Published in final edited form as:

ACS Chem Biol. 2018 August 17; 13(8): 2114–2120. doi:10.1021/acscchembio.8b00283.

An Assembly-activating Site in the Hepatitis B Virus Capsid Protein can also Trigger Disassembly

Shefah A. Qazi^{1,2}, Christopher J. Schlicksup¹, Jonathan Rittichier², Michael VanNieuwenhze², and Adam Zlotnick^{1,*}

¹Department of Molecular and Cellular Biochemistry, Indiana University, Bloomington, Indiana, 47405, USA

²Department of Chemistry, Indiana University, Bloomington, Indiana, 47405, USA

Abstract

The Hepatitis B Virus (HBV) core protein homodimers self-assemble to form an icosahedral capsid that packages the viral genome. Disassembly occurs in the nuclear basket to release the mature genome to the nucleus. Small molecules have been developed that bind to a pocket at the inter-dimer interface to accelerate assembly and strengthen interactions between subunits; these are under development as antiviral agents. Here, we explore the role of the dimer-dimer interface by mutating sites in the pocket to cysteine and examining the effect of covalently linking small molecules to them. We find that ligands bound to the pocket may trigger capsid disassembly in a dose-dependent manner. This result indicates that, at least transiently, the pocket adopts a destabilizing conformation. We speculate that this pocket also plays a role in virus disassembly and genome release by binding ligands that are incompatible with virus stability, “unwanted guests”. Investigating protein-protein interactions, especially large protein polymers, offers new and unique challenges. By using an engineered addressable thiol, we provide a means to examine the effects of modifying an interface without requiring drug-like properties for the ligand.

Graphical Abstract

INTRODUCTION

Over 240 million people worldwide are chronically infected with Hepatitis B Virus (HBV).² Though there is an effective HBV vaccine, there is no cure for those patients with a chronic

*Corresponding author Professor Adam Zlotnick, Department of Molecular and Cellular Biochemistry, Simon Hall, MSB, Indiana University, Bloomington, Indiana, 47405, (812) 856-1925, azlotnic@indiana.edu.

infection. Thus, there is an unmet need to develop new HBV therapies. Targeting HBV capsid assembly is a new and promising strategy.³⁻⁴

HBV is an enveloped virus with an icosahedral core. The protein shell of that core, the capsid, is formed from homodimers of the 183-residue core protein (HBcAg).⁵⁻⁶ *In vivo*, the core assembles on a complex of viral RNA and polymerase, and the viral DNA genome is reverse transcribed within the capsid while resident in the host cytoplasm. The overwhelming majority of capsids have T=4 icosahedral symmetry though a small fraction (ca. 5%) have T=3 symmetry. The core protein has a 149-residue assembly (Cp149) and a 34-residue disordered nucleic acid binding C-terminal domain. Cp149 can assemble into capsids *in vitro* and these are essentially identical to those seen in virus particles.⁷ Cp149 is a highly conserved protein,⁸ nonetheless it has three cysteines that are not necessary for function and can be replaced with alanine (Cp149-3CA) without impeding assembly activity.⁹ The HBcAg and resulting capsid have many functions beyond packaging viral RNA and serving as a container for reverse transcription. The capsid must display signals for intracellular trafficking, where the signals are on the C-terminal domain that are transiently exposed on the outside of the capsid.¹⁰ Capsids at nuclear pores uncoat to release the viral genome into the nucleus.¹¹ Thus, the capsid appears to be an extremely flexible complex.

In a capsid, a loop from one Cp149 fits into a groove in the neighboring subunit (Figure 1). For a T=4 capsid, there are four unique subunit environments A, B, C, and D that are filled by chemically identical but structurally distinct AB and CD dimers. Icosahedral fivefold vertices are formed by A-A interactions. Icosahedral fivefold vertices are formed by A-A interactions. Quasi-sixfold vertices are formed by two sets of B-C, C-D, and D-B interfaces (Figure 1). These interfaces are dominated by hydrophobic interactions that result in a weak pairwise association energy of about -3.5 kcal/mol;¹² as each subunit is tetravalent, the resulting capsids are very stable. The weak association energy prevents entrapment of defects by favoring dissociation of misassembled intermediates.¹³⁻¹⁵ *In vitro* assembly can be driven by increasing ionic strength, which may act to favor an assembly active state.¹⁶⁻¹⁷

Capsid assembly may also be driven by small molecules, known collectively as core protein allosteric modulators (CpAMs).³⁻⁴ CpAMs accelerate assembly and can stabilize protein-protein interactions by filling a pocket at the B-C and C-D interfaces.^{6, 18-19} CpAMs can also modify inter-subunit geometry leading to assembly of aberrant structures and distortion of pre-assembled capsids.^{18, 20}

To study the accessibility of the CpAM-binding pocket, we investigated the consequences of introducing a cysteine, along with a ligand capable of covalently capturing the cysteine thiol group, into the binding pocket. We created a series of pocket cysteine mutants L30C, I105C, and S106C, and V124C and characterized their assembly and reactivity *in vitro*, extending the approach pioneered by Wells and co-workers.²¹⁻²² By incorporating a covalent linkage to the ligand, we show that capsids can bind capsid-destabilizing factors. This effect suggests that the CpAM pocket may play a role in the mechanism of capsid disassembly, depending on its occupant. We also show that this site can be protected by CpAMs (i.e. HAP12).

MATERIALS

Cloning of Cp149–3CA mutants and protein purification

The HBV Cp149 3CA-pET11c construct was mutated to L30C, I105C, S106C, and V124C with a QuikChange mutagenesis kit (Stratagene). Cp149–3CA pocket mutant dimers were expressed in *Escherichia coli* BL21(DE3) grown in Terrific Broth with 50 µg/ml carbenicillin at 37°C and purified as previously described.²³ The extinction coefficient at 280 nm was 60,900 M⁻¹cm⁻¹, a standard extinction coefficient for the HBV assembly domain.²⁴

Calculation of the pseudocritical concentration by size exclusion chromatography (SEC)

CP149–3CA, L30C, I105C, S106C, and V124C dimers at varying concentrations from 4–40 µM were mixed with an equal volume of 50 mM HEPES, 600 mM NaCl, pH 7.5 buffer and incubated for 48 hours at room temperature to induce capsid formation. Samples were resolved using a Superose 6 column mounted on a Shimadzu HPLC equipped with a diode array detector. SEC chromatographs at 280 nm absorbance were integrated over the capsid and dimer elution volumes to determine the reaction products as a fraction of the input protein.

Visualization of particles by Electron Microscopy

Samples were adsorbed to glow-discharged carbon over paralodian copper grids (EM Sciences), negative stained with 2% uranyl acetate, and visualized with a JEOL 1010 transmission electron microscope equipped with a 4Kx4K Gatan CCD camera.

Labeling of pocket mutants

To BoDIPYm label CP149–3CA, L30C, I105C, S106C, and V124C, dimers at a concentration of 20 µM were incubated with 20 molar excess BoDIPY-FL maleimide (BoDIPYm) for 2 h in 4 °C. Samples were separated from free dye using (Zeba) desalting spin columns 7K MWKO. The amount of label in a sample was evaluated by absorbance, from the BoDIPYm/protein (504 nm /280 nm) absorbance ratio. The extinction coefficient of BoDIPYm is 78,000 M⁻¹ cm⁻¹ at 504 nm; the extinction coefficient is 1,300 M⁻¹ cm⁻¹ at 280 nm, which is negligible. To verify covalent binding of dye to protein, samples were run on 16% SDS-PAGE gel and imaged with a Typhoon Fluorescence imager excited at 495 nm. Gels were further stained with Coomassie blue to test for co-migration of BoDIPY and protein.

To test the effects of partial labeling, V124C capsids (15µM, 120µL) were incubated with BoDIPYm, Fluorescein maleimide (FAM), Fluorescein isothiocyanate (FITC), iodoacetamide, and N-ethyl maleimide at ratios of 0.25, 0.5, 1, 2, 4, 8, 16 reactant:dimer for 2 hours. Charcoal was used to remove excess dye and reactions were subsequently resolved by SEC in 300 mM NaCl, 50 mM HEPES, pH 7.5. Percent dissociation was determined as the integrated fraction of a SEC micrograph at 280nm eluting as free dimer.

To test for protection from BoDIPYm- induced dissociation V124C capsids (15 120 ^L) were incubated with various ratios of iodoacetmide, N-Ethyl maleimide, or HAP 12

overnight. The following day, capsids were treated with 20 molar excess BoDIPYm and incubated for 2 hours. Charcoal was used to remove excess dye before SEC.

RESULTS

To investigate the effect of filling the CpAM pocket without requiring a ligand that has intrinsically high affinity for the site, we created a series of pocket cysteine mutants L30C, I105C, and S106C, and V124C in the cysteine-free Cp149 construct, Cp149–3CA, to enable covalent capture of weak-binding ligands (Figure 1c).

Mutants were expressed and purified to yield soluble dimers. For initial characterization, mutant capsids were assembled in 300mM NaCl, pH 7.5 to determine their pseudocritical concentration, the maximal concentration of free Cp149 dimer in solution at equilibrium. Though all the mutants assembled, all had higher pseudocritical concentrations than the baseline Cp149–3CA. The mutant with the poorest assembly activity was S106C with a pseudocritical concentration of 10 μ M, followed by V124C at 6 μ M, and I105C and L30C each at approximately 4 μ M (Figure 2a-e). Transmission electron micrographs of the mutant capsids confirmed that the mutants were structurally similar to capsids of wild type protein (Figure 2f-i).

To be useful for investigating the effects of ligands on capsid assembly and stability, the cysteines had to be accessible to cysteine-reactive ligands in both free dimer and capsid forms. Dimer in low salt (50 mM NaCl) and capsid in moderate salt (300 mM NaCl) were treated with a cysteine-reactive fluorescent dye, BoDIPY-Fl-maleimide (BoDIPYm), which has an absorbance maximum at 504 nm. As a negative control, reactions were also performed with the cysteine-free parent protein, Cp149–3CA. After the reaction, free dye was removed, and absorbance spectra were recorded. In dimer form, all mutants except L30C were accessible for labeling (Figure 3a), possibly because L30C is more buried relative to the other mutants. We observed that the absorbance at 504nm was approximately twice the absorbance at 280nm for labeled dimer, which is expected for the doubly-labeled dimer as the dimer has two cysteines.

When labelling purified capsids, we observed that only the V124C capsid was maximally accessible for labeling (Figure 3). Other mutant capsids had low, sub-stoichiometric labeling, about 0.1 BoDIPYm per dimer. To ensure that labels were covalent, labeling efficiencies were confirmed by denaturing electrophoresis, using fluorescence and Coomassie staining to identify fluorophore and capsid protein respectively. In free dimer form, the HBV core protein and dye co-migrated for all species except L30C and the negative control Cp149–3CA (Figure 3a, inset). For preformed capsids, only V124C showed strong association with BoDIPYm (Figure 3b, inset).

Thus, we identified a CpAM pocket cysteine mutant which supports capsid labeling, V124C. In structural studies of capsids with bound CpAMs, only one CpAM pocket per dimer is fully accessible.⁶ Paradoxically, when V124C capsids were labeled with a 20-fold molar excess of BoDIPYm, the absorbance after removal of free dye indicated two BoDIPY m molecules per dimer. To our surprise, size exclusion chromatography (SEC) revealed that

V124C capsids had dissociated when conjugated to BoDIPYm (Figure 4a). This explains the paradox as both cysteines per dimer would be expected to be available. When examining the dose dependence of dissociation, we found that a reaction with two BoDIPYm molecules per dimer, presumably leading to 2 BoDIPYm bound per dimer, was sufficient to induce disassembly of more than 80% of the capsids (Figure 4). This observation establishes the CpAM binding pocket as a location from which disassembly could be triggered by small molecules.

To evaluate the features of these unwanted guests we considered a panel of cysteine reactive molecules against the V124C mutant core protein, and monitored the accessibility of the pocket and the ability of bound molecules to cause disassembly. BoDIPYm is a 414 Da hydrophobic molecule. To explore the role of the CpAM pocket on capsid disassembly, we evaluated disassembly induced by other thiol reactive compounds with varying size and polarity: fluorescein maleimide (FAM) (498 Da, large, polar), iodoacetamide (59 Da not including the iodine, small, polar), and N-ethyl maleimide (NEM) (125 Da, small, slightly polar). The pocket is planar, compact, and hydrophobic, so we suspected it would not tolerate polar groups or bulky groups. We used fluorescein isothiocyanate (FITC), which is amine reactive, as a negative control.

Only FAM caused disassembly like BoDIPYm, though at much higher concentrations. The high FAM concentration required for disassembly could reflect lower rate of capture due to accessibility of the FAM to cysteine residue. FITC did not induce disassembly. Thus, capsid could not withstand large bulky groups covalently bound to the CpAM pocket, though intact capsids can stably bind CpAMs, at least at the B and C pockets. Capsids could also be labeled with the small functional groups, iodoacetamide and NEM, independent of polarity. Notably, iodoacetamide led to modest disassembly, based on the accumulation of free dimer (Figure 4). Unlike the bulky fluorophores BoDIPYm and fluorescein, the mechanism for iodoacetamide-induced disassembly is likely not steric disruption, but rather due to the chemical properties of acetamide, which may weaken the hydrophobic protein-protein interactions between subunits – it has been observed the strength of dimer-dimer interactions varies linearly with the hydrophobic surface of residue 124 (Supplemental Figure 1 and reference ²⁵).

As neither iodoacetamide nor NEM have convenient chromophores to monitor labeling, we developed a protection assay to confirm capsid labeling. V124C capsids treated with different concentrations of iodoacetamide (Figure 5a) and NEM (Figure 5b) were subsequently treated with a 20-fold molar excess of BoDIPYm. In the absence of protection by alkylation of V124C, this amount of BoDIPYm completely dissociates capsid and labels each dimer at both of the two cysteine residues. Iodoacetamide progressively conferred protection from dissociation by BoDIPYm; however, it only protected about 75% of the protein, even at the highest concentrations tested (Figure 5a). On the other hand, NEM, which is much less polar based on water solubility, conferred nearly complete protection at a stoichiometric ratio of 2 ligands per dimer. This protection assay has general utility for screening compounds which bind to the inter-dimer interface.

As a proof of concept, we examined protection of the CpAM-binding site by HAP12. The HAP family of CpAMs bind strongly to the B-C and C-D inter-dimer pockets.^{6, 20} We observed that HAP12 provides protection against dissociation to the same extent as NEM (Figure 5c), despite not forming a covalent linkage to the protein. V124C capsids are completely protected from BoDIPYm induced disassembly by HAP12 at a 4:1 or higher molar ratio of dimer. Since HAP12 is a racemic mixture, this corresponds with two HAP12 molecules per dimer. Interestingly, as the capsids become saturated, and thus protected from disassembly, they accommodate a significant amount of dye in comparison to NEM (Figure 5b, c). This raises the question of how cysteine residues are accessible for labeling, despite a protected capsid that remains intact. A recent cryo-EM study of HAP-saturated capsids showed that HAPs cause quasi-sixfold vertices to flatten and fivefold vertices, composed of A subunits, to become more faceted, and potentially even break open.²⁰ The cysteines for each A subunit would correspond to 25% of the total cysteines at each A, B, C, D chain. Thus, we propose that at stoichiometric concentrations of the HAP12 the capsid is stabilized to prevent BoDIPYm-induced dissociation at the CpAM binding pocket (B, C chains), but A or D subunits are distorted in ways which become susceptible to labeling.

DISCUSSION

In vivo, HBV capsids assemble to package the viral genome then disassemble post-infection to release it. Filling the CpAM pocket can drive assembly *in vitro* and *in vivo*, leading to aberrant and empty particles.^{3-4, 26} These aberrant assemblies were the first mechanism identified to explain CpAM antiviral activity, though additional mechanisms are beginning to be explored. For example, the mechanisms of disassembly regulation in intact capsids is not understood but appears to also be sensitive to CpAMs. Here we incorporate a cysteine mutation, V124C, into the CpAM pocket, and demonstrate that it is accessible and reactive. We have also demonstrated that we can induce catastrophic dissociation of capsids by covalently modifying cysteine with a bulky ligand (Figure 4b). By attaching a polar ligand, i.e. alkylation with iodoacetamide, we modulate association energy and observe modest dissociation of the capsids. These observations lead us to suggest that a mechanical disruption, such as filling the HAP pocket with a guest incompatible with capsid stability, triggers virus disassembly.

It is curious that a capsid destabilizing ligand, like BoDIPYm, should bind so successfully to an intact capsid. The V124 site has predictable properties: located on a buried hydrophobic surface, the cysteine mutation modestly weakens association energy compared to wild type. This correlates well with other mutations made at this pocket (Supplemental Figure 1).²⁵ Thermodynamic linkage²⁷ leads to the prediction that destabilizing molecules should be preferentially excluded from the inter-dimer interface. Though BoDIPYm and FAM depend on a covalent bond to stably interact, linkage dictates that the capsid should rarely adopt states which are suitable for interaction with BoDIPYm. Yet, BoDIPYm can completely dissociate a population of capsids within two hours (Figure 4b). The effect of BoDIPYm and FAM are consistent with highly dynamic capsids, which are continuously breathing to allow CpAM pockets to transiently open and close. Without a covalent interaction, closure of the transient pocket at the inter-dimer interface should expel the destabilizing ligand. Indeed, HBV capsids are extremely flexible based on atomic force microscopy experiments,²⁸ an

observation recapitulated and defined at atomic detail by molecular dynamics simulations.^{29–30} The role of the pocket *in vivo* may be purely architectural: it facilitates T=4 architecture by permitting A-A and D-B inter-dimer contacts where the dimers are too close for a pocket to exist, but also B-C and C-D contacts where there is sufficient space in the pocket for it to be occupied by outside molecules. Given the observations presented in this paper, the pocket may very well provide a pathway towards capsid dissociation.

It remains striking that an assembly-stimulating site can also be used to stimulate capsid distortion and disassembly.^{6, 29} It is attractive to speculate that this site could have a critical role in the viral lifecycle, facilitating the breaking of the capsid to release the viral genome at the right time and place. Mature HBV capsids are notably unstable³¹ and release their DNA into the nucleus from the nuclear basket.¹¹ One explanation for capsid fragility is the pressure of the double stranded genome.³² Nonetheless, the trigger for dissociation has not been identified. The CpAM pocket is a highly conserved region in a highly conserved protein.^{8, 33} Once held in a closed environment, a low affinity ligand will be present at high effective concentration; thus the nuclear basket's nucleoporin proteins, known to interact with HBV cores,³⁴ are attractive candidates for the dissociative factor. The combination of high effective concentration and capsid breathing can allow a thermodynamically poor ligand, metaphorically an unwanted guest, to disrupt the capsid in an environment where it will not re-associate.

We (and others) have shown that the CpAM pocket can be used to drive assembly using mutations²⁵ and small molecules.^{18, 35–38} Indeed, any small molecule which can act on this site to regulate capsid morphology could provide the basis for an antiviral lead compound.³ In this work we show that the same site can be leveraged to drive disassembly (e.g. Figure 4). Furthermore, the HBV capsid has been investigated as a molecular delivery system.^{39–41} A targeted means of regulating capsid stability without resorting to high salt or denaturants may be valuable towards this end.

We envision an assortment of ligands which bind the core protein inter-dimer interface, and selectively modify the protein-protein interactions. Molecules which strengthen the interaction can drive assembly and stabilize capsid geometry as is the case for phenylpropenamide CpAMs.⁴² Alternatively, a high affinity guest may stabilize the protein-protein interface but distort geometry. This is the case for HAPs, which globally destabilize capsids to yield aberrant particles.²⁹ Our results introduce yet another perspective; that some molecules can completely destabilize the capsid structure, inducing premature disassembly. From a thermodynamic perspective, a destabilizing ligand, an unwanted guest, must bind the subunit better than the parent complex; here we have emulated that behavior. *In vivo*, where capsid disassembly is part of the life cycle of most viruses, a capsid destabilizing ligand may be an unrecognized form of receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Electron microscopy was performed at the Indiana University Electron Microscopy Center. The Typhoon Imager is part of the Physical Biochemistry Instrument Facility. AZ notes a potential conflict of interest arising from an interest in a biotech company, Assembly BioSciences.

Funding Sources

This work was supported by NIH grants R01-AI067417 and R01-AI118933 to AZ.

REFERENCES

1. Wynne SA; Crowther RA; Leslie AGW, The crystal structure of the human hepatitis B virus capsid. *Mol Cell* 1999, 3 (6), 771–780. [PubMed: 10394365]
2. Gish RG; Given BD; Lai CL; Locarnini SA; Lau JY; Lewis DL; Schluep T, Chronic hepatitis B: Virology, natural history, current management and a glimpse at future opportunities. *Antiviral Res* 2015, 121, 47–58. [PubMed: 26092643]
3. Diab A; Foca A; Zoulim F; Duranetel D; Andrisani O, The diverse functions of the hepatitis B core/capsid protein (HBc) in the viral life cycle: Implications for the development of HBc-targeting antivirals. *Antiviral Res* 2017.
4. Zlotnick A; Venkatakrishnan B; Tan Z; Lewellyn E; Turner W; Francis S, Core protein: A pleiotropic keystone in the HBV lifecycle. *Antiviral Res* 2015, 121, 82–93. [PubMed: 26129969]
5. Seeger C; Zoulim F; Mason WS, Hepadnaviruses In *Fields Virology*, Knipe DM; Griffin DE; Lamb RA; Martin MA; Roizman B; Straus SE, Eds. Lippincott Williams & Wilkins: Philadelphia, 2007; Vol. 2, pp 2977–3029.
6. Venkatakrishnan B; Zlotnick A, The Structural Biology of Hepatitis B Virus: Form and Function. *Annual Reviews of Virology* 2016, 3, 429–451.
7. Dryden K; Wieland S; Chisari F; Yeager M In *Structure of Native Hepatitis B Virus Particles by Electron Cryomicroscopy and Image Reconstruction*, American Society for Virology 21st Annual Meeting, Lexington, Ke, July, 2002; Lexington, Ke, 2002; pp P20–6.
8. Gunther S; Fischer L; Pult I; Sterneck M; Will H, Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999, 52, 25–137. [PubMed: 10384235]
9. Nassal M, Conserved cysteines of the hepatitis B virus core protein are not required for assembly of replication-competent core particles nor for their envelopment. *Virology* 1992, 190, 499–505. [PubMed: 1529550]
10. Li HC; Huang EY; Su PY; Wu SY; Yang CC; Lin YS; Chang WC; Shih C, Nuclear export and import of human hepatitis B virus capsid protein and particles. *PLoS Pathog* 2010, 6 (10), e1001162. [PubMed: 21060813]
11. Rabe B; Delaleau M; Bischof A; Foss M; Sominskaya I; Pumpens P; Cazenave C; Castroviejo M; Kann M, Nuclear entry of hepatitis B virus capsids involves disintegration to protein dimers followed by nuclear reassociation to capsids. *PLoS Pathog* 2009, 5 (8), e1000563. [PubMed: 19714236]
12. Ceres P; Zlotnick A, Weak protein-protein interactions are sufficient to drive assembly of hepatitis B virus capsids. *Biochemistry* 2002, 41 (39), 11525–31. [PubMed: 12269796]
13. Katen SP; Zlotnick A, Thermodynamics of Virus Capsid Assembly. *Methods in Enz.* 2009, 455, 395–417.
14. Perlmutter JD; Hagan MF, Mechanisms of virus assembly. *Annu Rev Phys Chem* 2015, 66, 217–39. [PubMed: 25532951]
15. Zlotnick A, To build a virus capsid. An equilibrium model of the self assembly of polyhedral protein complexes. *J Mol Biol* 1994, 241 (1), 59–67. [PubMed: 8051707]
16. Packianathan C; Katen SP; Dann CE, 3rd; Zlotnick A, Conformational changes in the Hepatitis B virus core protein are consistent with a role for allostery in virus assembly. *J Virol* 2010, 84, 1607–15. [PubMed: 19939922]
17. Stray SJ; Ceres P; Zlotnick A, Zinc ions trigger conformational change and oligomerization of hepatitis B virus capsid protein. *Biochemistry* 2004, 43 (31), 9989–98. [PubMed: 15287726]

18. Bourne C; Lee S; Venkataiah B; Lee A; Korba B; Finn MG; Zlotnick A, Small- Molecule Effectors of Hepatitis B Virus Capsid Assembly Give Insight into Virus Life Cycle. *J Virol* 2008, 82, 10262–10270. [PubMed: 18684823]
19. Zhou Z; Hu T; Zhou X; Wildum S; Garcia-Alcalde F; Xu Z; Wu D; Mao Y; Tian X; Zhou Y; Shen F; Zhang Z; Tang G; Najera I; Yang G; Shen HC; Young JA; Qin N, Heteroaryldihydropyrimidine (HAP) and Sulfamoylbenzamide (SBA) Inhibit Hepatitis B Virus Replication by Different Molecular Mechanisms. *Sci Rep* 2017, 7, 42374. [PubMed: 28205569]
20. Schlicksup CJ; Wang JC; Francis S; Venkatakrishnan B; Turner WW; VanNieuwenhze M; Zlotnick A, Hepatitis B virus core protein allosteric modulators can distort and disrupt intact capsids. *Elife* 2018, 7, pii: e31473. [PubMed: 29377794]
21. Buck E; Wells JA, Disulfide trapping to localize small-molecule agonists and antagonists for a G protein-coupled receptor. *Proc Natl Acad Sci U S A* 2005, 102 (8), 2719–24. [PubMed: 15710877]
22. Sadowsky JD; Burlingame MA; Wolan DW; McClendon CL; Jacobson MP; Wells JA, Turning a protein kinase on or off from a single allosteric site via disulfide trapping. *Proc Natl Acad Sci U S A* 2011, 108 (15), 6056–61. [PubMed: 21430264]
23. Zlotnick A; Ceres P; Singh S; Johnson JM, A small molecule inhibits and misdirects assembly of hepatitis B virus capsids. *J Virol* 2002, 76 (10), 4848–54. [PubMed: 11967301]
24. Wingfield PT; Stahl SJ; Williams RW; Steven AC, Hepatitis core antigen produced in *Escherichia coli*: subunit composition, conformational analysis, and in vitro capsid assembly. *Biochemistry* 1995, 34 (15), 4919–32. [PubMed: 7711014]
25. Tan Z; Pionek K; Unchwaniwala N; Maguire ML; Loeb DD; Zlotnick A, The Interface between Hepatitis B Virus Capsid Proteins Affects Self-Assembly, Pregenomic RNA Packaging, and Reverse Transcription. *J Virol* 2015, 89 (6), 3275–84. [PubMed: 25568211]
26. Venkatakrishnan B; Katen SP; Francis S; Chirapu S; Finn MG; Zlotnick A, Hepatitis B Virus Capsids Have Diverse Structural Responses to Small-Molecule Ligands Bound to the Heteroaryldihydropyrimidine Pocket. *J Virol* 2016, 90 (8), 3994–4004. [PubMed: 26842475]
27. Wyman J; Gill SJ, Binding and Linkage: Functional Chemistry of Biological Macromolecules. University Science Books; Herndon, 1990.
28. Roos WH; Gibbons MM; Arkhipov A; Uetrecht C; Watts NR; Wingfield PT; Steven AC; Heck AJ; Schulten K; Klug WS; Wuite GJ, Squeezing protein shells: how continuum elastic models, molecular dynamics simulations, and experiments coalesce at the nanoscale. *Biophys J* 2010, 99 (4), 1175–81. [PubMed: 20713001]
29. Hadden JA; Perilla JR; Schlicksup CJ; Venkatakrishnan B; Zlotnick A; Schulten K, Chemical-physical characterization and insight into cryo-EM resolution limits of the hepatitis B virus capsid revealed by all-atom molecular dynamics simulation. under review 2018.
30. Perilla JR; Hadden JA; Goh BC; Mayne CG; Schulten K, All-Atom Molecular Dynamics of Virus Capsids as Drug Targets. *J Phys Chem Lett* 2016, 7 (10), 1836–44. [PubMed: 27128262]
31. Cui X; Ludgate L; Ning X; Hu J, Maturation-associated destabilization of hepatitis B virus nucleocapsid. *J Virol* 2013, 87 (21), 11494–503. [PubMed: 23966388]
32. Dhason MS; Wang JC; Hagan MF; Zlotnick A, Differential assembly of Hepatitis B Virus core protein on single- and double-stranded nucleic acid suggest the dsDNA-filled core is spring-loaded. *Virology* 2012, 430 (1), 20–9. [PubMed: 22595445]
33. Klumpp K; Lam AM; Lukacs C; Vogel R; Ren S; Espiritu C; Baydo R; Atkins K; Abendroth J; Liao G; Efimov A; Hartman G; Flores OA, High-resolution crystal structure of a hepatitis B virus replication inhibitor bound to the viral core protein. *Proc Natl Acad Sci U S A* 2015, 112, 15196–201. [PubMed: 26598693]
34. Schmitz A; Schwarz A; Foss M; Zhou L; Rabe B; Hoellenriegel J; Stoeber M; Pante N; Kann M, Nucleoporin 153 arrests the nuclear import of hepatitis B virus capsids in the nuclear basket. *PLoS Pathog* 2010, 6 (1), e1000741. [PubMed: 20126445]
35. Stray SJ; Bourne CR; Punna S; Lewis WG; Finn MG; Zlotnick A, A heteroaryldihydropyrimidine activates and can misdirect hepatitis B virus capsid assembly. *Proc Natl Acad Sci U S A* 2005, 102 (23), 8138–43. [PubMed: 15928089]

36. Hacker HJ; Deres K; Mildenerberger M; Schroder CH, Antivirals interacting with hepatitis B virus core protein and core mutations may misdirect capsid assembly in a similar fashion. *Biochem Pharmacol* 2003, 66 (12), 2273–9. [PubMed: 14637185]
37. Feld JJ; Colledge D; Sozzi V; Edwards R; Littlejohn M; Locarnini SA, The phenylpropenamide derivative AT-130 blocks HBV replication at the level of viral RNA packaging. *Antiviral Res* 2007, 76 (2), 168–77. [PubMed: 17709147]
38. Guo F; Zhao Q; Sheraz M; Cheng J; Qi Y; Su Q; Cuconati A; Wei L; Du Y; Li W; Chang J; Guo JT, HBV core protein allosteric modulators differentially alter cccDNA biosynthesis from de novo infection and intracellular amplification pathways. *PLoS Pathog* 2017, 13 (9), e1006658. [PubMed: 28945802]
39. Lu Y; Chan W; Ko BY; VanLang CC; Swartz JR, Assessing sequence plasticity of a virus-like nanoparticle by evolution toward a versatile scaffold for vaccines and drug delivery. *Proc Natl Acad Sci U S A* 2015, 112 (40), 12360–5. [PubMed: 26392546]
40. Mohamed Suffian I. F. B.; Wang JT; Hodgins NO; Klippstein R; Garcia-Maya M; Brown P; Nishimura Y; Heidari H; Bals S; Sosabowski JK; Ogino C; Kondo A; Al-Jamal KT, Engineering hepatitis B virus core particles for targeting HER2 receptors in vitro and in vivo. *Biomaterials* 2017, 120, 126–138. [PubMed: 28056402]
41. Pumpens P; Grens E, Hepatitis B core particles as a universal display model: a structure- function basis for development. *FEBS Lett* 1999, 442 (1), 1–6. [PubMed: 9923592]
42. Katen SP; Chirapu SR; Finn MG; Zlotnick A, Quasi-equivalent binding pockets with multiple antiviral activities: Phenylpropenamide assembly effectors modify HBV capsid tertiary and quaternary structures. ms in progress 2012.

Figure 1. Locations of engineered cysteines at the dimer-dimer interface of the HBV core protein.

(a) 120 copies of homodimeric core protein (Cp) form T=4 icosahedral capsids. One icosahedral asymmetric unit is highlighted to show the four quasi-equivalent environments: A (light grey), B (dark grey), C (light blue) and D (medium blue). (b) The CpAM pocket, viewed from the capsid interior, highlighting the location of V124 (in red) at 5-fold and 6-fold vertices. (c) CpAM pocket mutants. Four single cysteines mutants were created at the pocket, L30C (purple), I105C (green), S106C (orange), and V124C (red). Residue V124 is in the final helix of the assembly domain; the loop just C-terminal of V124 fits into a groove in the neighboring subunit to support protein-protein interaction. The models shown here are based on PDB: 1QGT.¹

Figure 2. Biophysical characterization of pocket mutants.

(a-e) Pseudo-critical concentrations of assembly were determined for Cp mutants 300 mM NaCl by size exclusion chromatography. (f-i) Negative stain TEM images of assembled particles show morphologically normal capsids. The ionic strength was chosen to maximize the amount of assembly while minimizing the possibility of kinetically trapped incomplete capsids.

Figure 3. Accessibility of cysteines for labeling.

Absorbance spectra of purified dimer (a) and (b) capsid from reactions of Cp149–3CA cysteine mutants with BoDIPYm. Spectra show the absorbance of protein, 280 nm, and BoDIPYm, predominantly at 504nm. SDS-PAGE of dimer and capsid labeled with Coomassie blue (inset top panel) and imaged with a fluorescence detector (inset bottom) recapitulate the evaluation of labeling in the chromatographs. UV-vis spectra indicate that I105C, S106C, and V124C were all accessible in free dimer. Only V124C was accessible for labeling in assembled capsids.

Figure 4: Capsid disassembles when treated with BoDIPYm and is dose dependent. (a) Size exclusion chromatography of Cp149-3CAV124C: capsid (solid line) and after treatment with BoDIPYm (dashed line). (b) V124C capsids incubated with other ligands show dissociation varies. BoDIPYm (blue) has the greatest impact on dissociation followed by Fluoresceinmaleimide (red). Some dissociation was observed with iodoacetamide (cyan). Dissociation was not observed with FITC (green) or N-ethyl maleimide (magenta). Disassembly is considered to be site-specific, since no disassembly was observed with FITC.

Figure 5: V124C capsid can be protected from disassembly by ligands.

V124C capsids were incubated overnight with (a) iodoacetamide, (b) N-ethyl maleimide (NEM), or (c) HAP 12. The following day, samples were treated with a 20-fold molar excess of BoDIPYm for 2h, excess dye was removed, and samples were resolved by size exclusion chromatography to determine the percent dissociation (closed circles) and the percent of maximal BoDIPY labeling of the capsid peak (open circles). A molar excess of iodoacetamide is required to protect the Cp149–3CA-V124C from BoDIPYm labelling, indicating that it is not a preferred ligand. NEM and HAP12 protect V124C linearly with

concentration. For iodoacetamide and NEM there is good agreement between the amount of protection from dissociation and the amount of BoDIPYm labelling. For HAP12, there is a gap between the amount of labeling and the amount of protected capsid. At stoichiometric ratios of HAP, 2 HAPs/dimer, about 25% of the subunits are labeled, even though the capsids remain intact. Measurements were in triplicate except the first three points in panel a, where the low signal for capsid precluded calculation of percent dissociation, and the value was set to 100%.