

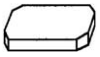
In vivo, retrovirus assembly begins with formation of a spherical provirion; for type C retroviruses this takes place on the cytoplasmic face of the host's cell membrane (Fig. 1D). The capsid is constructed from about 2000 copies of the Gag protein, a small number of Gag–Pol fusions, and two copies of viral RNA. Viral protease may be part of Gag or it may be expressed as Gag fusion, depending on the retrovirus. After assembly, the viral protease cleaves Gag and Gag fusions into their component proteins and the capsid collapses into the mature form: membrane binding matrix (MA) protein, an interior capsid (CA) protein (which has a cone-shaped quaternary structure in mature HIV), and the nucleocapsid (NC) protein, which binds RNA in part through two zinc fingers. We focus on results from two laboratories that investigate the role of the oligomeric state of Gag on capsid assembly.

An in vitro system has been developed to study Rous sarcoma virus (RSV) provirion formation, where the N-terminal membrane binding domain (MBD) and the C-terminal protease (PR) domain were deleted to produce ΔMBDΔPR (Yu et al., 2001). Similar to SINV CP, the resulting protein bound RNA or DNA oligomers to assemble into provirion-like particles (Fig. 1F). These retrovirus-like particles had about 1500 copies of ΔMBDΔPR. The basic residues of the NC domain were required for assembly, but zinc ions for the two zinc finger motifs were not.

The DNA length dependence for binding by ΔMBDΔPR was used to determine the number of nucleotides bound by a Gag protein (Johnson et al., 2002). In the presence of zinc, the site size was 8 nucleotides. In the absence of zinc, it was 11 nucleotides, suggesting that zinc induced a more compact conformation for NC. These studies also revealed that a DNA oligomer had to be at least two site sizes in length to drive assembly, even though shorter oligomers were bound by Gag without assembling. This result suggested that a Gag dimer might be an important intermediate in assembly.

This hypothesis was tested by replacing the NC and PR domains of RSV Gag with a GCN4 leucine zipper (Johnson et al., 2002), which forms a dimeric coiled-coil. The resulting protein was expected to form stable dimers that would bind membranes but not DNA. Expression of MA-CA-Zip

Table 1
Subunit geometry dramatically affects assembly

Model	12-mer of pentagons	30-mer of dimers	HBV	Capsid of trivalents	Capsid of trivalent$_2$
Subunit					
Contacts/subunit	5	4	4	3	4
Symmetry (s)	5	2	2	1 (or 3)	2
Subunits/capsid (N)	12	30	120	1500	750
Exponent	30/11	60/29	240/119	2225/1499	1500/749
	~3	~2	~2	~1.5	~2
Statistical term per subunit ($\pi S^{1/1-N}$)	0.25	0.56	0.52	1 (or 0.33)	0.50
$K_D$apparent ($K_{contact}{}^{-1}$ = 1 mM)	0.0016 μM	0.35 μM	0.46 μM	35 μM (12 μM)	0.49 μM

Note. The number of contacts per subunit (c), symmetry (s), and subunits per capsid (N) are used to calculate the $K_D$apparent, the threshold concentration for virus assembly. For calculating $K_D$apparent in our retrovirus models, we assume 1500 trivalent subunits or 750 trivalent$_2$ subunits per capsid. Calculation of the statistical term for the trivalent subunit assumes no symmetry or, parenthetically, threefold symmetry. Note that $K_D$apparent ~ $K_{contact}{}^{exponent}$, where the exponent is ([N*c]/2)/(N-1).

in insect cells resulted in the formation and budding of spherical capsids (Fig. 1E). A nearly full-length NC-containing Gag, lacking PR, formed similar particles. Changing the connection between the zipper and CA domains changed the morphology of the resulting particles from spheres to tubes. These results indicated that replacing the NC domain with a dimer-forming leucine zipper was sufficient to support assembly on a membrane and affect the morphology of the resulting product. The authors suggested that dimerization could trigger a conformational change enhancing Gag assembly (Ma and Vogt, 2002). (Other labs have replaced the NC domain of human immunodefficiency virus (HIV) with leucine zippers to produce virus-like particles, but they focused on the role of the remainder of Gag on assembly rather than the quaternary structure of NC.) As with SINV, one must also ask whether Gag dimers are serendipitous or an important assembly intermediate?

Studies of oligonucleotide-induced assembly of HIV show that more than dimerization is required for provirion assembly (Feng et al., 2002). First, it was observed that only a narrow range of DNA oligomer concentrations actually supported assembly; as with other Gag-DNA oligo studies, excess DNA inhibited formation of CLPs. Second, bound DNA freely exchanged with exogenous DNA. The effect of nucleic acid was further investigated with DNA oligos that have different assembly properties, based on the observation that HIV Gag has a sequence preference for TG repeats over A oligomers. With a 40-mer that had two 10 nucleotide TG sequences flanking either side of A_{20}, the oligomer had much the same behavior as a TG 40-mer or an A 40-mer. However, if the internal sequence was $(TG)_{10}$ with A_{10} ends, the oligomer had much stronger inhibitory effects than other oligomers. These data support two conclusions: (i) Protein–protein interaction is weaker than protein–DNA interaction, i.e., Gag will distribute on excess DNA rather than form assembly-active oligomers. (ii) DNA binding alone does not activate Gag assembly: excess DNA is inhibitory to assembly and overhanging DNA interferes with assembly in oligomers with a higher affinity (TG) center. So how does DNA (or RNA) induce assembly? The same lab observed that assembly of Moloney murine leukemia virus required RNA for assembly (Muriaux et al., 2001) and that it would use random host RNA in the absence of RNA with the Ψ packaging signal (Fig. 1D). The resulting particles however were readily destabilized by nucleases. The investigators suggested that RNA was acting as a scaffold.

We are left with seemingly paradoxical observations that dimerization is sometimes, but not always, sufficient for assembly.

Modeling a simplest case of assembly

Taking a step back, models are required for interpretation of complicated reactions. A model provides a means for calculation of reaction constants when the model predictions match experimental observations. When the reaction's behavior differs from that of the model, the sources of the difference can be identified and incorporated into a model that better reflects the system under study. We developed simplest case models based on geometric solids; two examples are a dodecahedron built from 12 pentagonal subunits and an icosahedron built from 30 rectangular "dimers" (Table 1) (Endres and Zlotnick, 2002; Zlotnick, 1994). In both cases, subunits were multivalent (five contacts per pentagon, four per dimer). The latter model was designed to resemble HBV.

These models were used to calculate equilibrium concentrations of reactants, intermediates, and products and to simulate assembly kinetics. The equilibrium constant between any two consecutive intermediates is proportional to the number of new intersubunit contacts, assuming that all contacts are (quasi-) equivalent and that one unit of association energy accrues per contact. Kinetic simulations require choosing a reaction path(s) and imposing rate constants; we have assumed that the capsid grows one subunit

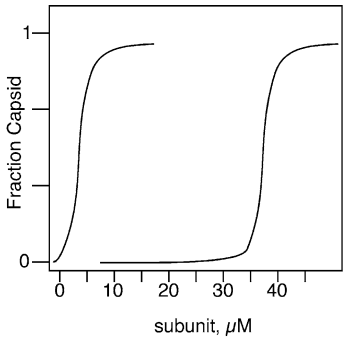


Fig. 2. Concentration dependence predicted for capsid assembly. These simulations show the effect of dimerizing subunit on assembly. The curves are for a capsid of 750 tetravalent (trivalent$_2$) subunits with $K_D$apparent = 0.5 μM (left) and a capsid of 1500 trivalent subunits with $K_D$apparent = 35 μM (right) but the same per contact association energy (see Table 1 for details). The graph shows the fraction of protein that has assembled into capsid at equilibrium as a function of total protein concentration.

at a time. Backward reaction rates are a function of the forward rate and the association constant. A small statistical coefficient modifies the microscopic rates and association constants to account for the numerous degenerate paths for capsid assembly.

These models predict that assembly reactions rapidly approach equilibrium and that the association energy between subunits must be restricted to a relatively narrow window for successful assembly. If the contact energy is too strong, there will be a kinetic trap with many partially assembled capsids and few of the free subunits that are required to complete the reaction. If it is too weak, subunits will not assemble. In the simplest case, assembly resembles a two-state reaction: almost entirely capsids and free subunits with very low concentrations of intermediates. Assembly reactions are predicted to be extremely concentration dependent (Fig. 2). Below a system dependent concentration, referred to as the $K_D$apparent, assembly is negligible; above it, most of the additional subunits are assembled. More formally, $K_D$apparent is the concentration where free subunit and capsid concentrations are equal. If the geometry of the capsid is known, it is a straightforward process to extract the average per contact association constant (K_{contact}) from K_{capsid} or $K_D$apparent (see equations below).

The equations below show that that subunits which have more contact surfaces will assemble at lower protein concentrations, i.e., the more contacts per subunit (whether the subunit is a monomer or an oligomer), the lower the $K_D$apparent and the more stable the capsid for a given K_{contact} (see Table 1). We can calculate K_{capsid} (eq. 1) from the concentrations of dimer and capsid (HBV capsids are assembled from $N = 120$ dimeric subunits). Because of the size of the numbers, all calculations are performed using logarithms. Since each of the N subunits has four contact surfaces (c), we can say that K_{capsid} is the product of (Nc)/2 "equivalent" units of K_{contact}, or K_{contact}^{240} for HBV (Eq 2). Of course, K_{contact} will be an average value when interactions between subunits are not perfectly equivalent. The symmetry of the subunit (s), typically one- two-, or five-fold (see Table 1 for examples), has a direct effect on the degeneracy of the association reaction (πS, Eq 3), analogous to reactions where there are multiple independent binding sites (Zlotnick, 1994). However, πS makes a relatively small contribution to K_{capsid} (Eq. 2). K_{D}apparent is intimately related to K_{contact} and K_{capsid} (eq 4):

$$K_{\text{capsid}} = [\text{capsid}]/[\text{subunit}]^N \quad (1)$$

$$K_{\text{capsid}} = (\pi S K_{\text{contact}}^{Nc/2}) \quad (2)$$

$$\pi S = s^{N-1}/N \quad (3)$$

$$K_D\text{apparent} = K_{\text{capsid}}^{1/1-N} = \pi S^{1/1-N} K_{\text{contact}}^{(Nc/2)/(1-N)} \quad (4)$$

Assembly of HBV capsids is driven by weak association energy

In vivo, the HBV capsid assembles around a complex that includes viral RNA and viral reverse transcriptase to form the HBV core (Fig. 1G). In vitro assembly of empty capsids using the dimeric 149 residue assembly domain of the core protein can be induced by high NaCl concentration (Fig. 1H). In HBV, subunit dimers are stable in solution. Assembly of HBV conforms to thermodynamic and kinetic predictions of the simplest case assembly models (Ceres and Zlotnick, 2002; Zlotnick et al., 1999). Assembly reactions appear to contain only dimer and capsid and show the predicted steep concentration dependence. The $K_D$apparent for HBV, 0.5 to 15 μM depending on assembly conditions, corresponds to a value for $1/K_{\text{contact}}$ of 1 to 5 mM. This is a remarkably weak association constant, yet capsids assemble because subunits are multivalent. The temperature dependence of assembly indicates that the reaction is entropy driven, which is consistent with the buried hydrophobic surface observed in the crystal structure. Capsids appear even more stable than indicated by $K_D$apparent because of their hysteresis to dissociation, i.e., a steep energy barrier inhibits dissociation, which suggests the presence of a catalyst in vivo (Singh and Zlotnick, 2003). The principles demonstrated with HBV can be extended to other systems. Deviation from the predictions of the basic model can identify important features of an experimental reaction and can be incorporated into more specific and sophisticated models.

Models show why dimerizing subunits in alpha- and retroviruses can induce assembly

The above studies demonstrate cases where dimers of subunits support assembly under conditions where mono-

mers do not. However, it does not follow that dimers are a fundamental building block in the assembly process. The assembly models show that there is a physical basis by which dimerization would be expected to enhance assembly.

To examine the effect of dimerization, we first need a model of subunit geometry. The geometry of the different subunits in Table 1 is representative of different capsid proteins. The pentagonal subunit is similar to a picornavirus 14S complex of five Vp0-Vp1-Vp3 heterotrimers or a papovavirus penton. The tetravalent rectangular subunit is topologically similar to the HBV dimer beside it. As a model for retroviral Gag, we suggest a trivalent subunit, though this may understate the true number of intersubunit contacts of Gag. A dimer of trivalent subunits topologically reflects the contacts seen in image reconstructions of CA oligomers (Li et al., 2000; Mayo et al., 2003).

To examine how different geometries affect assembly, we need only calculate $K_D$apparent, the threshold concentration required for efficient assembly. This requires values for K_{contact}, the number of contacts per subunit (c), the number of subunits per capsid (N), and reaction degeneracy (πS), as outlined in Eq. 1 to 4. For Table 1, we use a weak $(K_{\text{contact}})^{-1}$ of 1 mM, about the same as the value experimentally measured for HBV.

The more contacts a subunit makes, the more stable the resulting capsid ($K_D$apparent, Table 1). For $(K_{\text{contact}})^{-1} = 1$ mM, the $K_D$apparent changes by a factor of about 100 for each additional contact per subunit, from 1.6 nM for a pentavalent subunit up to 35 μM for a asymmetric trivalent subunit. Dimerizing two trivalent subunits generates a tetravalent subunit, decreasing the number of subunits per capsid by a factor of 2 and decreasing $K_D$apparent by a factor of 70 to 0.49 μM. This hypothetical reaction correlates with the dimerization of SINV or retrovirus subunits to activate assembly. Stronger K_{contact} enhances the effect of "number of contacts" on $K_D$apparent. By comparison to the number of contacts per subunit, the number subunits per capsid has a very modest effect on $K_D$apparent; consider the two examples of tetravalent subunits in Table 1: a 30-mer and HBV with 120 subunits.

Summary

Weak protein–protein interactions appear to be a common theme in virus assembly. Weak interactions can be enhanced by binding subunits to a scaffold or by oligomerizing them. For any virus built of monomers, there will be many ways that subunits can interact as dimers. Dimers may exist only in the context of the capsid or may be fundamental to assembly, as is the case with HBV. For SINV, recent evidence suggests that the crosslinked dimer is not the fundamental building block of assembly. However, SINV dimers will still be important in the assembly process.

Are the dimers formed by Gag and DNA oligonucleotides fundamental to assembly or do they mimic a transient intermediate in the reaction? Given our improved understanding of the effect of dimerization, the two views on NA-induced retroviral assembly can be reconciled. Dimerization of Gag (and SINV CP) can be enhanced by binding to NA, in part by elevating the local protein concentration and in part by correctly orienting the protein for dimerization. In this way, NA acts as a scaffold that promotes local and global quaternary interactions: dimerization and capsid formation, respectively. Similarly, HIV assembly was enhanced when a Gag–Gag interaction domain of Mason–Pfizer monkey virus, termed the internal scaffolding domain, was incorporated in HIV Gag (Sakalian et al., 2002). If dimerization truly activates Gag for assembly, K_{contact} (proportional to the net association energy per contact) will be stronger for dimer than monomer. On the other hand, if dimerization represents the strongest of several distinct contacts, the average K_{contact} measured for assembly with preformed dimer will actually be weaker than a K_{contact} measurement that includes dimer formation. Only a quantitative analysis will be able to discern whether Gag dimers are a fundamental species or if the decreased K_D apparent observed with preformed dimers (by leucine zippers or oligonucleotides) is only an effect of increased contact number. However, the underlying observation for the viruses discussed in this minireview (and most others) is that their capsid proteins associate through locally weak interactions to form globally stable structures. Weak interactions between capsid proteins minimize formation of kinetic traps, allow a greater degree of regulation of assembly, and may be essential for viruses where dissociation is part of the virus life cycle.

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