Kinetic Models of Assembly*

Observed Hysteresis of Virus Capsid Disassembly Is Implicit in Kinetic Models of Assembly*

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For many protein multimers, association and dissociation reactions fail to reach the same end point; there is hysteresis preventing one and/or the other reaction from equilibrating. We have studied in vitro assembly of dimeric hepatitis B virus (HBV) capsid protein and dissociation of the resulting T = 4 icosahedral capsids. Empty HBV capsids composed of 120 capsid protein dimers were more resistant to dissociation by dilution or denaturants than anticipated from assembly experiments. Using intrinsic fluorescence, circular dichroism, and size exclusion chromatography, we showed that denaturants dissociate the HBV capsids without unfolding the capsid protein; unfolding of dimer only occurred at higher denaturant concentrations. The apparent energy of interaction between dimers measured in dissociation experiments was much stronger than when measured in assembly studies. Unlike assembly, capsid dissociation did not have the concentration dependence expected for a 120-subunit complex; consequently the apparent association energy systematically varied with reactant concentration. These data are evidence of hysteresis for HBV capsid dissociation. Simulations of capsid assembly and dissociation reactions recapitulate and provide an explanation for the observed behavior; these results are also applicable to oligomeric and multidomain proteins. In our calculations, we find that dissociation is impeded by temporally elevated concentrations of intermediates; this has the paradoxical effect of favoring reassembly of those intermediates despite the global trend toward dissociation. Hysteresis masks all but the most dramatic decreases in contact energy. In contrast, assembly reactions rapidly approach equilibrium. These results provide the first rigorous explanation of how virus capsids can remain intact under extreme conditions but are still capable of “breathing.” A biological implication of enhanced stability is that a triggering event may be required to initiate virus uncoating.

Hysteresis, the lagging of effect behind cause (1), operationally defined as a failure of opposing reactions to equilibrate, can be an impediment to understanding the stability of macromolecular complexes. Examples of hysteresis include DNA melting and annealing as well as association-dissociation reactions for trimeric collagen fibrils (2), SNARE (soluble N-ethylmaleimide factor attachment protein receptor) complexes (3), and viruses (4, 5). In the course of investigating assembly of hepatitis B virus (HBV),† we have observed a marked hysteresis between association and dissociation and identified a mechanism for hysteresis that is internally consistent with our understanding of the assembly process.

HBV is an enveloped DNA virus with an icosahedral core. Although it is found in two sizes (6), most HBV capsids (the protein shell of the core) are complexes of 120 homodimeric capsid proteins arranged with T = 4 quasi-symmetry (7, 8). A relatively rare smaller capsid is composed of 90 dimers. Image reconstruction of cores from HBV and homologous hepatitis viruses are essentially identical to capsids from a bacterial expression system (9) and to capsids assembled in vitro from the assembly domain of the capsid protein (10). The full-length capsid protein has 183 residues including the C-terminal RNA binding domain, which has 34 residues. We refer to dimers of the first 149 residues, which include the linker sequence that connects assembly and RNA binding domains (11), as Cp1492. The dimers are tetravalent; in the T = 4 capsid they are arranged as a network of 5-fold and quasi-6-fold vertices held together by 240 very similar quasi-equivalent contacts (12).

We are interested in the mechanisms of capsid assembly and dissociation. To interpret experimental observations, we have developed testable models of assembly reactions (13–15); capsid assembly is not well described using mathematical models developed for crystal or filament formation (cf. Ref. 16). Our models describe assembly as a cascade of low order reactions leading to formation of a closed polymer of specified size.

In vitro HBV capsid assembly is consistent with the predictions of the simplest case of the assembly model. Cp1492 assembles into capsids spontaneously in response to protein concentration and ionic strength (8, 10, 14). Assembly can be observed by light-scattering, fluorescence, and size exclusion chromatography (SEC). As predicted from the model, intermediates are rare and kinetics are sigmoidal; the sigmoidal shape occurs because of the time required to accumulate intermediates necessary to support assembly of subsequent intermediates and eventually capsid. A model-based analysis of assembly kinetics indicates that assembly is nucleated by a trimer of Cp1492 (14), which is particularly compatible with the HBV capsid geometry. Model studies predicted that weak interaction energies between multivalent subunits would be sufficient to form a stable particle and that assembly reactions can closely approach equilibrium (13, 15). We found that the interaction energy between adjacent dimers is on the order of −3 to −4 kcal mol−1 (17), a value that is in qualitative agreement with the prediction from the model and the energy calculated from the HBV crystal structure (12).

In the course of our studies on HBV, we became interested in

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† The abbreviations used are: HBV, hepatitis B virus; SEC, size exclusion chromatography; GuHCl, guanidine HCl; LS, light scattering.
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EXPERIMENTAL PROCEDURES

Capsid Preparation—The dimeric HBV capsid protein assembly domain, Cp149, was expressed in Escherichia coli and purified as described (18). Capsids for dissociation experiments were assembled by adding NaCl to Cp149 to final concentrations of 25 μM Cp149, 25 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.5 M NaCl (assembly buffer) then incubating at 4 °C for 24 h. Capsids were isolated by SEC using a Superose-6 column equilibrated with assembly buffer. Fractions were pooled and concentrated using Centricon YM-30 filters (Amicon Biosciences, Millipore Corp., Bedford, MA) and dialyzed against assembly buffer. Protein concentration was determined by absorbance using ε280 of 60,900 M−1 cm−1.

Dilution Experiments—Purified capsids were diluted to final protein concentrations of 0.5 and 30 μM in 25 mM HEPES, pH 7.5, 0.15 M NaCl, 5 mM dithiothreitol and incubated at 21 °C for 2–5 days. The capsid concentration was quantified using SEC using a Superose-6 column equilibrated with the same buffer on a SMART chromatography system (Amersham Biosciences).

Capsid Dissociation by Chaotropes—Guandine HCl (GuHCl) or urea was added to purified capsids in the presence of specified NaCl concentrations and incubated for up to 48 h. In practice, most experimental measurements were made after 24 h; no significant change was observed at longer times. Experiments with urea were conducted in 50 mM Tris-HCl, pH 7.5, rather than HEPES to scavenge reactive cyanate from decomposing urea.

Trypsin fluorescent at 21 °C was observed with a SPEX Fluoromax fluorometer (Edison, NJ) using a 3-mm path length cuvette (Hellma, Forest Hills, NY). Excitation and emission wavelengths were 290 and 324 nm, respectively. Light-scattering (LS) data were recorded with excitation and emission wavelengths at 320 nm. A 1.0 neutral density filter (Melles Griot, Irvine, CA) was in the excitation path for all experiments.

Circular dichroism spectra were measured using a JASCO J-715 spectropolarimeter. All spectra were obtained with 10 μM Cp149 in 25 mM HEPES at 21 °C using a 1-mm cuvette (Hellma).

Data Analysis—Raw data from trypsin fluorescent, LS, and SDV data could not be directly compared because fluorescent and LS were differentially sensitive to chaotrope concentration. Scaling data sets was facilitated by treating dissociation as a two-state mixture of capsid and dimer. This simplification is supported by data described under "Results." The normalized and base line-corrected data are expressed in terms of mass fraction capsid (mfc),

\[
\text{mfc} = \frac{F_{\text{observed}} - F_{\text{denatured}}}{[\text{denatured}](f_{\text{capsid}} - f_{\text{dimer}})}
\]

(Eq. 1)

where \(F_{\text{observed}}\) is the fluorescence intensity measured at a given concentration of urea or guandine HCl. The coefficients \(f_{\text{capsid}}\) and \(f_{\text{dimer}}\) were the fluorescence of capsid and dimer per concentration of denaturant; these were obtained by linear extrapolation of pre- and post-transition base lines, respectively. Because of the inherent noise of the raw data, the coefficients for the background subtraction were averaged from seven experiments. An identical treatment was used for LS data.

The capsid and dimer concentrations calculated from the mass fraction capsid were used to determine the values for the capsid association constant, \(K_{\text{associ}}\), and the per contact association constant, \(K_{\text{contact}}\). \(K_{\text{associ}}\) was calculated based on the equilibrium expression for assembly of capsid from 120 dimers (Equation 2). This was more conveniently handled in logarithmic form (Equation 3). Calculation of \(K_{\text{contact}}\) (Equation 4) is based on the geometry of the capsid (13) and the assumption that quasi-equivalent contacts form with about the same association constant (17). The 120 tetraivalent dimers in the HBV capsid share 240 contacts; the degeneracy inherent in capsid assembly is (2119/120) (13, 15). In the equations below, \(R\) is the gas constant of 1.987 cal/mole degree, and \(T\) is 294 K for these experiments.

\[
K_{\text{associ}} = \frac{[\text{capsid}][\text{Cp149}]}{[\text{Cp149}][\text{dimer}]} \quad \text{(Eq. 2)}
\]

\[
\ln K_{\text{associ}} = \ln([\text{capsid}]) - 120 \times \ln([\text{Cp149}]) \quad \text{(Eq. 3)}
\]

\[
K_{\text{contact}} = \exp(\ln K_{\text{associ}} - \ln([\text{Cp149}]/120))/240 \quad \text{(Eq. 4)}
\]

\[
\Delta G_{\text{contact}} = \frac{RT\ln K_{\text{contact}}}{f} \quad \text{(Eq. 5)}
\]

\[
\Delta G_{\text{contact}} = \Delta G_{\text{contact}} + \ln m[\text{urea}] \quad \text{(Eq. 6)}
\]

For comparison of data sets it was convenient to fit the dissociation curves to a unimolecular equation,

\[
mfc = 1 - 1 + e^{-\Delta G_{\text{contact}} m[\text{urea}] / RT} \quad \text{(Eq. 7)}
\]

where \(m\) is mass fraction capsid, and \(\Delta G\) is an empirically fit pseudo-energy term. Equation 7 was only used for visual comparison of curves.


Simulations of Dissociation Reactions—Simulations were based on the mathematical model developed for assembly of a 30-subunit icosahedron developed in previous studies, where the subunits topologically resemble those of HBV (15). The system of equations included a trimeric nucleation step, as does HBV (14). The microscopic forward rate constant for nucleation was 103 (m−1 s−1) compared with 102 for elongation. The rates used in simulations were based on a crude fit to HBV assembly kinetics (not shown). Numerical integrations were calculated using BERKELEY MADONNA (Berkeley Software, Berkeley, CA) with the Rosenbrock numerical integration methods. For simulations, we simultaneously calculated the concentrations of all intermediates, capsid, and free subunit. The concentration of a given intermediate \(n\), with \(m\) subunits, was calculated by numerical integration of rate equations (Equation 8).

\[
[n] = [k_{\text{on}} n - 1][1] - k_{\text{off}} [n][1] - k_{\text{diss}} [n] + k_{\text{on}} + 1[n + 1] \quad \text{(Eq. 8)}
\]

In Equation 8, \(k_{\text{on}}\) is the forward rate constant for production of \(n\) from an intermediate with \((n - 1)\) subunits and monomer, \(k_{\text{off}}\). This rate constant includes statistical coefficients as previously detailed (13). Dissociation rates \(k_{\text{diss}}\) were calculated from the forward rate and the association constant \(K_{\text{ass}} = k_{\text{off}} / k_{\text{on}}\), where the association constant is a function of the number of intersubunit contacts, the per contact microscopical association constant (see Equation 4), and a statistical factor reflecting reaction degeneracy (12). Cp149, observed for HBV is 200–2000 s−1, depending on conditions. The \(K_{\text{contact}}\) for the simulations described in this paper was varied from 20 to 10,000 s−1. Equation 8 emphasizes that association reactions are dependent on significant concentrations of intermediates and monomers, whereas the dissociation rate is strongly affected by the number of bonds to be broken.

For initial simulations, we assumed that all contacts made by a given subunit were formed or broken simultaneously. To mimic independent breakage of contacts on a single subunit, we inserted a probability factor; for an intact capsid with 30 tetraevalent subunits forming 60 contacts, the odds of a second contact breaking on a subunit with one already broken contact were 3/59. For computational simplicity in these simulations, we adopted a simplest case rule requiring that two contacts be broken to release a subunit. Light scattering for simulations was calculated by assuming that the contribution of each intermediate to the signal was proportional to its mass and concentration, without any size- or shape-dependent form factor.
RESULTS

Capsid Dissociation Displays Hysteresis—In agreement with model studies, we have observed that capsid assembly is very concentration-dependent (14, 17). This is expected for any system that incorporates 120 independent subunits. Simulations show that assembly reactions approach equilibrium rapidly. In practice, we found that HBV assembly reactions equilibrated within 24 h (17), which allowed us to calculate the per contact association energy, $\Delta F_{\text{contact}}$. Based on assembly studies in 0.15 M NaCl (17), we expected that dilution of Cp1492 capsids would lead to a broad dissociation transition between about 15 and 40 $\mu$M total Cp1492. The midpoint of the transition, with 50% capsid, was expected at 20 $\mu$M total protein and nearly complete dissociation was expected below 14 $\mu$M. However, we observed little dissociation of capsids even after incubation in 0.15 M NaCl, 21 °C for up to 5 days (Fig. 1). A small amount of free Cp1492 was present in all samples, but the mass fraction of capsid remained approximately constant at >90% to at least 5 $\mu$M total protein. What little dimer was observed after 5 days was essentially identical to concentrations observed after 2 days (data not shown). Even at 0.5 $\mu$M, the lowest protein concentration examined in this study, less than a third of the protein was free dimer. In contrast, assembly studies led to the prediction that dissociation would be nearly quantitative after dilution to less than 15 $\mu$M. Our data give the appearance that the concentration-dependent dissociation transition will occur at lower initial [capsid], but we could not reliably measure these very low concentrations. These results are a first demonstration of hysteresis with HBV; that is, capsid dissociation and assembly reactions are not in equilibrium.

Denaturants Induce Two Transitions—An alternative approach to measuring stability of a protein complex is with chaotropes such as GuHCl and urea. The effect of GuHCl on Cp1492 capsids was measured by intrinsic tryptophan fluorescence, LS, SEC, and circular dichroism (CD). After normalization for denaturant-dependent changes in LS and fluorescence signal (see “Experimental Procedures”), titrations observed by fluorescence, LS, and SEC measurements were coincident between 0 and 4 M GuHCl (Fig. 2A). SEC measurements showed a dissociation transition between 1 and 2 M GuHCl during which the concentration of Cp1492 increased at the expense of capsid. Only two major species, capsid and Cp1492, were observed throughout the dissociation transition. In light of the SEC observations, the concurrent decreases in LS, which is proportional to the average molecular weight, and fluorescence can be interpreted in terms of fraction of capsid (see “Experimental Procedures,” Equation 1). Although fluorescence intensity decreased by a factor of 2 during dissociation, the emission maximum remained at 324 nm. This suggests that the environment of the tryptophan residues did not change dramatically through the transition and that Cp1492 remained folded (Fig. 2B). CD spectra support this last assertion; no significant change in secondary structure was observed between 0 and 2.5 M GuHCl (Fig. 2C); there is a small difference between CD spectra of capsid and dimer (8).

Based on SEC and LS, there was no capsid remaining beyond 2.5 M GuHCl. At higher GuHCl, Cp1492 undergoes a second transition observable by fluorescence (Fig. 2B) and CD (Fig. 2C). From 3 to 5 M GuHCl, the fluorescence intensity increased, and the emission maximum shifted from 324 to 340 nm (Fig.
Chaotrope Studies Also Indicate Hysteresis of Dissociation—Chaotrope titrations allowed investigation of the concentration dependence of dissociation as well as capsid stability. Capsid stability is more easily understood in terms of the average bimolecular association constant between pairs of subunits, $K_{\text{contact}}$, than the overall association constant based on the $K_{\text{capsid}}$, which is in units of M$^{-1}$ (see “Experimental Procedures,” Equations 2 and 4). From assembly studies, we know that $K_{\text{contact}}$ is very weak at 21 °C and 0.3 M NaCl, corresponding to a $\Delta G_{\text{contact}}$ of $-3.7$ kcal mol$^{-1}$ (17).

The value of $\Delta G_{\text{contact,H}_2\text{O}}$ was determined from urea titrations by measuring $\Delta G_{\text{contact}}$ at different urea concentrations and extrapolating to 0 mM urea (19, 20). For these extrapolations, a single $m$ value of 1.04 ± 0.19 (kcal mol$^{-1}$/M [urea]) was used. This $m$ value is the average from 15 titrations at 4 protein concentrations measured by fluorescence and LS (Fig. 3, A and B). The $m$ value has been related to the change in the amount of hydrophobic surface exposed in the unfolding (or dissociation) transition (22), which should be independent of concentration or ionic strength. Note that the data presented (Fig. 3) are in terms of mass fraction of capsid; at the midpoint of the transition, when capsid and dimer mass fraction are equal for 2 μM total Cp1492, $\Delta G_{\text{contact}}$ is $-3.8$ kcal mol$^{-1}$ (see Equations 3–5).

The calculated $\Delta G_{\text{contact,H}_2\text{O}}$ was roughly twice the value determined in assembly studies (Table I) (17). The $\Delta G_{\text{contact,H}_2\text{O}}$ determined from urea dissociation of 1.25 mM Cp1492 in 0.15 M NaCl was $-5.8$ kcal mol$^{-1}$. This result corresponds well with the data in Fig. 1, where capsid was dissociated by dilution. This substantial difference between association energy measured in assembly reactions and by dissociation further demonstrates hysteresis.

Unlike $\Delta G_{\text{contact}}$ from assembly studies (17), $\Delta G_{\text{contact,H}_2\text{O}}$ was not constant at different protein concentrations. Purified Cp1492 capsids at 2, 4, 8, and 16 μM concentrations in 0.3 M NaCl were incubated with urea (Fig. 3, A and B). The systematic variation of $\Delta G_{\text{contact}}$ indicates that the dissociation reaction was not well represented by an equilibrium between capsid and 120 dimers. This is demonstrated by comparing calculated and observed dissociation curves. Given a $\Delta G_{\text{contact,H}_2\text{O}}$ and an $m$ value, it is straightforward to back-calculate the mass fraction of capsid at any urea concentration (Fig. 3C, dashed lines). The dissociation curve calculated for 2 μM Cp1492 fit that data. However, the curve calculated for 16 μM Cp1492 using the $\Delta G_{\text{contact,H}_2\text{O}}$ derived from the 2 μM data does not fit. In summary, HBV capsid dissociation can be induced by chaotropes, but it does not show the predicted concentration dependence expected for assembly of a 120-mer (Equation 2). Therefore, although the dissociation energies determined in urea titrations are much greater than those determined from assembly,
they are at least consistent with an effort to evaluate dissociation by dilution (Fig. 1).

Examination of HBV dissociation kinetics was not straightforward. Dissociation by urea or GuHCl was largely complete within a few hours; there was no measurable difference between the degree of dissociation measured at 24 and 48 h (data not shown). Early times in a dissociation reaction could be observed by fluorescence and LS; however, these results could not be interpreted because of the undeterminable relative contributions of capsids and intermediates to the signal (see results of simulations, this section). In contrast, assembly reactions have very low concentrations of intermediates (13).

Capsid Dissociation Is Reversible—Equilibrium between HBV capsid and dimer implies a flux between the two states. This leads to the predictions that capsids will assemble in urea and that dissociation, like assembly, will be ionic strength-dependent (8, 14, 17). Conversely, if capsids assemble irrevocably, one would expect dissociated dimers to be inert and that there would be no ionic strength dependence of dissociation.

Urea-induced capsid dissociation was observed at 0.15, 0.325, and 0.5 M NaCl (Table I). The midpoint of dissociation in these reactions was influenced by the ionic strength, varying from 1.75 to 3.25 M urea, although curve shape was not changed. The m value of 1.04 (kcal mol⁻¹)/[urea] fitted data at all three salt concentrations. The effect of [NaCl] on dissociation and assembly was qualitatively the same. This suggests that dissociation was inhibited by high salt in the same manner that assembly is induced by it.

Cp1492 derived from urea-dissociated capsid readily reassembles. In fact, urea dissociation and reassociation are part of the purification protocol for assembly active Cp1492 (8, 10, 14). We determined the ΔG_contact in the presence of urea from reassembly experiments for comparison to values determined from dissociation. Assembly of 10 μM Cp1492 in varying concentrations of urea was driven by the addition of NaCl to 0.3 M (Fig. 4). We found that 0.3 M NaCl was sufficient to drive assembly of an appreciable amount of capsids in 0 to 0.5 M urea. The observed concentrations of capsid and dimer in 0 M urea, 2.7 and 2.3 μM, respectively, were in good agreement with the concentrations expected for these assembly conditions (17). At urea concentrations up to 1 M, some capsid was detectable but could not be reliably quantified. The effect of [urea] on ΔG_contact was observed in these assembly experiments corresponds to an m value of -0.4 (kcal mol⁻¹)/[urea], much less than the 1.04 (kcal mol⁻¹)/[urea] value from the dissociation data. These data demonstrate that capsids are stable at urea concentrations that are too high to support de novo assembly, another example of a hysteresis cycle.

Simulations of Dissociation Kinetics Predict Hysteresis—By themselves, the preceding results only show that association and dissociation reactions are distinctly different and that dissociation does not conform to most expectations for a reaction approaching equilibrium. Assembly does conform to those expectations (17). The difference between association and dissociation can be reconciled by examining dissociation of a 30-subunit model. We found that simulations of dissociation reactions allowed us to identify reaction features that resulted in hysteresis, suggesting that the source of the hysteresis is kinetic.

We initially expected that capsids would collapse as soon as they began the process of dissociation. A simple analogy is the effect of removing one brick from an arch. Contrary to this expectation, the estimated LS calculated from dissociation simulations did not generate first order kinetics (Fig. 5A). A closer inspection of the kinetic trajectories for [capsid] shows that the decrease in concentration was not monotonic. Initially, [capsid] decreased rapidly; then [capsid] increased slightly; then there was a gradual decline (Fig. 5B). An advantage to model reactions is that all populations can be examined. What has happened in these dissociation simulations? A population of 30-mer capsids dissociated to generate a variety of intermediates and free subunit. For some intermediates, in particular the 29-mer, the resulting concentration of intermediate and free subunit. For some intermediates, in particular the 29-mer, the resulting concentration of intermediate and free subunit was a gradual decline (Fig. 5). An advantage to model reactions is that all populations can be examined. What has happened in these dissociation simulations? A population of 30-mer capsids dissociated to generate a variety of intermediates and free subunit. For some intermediates, in particular the 29-mer, the resulting concentration of intermediate and free subunit was a gradual decline (Fig. 5). An advantage to model reactions is that all populations can be examined. What has happened in these dissociation simulations? A population of 30-mer capsids dissociated to generate a variety of intermediates and free subunit. For some intermediates, in particular the 29-mer, the resulting concentration of intermediate and free subunit was a gradual decline (Fig. 5).

As a result of this “local” barrier to dissociation, these simulations were very slow to reach equilibrium. Simulations of assembly reactions rapidly reached equilibrium; there was no divergence between calculated equilibrium concentrations of capsid and subunit for a given ΔG_contact and the concentrations observed after a 24-h simulation (see Fig. 6). Shorter simulations of assembly (~2 h) resulted in a close approximation of the equilibrium value (not shown); the presence of a stochastic equilibrium between intermediates and capsid has been demonstrated by the effect of altering the dissociation rate (14, 15). However, simulations of dissociation reactions using the same model, beginning with “pure” capsid, showed an obvious...
hysteresis. Very long simulations marginally decreased the hysteresis.

The initial simulations of association and dissociation incorporated an assumption that all contacts made by a given subunit were made and broken simultaneously. There was a dramatic enhancement of hysteresis when we relaxed this assumption by allowing contacts to break independently of one another and requiring that a subunit have at least two broken contacts to dissociate (Fig. 6). This was modeled by incorporating a probability coefficient into the model (see “Experimental Procedures”). Because it did not alter contact energy, this two-contact rule had no significant effect on the rate or extent of association. In the simulations, the disparity between association and dissociation is attributable to hysteresis; it is not a result of insufficient equilibration time.

Dissociation of HBV (Fig. 3) and other viruses (4, 5) shows a much weaker than expected concentration dependence for dissociation. The hysteresis observed in simulations mirrored this effect (Fig. 6C). Simulations for assembly and dissociation were conducted for total subunit concentrations of 2, 4, 8 and 16 μM to parallel the experimental work. Instead of urea concentration, we varied the input ΔG_contact to mimic the effect of urea. As expected, the assembly reactions showed a high degree of concentration dependence, whereas dissociation occurred at lower energies (corresponding to higher concentrations of urea) and reduced concentration dependence. The greater the hysteresis of dissociation, the weaker was its concentration dependence.

**DISCUSSION**

HBV capsids assemble from 120 copies of the dimeric capsid protein. Kinetic simulations predict that the assembly reaction will approach the concentrations predicted from reactant concentration and association energy, whereas dissociation will demonstrate marked hysteresis. Observations of assembly and dissociation support this prediction. Assembly reactions show the expected steep concentration dependence (17). Dissociation reactions do not reach the same concentration of products as assembly reactions nor do they show the expected concentration dependence. However, the free subunits collected from dissociation reactions are not denatured and are competent to reassemble. The per contact association energy estimated from denaturation studies yields a result that is consistent with dissociation by dilution. We conclude that hysteresis is isolated to the dissociation reaction.

We demonstrate the most likely source of hysteresis by taking advantage of kinetic simulations that were originally developed to describe assembly (13, 14, 17). Hysteresis is observed in simulations of dissociation but not association, although they are constrained to the same path. Consequently, hysteresis does not violate the principle of microscopic reversibility (23, 24). Kinetic simulations reveal dissociation to be a complex reaction where accumulation of temporally stable intermediates results in kinetic traps that impede the reaction, creating an energy barrier to dissociation. During dissociation, intermediates may reach concentrations that are orders of magnitude greater than at equilibrium; however, they may still be difficult to quantify experimentally. This last effect leads to the two-state appearance of assembly and dissociation reactions observed experimentally and in simulations.

An important concern with simple models is whether they can accurately reflect the behavior of a complex system. In dissociation simulations, we observed that the rate-controlling intermediates were nearly complete capsids (especially 29-mers). This is largely because they are slow to dissociate further but readily re-associate with excess monomer. Because all dissociation paths necessarily begin with intermediates of N-1 subunits, we suggest that our simplest case model of dissociation, with its single path, results in a reasonable representation more complex dissociation paths.

Association and dissociation reactions with their many possible paths can be considered in terms of an energy landscape (25–29). As a general rule, each successive intermediate in an association reaction is progressively more stable because a greater number of intersubunit contacts are formed (13, 14). The resulting landscape can be characterized as a steep downhill slope with capsid as the only significant minimum. The landscape for dissociation of the 30-mer model (and presumably HBV capsules) is very different. In order for the first few tetravalent subunits to dissociate from the capsid, three or four contacts must be broken; in contrast, the capsid stability is roughly proportional to the energy of two contacts (15). This last effect is analogous to the hysteresis observed in molecular
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dynamics simulations of a dimer (30). In our model studies, metastable intermediates contribute to a kinetic and energetic barrier for capsid dissociation. Early in dissociation, these species are present at concentrations that are high enough to favor reassembly, although these same conditions will not support de novo assembly. Dissociation proceeds because a fraction of the metastable intermediates dissociates irretrievably to smaller unstable forms, but is much slower than would be predicted for a simple two-state reaction.

Hysteresis is inherent in capsid dissociation. It arises from the closed geometry of an icosahedral particle, or any nominally symmetrical oligomer. It can be traced to intermediate reactions where association is favored over the globally favored dissociation. It is clear that hysteresis would be accentuated if subunits were constrained by a membrane or tethered by nucleic acid. Simulations described in this study also suggest that the independent non-cooperative breaking of intersubunit contacts may make a significant contribution to the hysteresis of dissociation. We deduce that hysteresis can also be increased by any mechanism that introduces differentially stabilized intermediates that would form additional minima in the energy landscape of dissociation.

Weber et al. (31) suggest an alternative explanation for hysteresis by postulating that populations of capsids (and other multimers) dissociate irreversibly. Irreversible dissociation would lead to a relatively narrow transition that could be broadened if the oligomer were heterogeneous and each sub-species had a slightly different stability. In the case of HBV, we have shown that dissociation is reversible. Any heterogeneity of the capsids would broaden the dissociation transition and actually decrease the magnitude of the calculated association energy.

We suggest that hysteresis has a biological role. Capsid proteins may associate with modest per contact energy to minimize kinetic trap formation during assembly (13, 14) yet still yield a stable particle. The hysteresis effect would then preserve capsids under non-ideal conditions, for example, between hosts or at low concentrations within a new host. This scenario is appealing because the combination of weak inter-subunit contact energy combined with independent disruption of contacts can lead to the breathing observed in capsids of several viruses (32–34), where intersubunit contacts break and re-annel to transiently expose buried protein segments to antibodies and proteases. Thus, capsids remained stable even where individual contacts were not. Hysteresis can mask the underlying fragility of a virus. A particularly revealing observation that supports this argument is that poliovirus receptor acts as transition state catalyst to lower the activation energy for conversion of 160 S poliovirus to an infectious intermediate (35). This final point also suggests that there is hysteresis, a trigger may be required for uncoating, giving the virus an important regulatory control.

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