pH-Induced Conformational Changes of Human Bocavirus Capsids

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Abstract

Human bocavirus 1 (HBoV1) and HBoV2-4 infect children and immunocompromised individuals, resulting in respiratory and gastrointestinal infections, respectively. Using cryo-electron microscopy and image reconstruction, the HBoV2 capsid structure was determined to 2.7 Å resolution at pH 7.4 and compared to the previously determined HBoV1, HBoV3, and HBoV4 structures. Consistent with previous findings, surface variable region (VR) III of the capsid protein VP3, proposed as a host tissue-tropism determinant, was structurally similar among the gastrointestinal strains HBoV2-4, but differed from HBoV1 with its tropism for the respiratory tract. Towards understanding the entry and trafficking properties of these viruses, HBoV1 and HBoV2 were further analyzed as species representatives of the two HBoV tropisms. Their cell surface glycan-binding characteristics were analyzed, and capsid structures determined to 2.5-2.7 Å resolution at pH 5.5 and 2.6, conditions normally encountered during infection. The data showed that glycans with terminal sialic acid, galactose, GlcNAc or heparan sulfate moieties do not facilitate HBoV1 or HBoV2 cellular attachment. With respect to trafficking, conformational changes common to both viruses were observed at low pH conditions localized to the VP N-terminus under the 5-fold channel, in the surface loops VR-I and VR-V and specific side-chain residues such as cysteines and histidines. The 5-fold conformational movements provide insight into the potential mechanism of VP N-terminal dynamics during HBoV infection and side-chain modifications highlight pH-sensitive regions of the capsid.

Importance

Human bocaviruses (HBoVs) are associated with disease in humans. However, the lack of an animal model and a versatile cell culture system to study their life cycle limits the ability to develop specific treatments or vaccines. This study presents the structure of HBoV2, at 2.7 Å resolution, determined for comparison to the existing HBoV1, HBoV3, and HBoV4 structures, to enable the molecular characterization of strain and genus-specific capsid features contributing to tissue tropism and antigenicity. Furthermore, HBoV1 and HBoV2 structures determined under acidic conditions provide insight into capsid changes associated with endosomal and gastrointestinal acidification. Structural rearrangements of the capsid VP N-terminus, at the base of the 5-fold channel, demonstrate a disordering of a “basket” motif as pH decreases. These observations begin to unravel the molecular mechanism of HBoV infection and provide information for control strategies.

Introduction

The genus *Bocaparvovirus,* belonging to the *Parvovirinae* subfamily of the *Parvoviridae* family (1), was named after the first two virus members, bovine parvovirus (BPV) reported in 1961 (2, 3) and canine minute virus (CnMV) isolated in 1967 (4). In 2005, a third member, human bocavirus 1 (HBoV1) was discovered in children (<2 years of age) suffering from acute respiratory tract infections (5). Three more human bocaviruses, HBoV2, HBoV3, and HBoV4, were subsequently isolated from pediatric patients with acute gastroenteritis (6-8). HBoV infections have been described worldwide in young children (7, 9-13), resulting in high seroprevalences of up to 80% for HBoV1 and 50% for HBoV2 already at the age of 6 years (14, 15).

The HBoV genomes contain three open reading frames (ORFs) (16, 17). ORF1 encodes a non-structural protein 1 (NS1) involved in genomic DNA replication (18). ORF2 encodes a phosphorylated nucleoprotein (NP) also important for DNA replication, capsid protein expression, and interferon signaling (19-21). ORF3 encodes three overlapping viral proteins, VP1 (~74 kDa), VP2 (~64 kDa), and VP3 (~60 kDa) (16, 17), which assemble the capsid, with T=1 icosahedral symmetry, from 60 VPs in an approximate 1:1:10 ratio (22). The VPs are expressed from different start codons within the same transcript and therefore share a common C-terminus (23-25). However, 60 copies of the common VP3 are capable of assembling capsids (26). Importantly, VP1 contains a unique N-terminal region (VP1u) containing a phospholipase A2 (PLA2) domain conserved in most parvoviruses (27). Its enzymatic activity is required for the capsid to escape the endo/lysosomal pathway after entry into host cells. The structure of the VP3 common region contains two α-helices (αA and αB) and an eight-stranded anti-parallel-β barrel (βB to βI) that forms the conserved core of the capsid. Large loops inserted between the β-strands form the exterior capsid surface (28, 29). These loops display the highest amino acid (aa) sequence variability and structural diversity among HBoV1, HBoV3, and HBoV4 capsids,

and contain regions defined as variable regions (VRs) (28, 29). Characteristic features of the capsids include depressions at the icosahedral 2-fold axes and surrounding a channel at the 5-fold axes, three separate protrusions that surround the 3-fold axes, and raised capsid regions between the 2- and 5-fold axes, termed the 2-/5-fold wall (28, 29).

The HBoV capsid, as with other parvoviruses, serves as a protective coat for the packaged genome during the infection process that initiates with receptor attachment and cellular entry, involves trafficking in the endo/lysosomal pathway, nuclear entry, genome release and replication, transcription and translation, and ultimately capsid/virion assembly (30-32). For host cell recognition the capsid attaches to specific receptor(s) on the cell surface. With the exception of bovine parvovirus (BPV), reported to bind glycophorin A (GPA) with terminal α-2,3 O-linked sialic acid on erythrocytes, nothing is known about cellular entry receptor usage by other bocaviruses (33). As shown for many other parvoviruses, the capsid traffics through the endo/lysosomal pathway, to the nucleus for genome replication, after cellular entry, and requires a pH gradient from the early endosome (pH 6.0), to late endosome (pH 5.5), and lysosome (pH 4.0) conditions for successful infection (32). This acidification is reported to serve as a trigger for virus escape from the transport vesicles, utilizing the VP1u PLA2 enzymatic activity (31, 34). Similar to other parvoviruses, the bocavirus VP1u is postulated to become externalized through the 5-fold channel of the capsid, from the interior of the capsid, to enable its PLA2 function upon acidification of the endosomes (35-37).

In this study, the capsid structure of HBoV2 was determined to 2.7 Å resolution by cryo-electron microscopy (cryo-EM) and image reconstruction, and compared to the available capsid structures of HBoV1, HBoV3, and HBoV4 determined with the same method, to 2.9, 2.8, and 3.0 Å resolution, respectively (28). The HBoV2 structure is similar to those of HBoV3 and 4, the other enteric viruses. All four structures, determined at neutral pH, exhibit surface loop topology differences in the VRs associated with tropism disparities (among parvoviruses), and all display

a “basket” motif formed by the N-termini of VP3, extending the 5-fold channel into the capsid interior. The cell surface glycan-binding properties of HBoV1 and HBoV2 were also investigated and their structures determined at pH 5.5 and 2.6, to characterize the effect of pH acidification encountered during trafficking on the capsid structure. Column and cell binding-based assays failed to identify potential interacting glycans either because the cell attachment receptor does not contain a glycan, that the glycan recognized was not present in the array tested or the attachment site is not present in VP3. Comparison of the structures at pH 7.4, 5.5, and 2.6 showed analogous disordering of the basket motif under the 5-fold channel, in addition to conformational changes at several surface loops, and modifications of cysteine and histidine amino acid side chains as pH drops. These changes suggest potential re-arrangements required for successful virus trafficking. Significantly, capsid stability measurements showed the highest thermal stability (T_m) at pH 5.5, suggesting that concurrent stabilization and re-arrangements drive the capsid transitions required for infection, including the disordering of the 5-fold basket motif, predicted to be necessary for externalization of the PLA2 function at low pH. This study provides insight into the mechanism(s) of HBoV infection and potential means to control HBoV spread.

Results and Discussion

The HBoV2 structure conserves features of other bocavirus members. HBoV2 VP3 virus-like particles (VLPs) were generated using the baculovirus/*Sf*9 expression system, and produced and purified as previously reported (28) (see Methods section) in quantities and quality suitable for structural studies (not shown). The structure of HBoV2, at pH 7.4, was determined from a total of 188,857 capsid images, extracted from 2,921 micrographs. The individual capsid images were combined to reconstruct the capsid structure to 2.7 Å resolution (Fourier shell correlation (FSC) = 0.143 (Table 1)). The HBoV2 capsid density map exhibits surface capsid topology features shared by other known *Parvovirinae* subfamily members, including: a depressed region at the 2-fold axes; three protrusions surrounding the 3-fold axes; a cylindrical channel at the 5-fold axes, surrounded by a wide depressed region; and a "wall" between the 2- and 5-fold axes (22, 31) (Fig. 1A). A cross-sectional view of the HBoV2 capsid showed a “basket" beneath the 5-fold axes, a structural feature observed for BPV (29), HBoV1, HBoV3, and HBoV4 (28) (Fig. 1B). The HBoV2 amino acid side chains were well ordered in the core secondary structure (Fig. 1C) and most surface loop regions enabling most of VP3 to be built. Exceptions were missing side-chain densities for some residues at the apexes of some surface loops and acidic residues known to be most susceptible to radiation damage in cryo-EM (38). Furthermore, the first 32 residues of VP3 could not be built into the density map due to lack of ordering of the N-terminus. Similar to the other HBoV structures (28), the first ordered N-terminal residue of VP3, residue 33, is located under the 5-fold channel projecting into the basket density extending into the interior of the capsid (Fig. 1D). The refined model, from residue 33 to the C-terminal residue 542, has a correlation coefficient (CC) of 0.83 to the reconstructed density map. The refinement statistics for the model are summarized in Table 1. The VP3 structure of HBoV2 displays the conserved core features of other parvoviruses (29), including an eight-stranded anti-parallel β-barrel (βB - βI) that is organized in two β-sheets,

149 BIDG and CHEF with an additional βA strand that is anti-parallel to βB, and an α-helix (αA)
150 between βB and βC (Fig. 2A). In addition, a second α-helix (αB) exists in the HBoV2 VP3
151 structure located in one of the surface loops between the βE and βF (Fig. 2A), similar to other
152 known bocavirus structures (28, 29).

153

154 **Comparison of HBoV VP3 structures at pH 7.4 supports a role for VR-III as a tropism**
155 **determinant.** The HBoV VPs have an amino acid sequence identity that ranges from 77 to 90%
156 (7). Structural comparison of the different HBoV capsid structures showed that their core
157 regions are nearly perfectly superposable (Fig. 2B). Furthermore, several VRs exhibit similar
158 conformations, including VR-II, VI, VII, VIII, and IX (Fig. 2B). The remaining VRs, VR-I, VR-III,
159 VR-IV, and VR-VIIIB, showed differences, with VR-III and VIIIB differing in HBoV1 due to a 4 aa
160 insertion and an alternative conformation, respectively, and VR-IV in HBoV4 due to a 2 aa
161 insertion as previously reported (28). Variable region I and VR-V differed among all HBoV
162 strains despite the lack of insertions or deletions. Overall, the HBoV2 VRs resemble those of
163 HBoV3 with a minor difference in VR-V (Fig. 2B), with an overall Cα RMSD of 0.66 Å for their
164 superposed VPs. HBoV4 displays structural differences to HBoV2 in VR-I, IV, and V (Fig. 2B),
165 and is the next most structurally similar with an overall VP Cα RMSD of 0.78 Å. HBoV1, has the
166 lowest sequence similarity and a different tissue tropism compared to the gastrointestinal
167 HBoVs and also exhibits the greatest VP Cα RMSD of 1.13 Å compared to HBoV2. Previously,
168 it was postulated that VR-III, displaying the highest structural difference between the respiratory
169 and the gastrointestinal HBoVs, might be a determinant for tissue tropism (28). HBoV2
170 conserves the structure of this loop in the gastrointestinal HBoVs. In contrast to the other
171 surface loops, the aa sequence of VR-III, residues 196-IHELAEMED(A/S)NAVEKAI-212, is
172 highly conserved among HBoV2, HBoV3, and HBoV4. This further highlights the potential
173 importance of VR-III for tissue tropism. VR-III is located at the 2-5-fold wall of the capsid where

other parvoviruses are also known to bind to glycan receptors, e.g. AAV1 to sialic acid (39) or AAV9 to terminal galactose (40). This capsid region along with VR-I, VR-IV, VR-V, and VR-VIII also serve as dominant antigenic regions (41). HBoV-based vectors are currently being developed as vectors for gene therapy applications (42). The structure information for HBoV2, along with those already available for the other HBoV strains (28), will assist future engineering of vectors to reduce the detrimental effects of pre-existing immunity, and improve or alter the tropism as recently shown for HBoV1 (43).

HBoV1 and HBoV2 VP3 VLPs do not bind common cell surface glycans. To date, no viral entry receptors have been identified for any of the HBoVs. To characterize whether the HBoVs use glycans as receptors, HBoV1 and HBoV2, the two most widely distributed HBoVs representing the two HBoV tropisms and disease phenotypes (5, 7), were analyzed. Heparin-binding assays, to mimic heparan sulfate proteoglycan (HSPG) interactions, utilizing VLPs, were performed (Fig. 3A) in addition to cell-binding assays with fluorescently labeled VLPs (Fig. 3B). AAV2 VLPs known to bind HSPG (44) and AAV5 VLPs that do not bind to HSPGs but bind to terminal sialic acid (45), were used as controls. In the heparin-binding assays, AAV2 VLPs appeared in the elutions fraction consistent with binding while AAV5 and the HBoV1 and HBoV2 VLPs were detected in the wash and flowthrough fractions indicating lack of binding (Fig. 3A). Cell-binding assays, to analyze binding to terminal sialic acid, galactose, or N-acetylglucosamine, utilized differential glycan-presenting CHO cell lines. The parental CHO-Pro5 cell line displays terminal sialic acid, the mutant Lec2 cell line displays terminal galactose, and the Lec8 cell line displays N-acetylglucosamine, resulting from mutations in specific genes required for glycan biosynthesis (46). AAV5 that is known to bind to sialic acid (45) only bound to Pro5 cells whereas AAV2, which binds to HSPG (44) and does not require any of the glycans affected by the mutations of the CHO cell lines, bound to all three cell lines with approximately

equal efficiency (Fig. 3C). In contrast, both HBoV1 and HBoV2 showed no significant binding to any of the three cell lines, indicating that they do not use terminal sialic acid, galactose, or N-acetylglucosamine as receptors (Fig. 3C). In addition, a series of colon cell lines: HT-29, CaCo-2, and CMT-93 and the lung cell line A549, were analyzed (Fig. 3C). While AAV2-VLPs bound efficiently to all these cells, AAV5 showed weak to medium binding phenotypes and both HBoV1 and HBoV2 showed no significant binding (Fig. 3C). These observations indicate that these viruses might require additional cofactors for cellular attachment such as bile salts or microbial molecules as described recently for noroviruses (47). It could also be that they do not utilize glycans for cellular entry or that, unlike AAV2 and AAV5, VP3 alone does not contain the glycan binding determinant and VP1 is required, as reported for B19 (48).

HBoV1 and HBoV2 capsids reveal strain-specific as well as common phenotypes under pH environments associated with infection. During infection and trafficking, HBoVs encounter different cellular environments. HBoV1, is exposed to the mild acidic pH conditions in the respiratory tract ranging from pH 5.5 to 6.7 (49). In contrast, HBoV2-HBoV4, associated with gastrointestinal infections, are initially exposed to the acidic extracellular environment of the stomach, ranging from pH 1.5 to 3.5 (50). While experiencing these varied extra-cellular pH conditions the capsids have to remain structurally intact to infect cells. In addition, the HBoVs all also experience varied pH conditions after cellular internalization while trafficking through the early (pH 6) and late endosome (pH 5.5), lysosome (pH 4), and then nucleus (pH 7.4). In order to confirm that HBoV1 and HBoV2 capsids are indeed intact at these conditions the VLPs were dialyzed into different pH environments and analyzed by negative-stain EM. The result showed that the capsids were visually intact in all the pHs tested, even with multiple changes of pH conditions (from pH 7.4 to 2.6 and back to 7.4) (Fig. 4A and B).

To further analyze the stability of the capsids at the different pH environments the melting temperature (T_M) of the HBoV VLPs were determined by differential scanning fluorimetry (DSF) with previously characterized AAV5 VLPs for direct comparison. The T_M of AAV5 at pH 7.4 has been reported to be 89.0 ± 0.1°C (in Universal buffer, see methods for ingredients) (51) and confirmed here (T_M of 89.5°C, Fig. 4C). At lower pH conditions the T_M of AAV5 gradually decrease; 89.5°C at pH 7.4, 88.0°C at pH 6.0, 80.5°C at pH 5.5, 78.0°C at pH 4.0, and 71.5°C at pH 2.6. The thermal stability of AAV5 capsids has also been analyzed in citric phosphate buffer at pH7.4, 6.0, 5.5, and 4.0 with comparable results (37). The thermal stability profile of HBoV2 followed a similar trend to AAV5, however at lower temperatures, with the T_M decreasing with pH; 73.2°C at pH 7.4, 70.3°C at pH 6.0, 70.0°C at pH 5.5, 67.8°C at pH 4.0, and 43.5°C at pH 2.6 (Fig. 4C). The HBoV1 profile differed in that it's T_M increased as pH dropped and then decreased again: 65.3°C at pH 7.4, 68.7°C at pH 6.0, 69.7°C at pH 5.5, and then reduced to 67.0°C at pH 4.0 and 41.3 °C at pH 2.6. A similar trend was previously observed for AAV1, AAV2, and AAV8 (37). The capsid stability is reversible, for example, when dialyzed from pH 2.6 to 7.4 (Fig. 4C).

The T_M of the two HBoVs differed most at pH 7.4, by ~8°C, and by only ~2°C from pH 6 to 2.6, with HBoV2 being the most stable. The difference at pH 7.4 is consistent with the utility of T_M for identifying AAV serotypes (52) and suggests that the HBoVs can be similarly differentiated by T_M. Notably, the VP1u and VP1/2 common regions of VP1 and VP2 do not contribute to AAV capsid stability, and this phenotype is likely the same for the HBoVs. Despite the lack of minor VPs in the HBoV VLPs, the maintained stability of the capsids at pH 6.0 and 5.5 is consistent with the need to preserve integrity under the conditions where the PLA_2 domain within the VP1u is proposed to be externalized for endo/lysosomal escape (53). The reversibility of the pH effects on the capsid, from a destabilized low pH environment to pH 7.4, provides

insight into the requirements to escape into the cytoplasm and travel into the nucleus while protecting the genome.

The capsid structure of HBoV1 and HBoV2 at pH 2.6 and 5.5 reveals capsid dynamics associated with endo/lysosomal escape. To further analyze the observed contrasting capsid stabilities at pH 2.6 and 5.5, the structures of HBoV1 and HBoV2 were determined at these pHs to identify any associated structural differences. VLPs were dialyzed to the two pH conditions and vitrified on EM grids for data collection. For HBoV1 50,342 capsids were selected at pH 2.6 and 66,471 capsids at pH 5.5 resulting in a three-dimensional (3D) reconstructed capsid structure at 2.54 and 2.74 Å resolution, respectively (Table 1). Similarly, for HBoV2, 19,648 and 30,308 capsids were selected at pH 2.6 and pH 5.5 for a 3D-reconstructed capsid structure at 2.51 and 2.74 Å resolution, respectively (Table 1).

Comparison of the HBoV1 and HBoV2 maps at pH 2.6 and 5.5, with the previously published pH 7.4 HBoV1 map (28) and the map of HBoV2 described above, showed minor structural differences on the capsid exterior surface but significant movement of the VP3 N-terminus (Fig. 5, 6 and 7). The capsid surface differences are located at the 2-5-fold wall (VR-I) and 3-fold protrusions (VR-V) of the HBoV1 and HBoV2 capsids at pH 2.6 compared to pH 7.4 and 5.5 (Fig. 6), with HBoV2 showing additional density at the 3-fold symmetry axis at pH 2.6 (Fig. 5). On the capsid interior, the 'basket' structure beneath the 5-fold axis at pH 7.4 becomes less pronounced at pH 2.6, suggesting that low pH induces movements of the VP3 N-terminus for both HBoV1 and HBoV2 (Fig. 5). Interestingly, low resolution cryo-EM structures of both capsids returned to pH 7.4 from 2.6 showed the reappearance of the basket structure and loss of the 3-fold density in HBoV2 (data not shown), implying that these structural observations are reversible. This would be consistent with the requirement to maintain capsid integrity following VP1u externalization to protect the encapsulated genome en route to the nucleus for replication.

Similar to pH 7.4, the low pH structures were ordered from residue 33 to the C-terminus, residue 542 in HBoV1 and 537 in HBoV2. The CCs for the models fitted into their respective maps ranged from 0.80 to 0.87 (Table 1). The RMSDs for HBoV1 are 0.6 Å (pH 5.5) and 0.7 Å (pH 2.6) for Cα atoms compared to the pH 7.4 structure. As reported previously, two regions of the HBoV1 pH 7.4 map, residues 204-209 (VR-III) and 333-338 (VR-V), were poorly ordered, and only the main chain interpreted. Similarly, the HBoV1 map at pH 2.6 displayed disordered density in these loops and as well as residues 78-84 (VR-I) (Fig. 6A and C). Despite the high level of disorder in these loops, the main chain was interpretable (Fig. 6A and C). In contrast to the pH 2.6 and pH 7.4 HBoV1 maps, the pH 5.5 density was better ordered in these loops with most side-chain densities observed (Fig. 6A and C). Overall, the HBoV1 VP structure topology was similar at all three pH conditions analyzed except for conformational shifts in VR-I and VR-V (Fig. 6E). These structural shifts were more pronounced for pH 2.6 with main chain movements of ~9 Å in both VR-I and VR-V compared to pH 7.4, and ~3 Å in VR-I and ~7 Å in VR-V between pH 5.5 and 7.4. These observations identify the 2/5-fold wall and 3-fold protrusions of the capsid as being involved in the capsid dynamics associated with low pH-mediated transitions required for infection.

For the HBoV2 pH 5.5 and 2.6 structures the overall RMSDs for their VP superposed onto the pH 7.4 coordinates is 0.4 and 0.7 Å, respectively, for Cα atoms. The HBoV2 surface loops were generally more ordered at pH 7.4 and 5.5 compared to those of HBoV1 (Fig. 6A-D). At pH 2.6, VR-V was slightly disordered and only the main chain interpreted (Fig. 6D). Similar to HBoV1, the overall capsid structure was maintained with low pH-dependent shifts of the main chain observed in VR-I and VR-V. These shifts are also more pronounced at pH 2.6, with main chain movements of ~4 Å in VR-I and ~6 Å in VR-V compared to pH 7.4, and <2 Å in VR-I and <3 Å in VR-V at pH 5.5 compared to pH 7.4. Thus, the HBoV2 capsid is susceptible to reduced pH-induced conformational changes compared to HBoV1. This could be an adaptation to the

297 potential fecal-oral transmission route involving the acidic conditions of the stomach. In addition,
298 the pH-sensitive regions of the HBoV capsids, VR-I and VR-V, located at the side of the 2/5-fold
299 wall and 3-fold protrusions, is known to bind to various receptors in other parvoviruses (39, 40,
300 54, 55). The pH-induced movement of these loops may enable the capsid to detach from its
301 receptor after cell entry and acidification of the endosomes.

302

303 **The HBoV VP N-terminus experiences pH-induced conformational changes**. The basket-
304 like structure of the HBoVs under the 5-fold channel (Fig. 1D) is formed by the glycine-rich N-
305 terminus of VP3 (28). The high number of glycine residues grouped at the N-terminus of the
306 major VP is a common element among the parvoviruses (22). However, among the parvoviral
307 structures determined to date only the bocaviruses (28, 29), AAV5 in complex with an antibody
308 (56), and Penaeus monodon metallodensovirus (57) display this feature. The clustering of
309 glycines is believed to provide flexibility to the N-terminus for the externalization of the VP1u
310 region with its PLA2 domain through the 5-fold channel. The presence of the basket could thus
311 be thought to prevent premature externalization, acting as a "cork" to the 5-fold channel, or as
312 the region positioned to initiate the process. However, the basket is still present at pH 5.5 (Fig. 5
313 and 7), the condition encountered by capsids during endosomal acidification and is thought to
314 be associated with the externalization of the VP1u region. At pH 2.6 the basket under the 5-fold
315 channel disappears, with density observed leading away from the 5-fold channel, on the capsid
316 interior surface, interpreted as aa24-29 of the VP3 N-terminus (Fig. 5 and 7). This density runs
317 antiparallel to the βG strand in both HBoV1 and HBoV2 (Fig. 7B and D) and possesses several
318 glycines on both ends. It is possible that this movement away from the 5-fold channel (Fig. 7E
319 and F) is a mechanism to prevent premature externalization of VP1u, or that externalization is
320 via a different portal, for example, the 2-fold axis. However, these studies were performed on
321 HBoV VP3-only VLPs. The wild-type capsid contains, on average, 50 copies of VP3, 5 of VP1,

and 5 of VP2 with extended N-terminus of 129 and 35 aa, respectively. Due to the incompatibility of the number of VP1 and VP2 with icosahedral symmetry (imposed during structure determination), their structures would also be absent in the density map of wild-type capsids (22). Nonetheless, in future studies, it would be interesting to see whether VP1 and VP2 has an effect on the basket structure under the 5-fold channel.

pH-induced residue level changes targets cysteines and histidines. In addition to structural shifts described above a number of side chain rotamer changes and unassigned densities proximal to side chains were observed in the HBoV1 and HBoV2 pH 2.6 and 5.5 maps compared to pH 7.4 (Fig. 8). The amino acids affected the most are cysteines and histidines. The HBoV1 and HBoV2 VP3 contains a total of 10 and 8 cysteines, respectively. None of these are in close enough proximity to form disulfide bonds. However, in HBoV1, at pH 5.5 and 2.6, two and eight cysteines, respectively, exhibited modifications or additional density, not consistent with the cysteine side chain alone, as seen at pH 7.4 (Fig. 8A and B, Table 2). Similarly, the same observation was made for HBoV2, with three cysteines at pH 5.5 and five at pH 2.6 exhibiting additional density (Fig. 8A and B, Table 2). In some cases, the additional density on the cysteines caused steric clashes with neighboring amino acids resulting in conformational shifts of amino acid side chains. For example, conserved C159's modification flips the orientation of a nearby phenylalanine (F234 in HBoV1; F239 in HBoV2) at pH 5.5 and 2.6 (Fig. 8A). Generally, conserved cysteines showed modifications in both HBoVs except for C473 in HBoV2 that did not show any additional densities unlike structurally equivalent C477 in HBoV1 (Fig. 8B). This density extends to nearby C104, not conserved in HBoV2 (Table 2), and M177 in HBoV1. Vice-versa there were also unique cysteine densities in HBoV2, e.g., C393 (valine in HBoV1), modified by additional density. This cysteine is located near the 3-fold symmetry axis (Fig. 8B). The three symmetry-related cysteines created the large extra density

seen at the center of the 3-fold axis (Fig. 5 and 8B). Generally, the cysteines with modifications in the HBoV1 and HBoV2 capsids are located either on the exterior or interior surface of the capsid (Fig. 8C and D) and are located in hydrophobic environments. In contrast, unmodified cysteines are either buried or situated in less hydrophobic regions (not shown).

The cysteine modification phenomenon appears to be independent of the buffer used, because glycine-HCl was used at pH 2.6 and universal buffer at pH 5.5 (see methods for ingredients). It is important to note that pH-induced cysteine modifications have not been previously described. Deposited EM maps in the electron microscopy data bank (EMDB) mostly described protein structures at neutral pH and the few maps at lower pH do not possess the necessary resolution required for this level of comparison with one exception. EMD-4063 (PDB accession number 5LK7) is the structure of the slow bee paralysis virus virion at pH 5.5 (in sodium acetate buffer) at a resolution of 3.42 Å. This structure contains five cysteines. While not discussed in the publication, two of these cysteines, Cys117 and Cys241 in chain A, show similar modifications as seen in the HBoV maps. The fact that these additional densities were not observed at pH 7.4 indicates that the low pH may be responsible through the reaction of glycine or acetate ions react with the thiol group. In future studies, mass spec analyses may clarify the nature of these cysteine modifications. A low-resolution structure of HBoV2 determined from a sample dialyzed to 2.6 and then back to pH 7.4 did not show the extra density at the center of the 3-fold axis. This indicates that these modifications are reversible. The biological significance of these cysteines and their modifications needs to be evaluated especially given that Cys159 and Cys247/243 showing these densities at pH 2.6 and 5.5 (Fig. 8A, Table 2) are conserved in all HBoVs (28).

HBoV1 and HBoV2 both contain 12 histidines within their VP3 sequence (Table 2). Among these, there are two conserved pairs with continuous density between them: H156:H230/226 and H163:H443/439 for HBoV1 and HBoV2, respectively (Fig. 9A, Table 2). At

pH 2.6, additional density occurs between the H156:H230/226 pair in HBoV1 and HBoV2, but not between the H163:H443/439 pair (Fig. 9A). These "joined" histidine pairs are connected within the same VP monomer and are conserved in all HBoVs. In addition, a series of “single” histidines also exhibited modifications at pH 2.6 (Table 2). Conserved histidines in both HBoVs behaved identical with either both having additional density at pH 2.6, e.g. H497/493 (Fig. 9B), or both not having additional density, e.g. H515/511. In some cases, aa near histidines with extra density underwent a conformation change, such as K459 and Y462 near H70 in HBoV2 (Fig. 9B). Interestingly, these residues did not make the same conformational rotation in HBoV1 despite being conserved. In contrast to the cysteines mentioned above, histidines with and without additional density are found on both the capsid interior and exterior surfaces (Fig. 9C). Histidines have been shown to change their orientation at different pHs in AAV8 (58). However, additional density was not reported at low pH in this past study that utilized X-ray crystallography. Here it is only seen at pH 2.6 (Fig. 9A and B). To our knowledge, no cryo-EM structure of sufficient resolution has been published at this pH of other parvoviruses and thus relevance requires further studies beyond the scope of this work.

In summary, the HBoV2 capsid structure presented here completes the panel for the currently available HBoV strains. As anticipated, HBoV2 was structurally similar to the other gastrointestinal HBoVs, HBoV3 and HBoV4, and differs from HBoV1 at VR-III, supporting a role for this VP region in tissue tropism as originally proposed (28). The capsid dynamics associated with endo/lysosomal trafficking was documented with HBoV1 and HBoV2 as representatives of the respiratory and gastrointestinal strains, respectively. Both show similar N-terminal VP rearrangements and residue level, cysteine and histidine, modifications at specific positions on the interior and exterior capsid surface. The N-terminal changes support progression towards the externalization of the PLA2 enzyme required for phospholipid cleavage and escape to the cytosol or interaction with the nuclear membrane. The modifications are reversible, suggesting

that they may be required for interaction with other molecules in vesicle membranes. Following escape from the endo/lysosomal pathway, the HBoV capsid reverts to protecting the packaged genome and trafficking to the nucleus for genome replication.

Methods

HBoV VLP generation and purification. The baculovirus-expression system in *Sf9* (*Spodoptera frugiperda*) cells was used to express VLPs of HBoV1 and HBoV2**.** Briefly, 250 ml of *Sf9* cells (1.7~2.0x10^6 cells/ml) were infected by a recombinant baculovirus expressing HBoV1 or HBoV2 VP3 at a multiplicity of infection of 5 and incubated at 28°C for 3 days. The infected cells were lysed by three rounds of freeze/thaw cycles in TNET buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100). After the third cycle, the completely thawed pellet was treated with Benzonase (250 U/µl, 1 µl/ml sample) to remove nucleic acid. The VLPs were pelleted through a 20% sucrose (w/v, in TNET) cushion centrifugation at 45,000 rpm for 3 h at 4°C. Subsequently, the pelleted VLPs were resuspended in TNTM buffer and further purified by sucrose gradient (5% to 40% sucrose [wt/vol] in TNTM buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 0.2% Triton X-100), centrifugation at 35,000 rpm for 3 h at 4°C. 20-25%, 25-30%, and 30-35% fractions of sucrose gradient were collected and analyzed for the presence of VLPs. The fractions were dialyzed three times into 1×PBS (phosphate buffer saline, pH 7.4) at 4°C for 3 h each and concentrated to ~1-2 mg/mL using an Apollo concentrator column. SDS-PAGE and negative-stain EM using a Tecnai G² Spirit TEM (FEI Co.) were used to analyze the purity and VLP integrity, respectively, as described previously (56). Alternatively, the purified HBoV1 or HBoV2 samples were dialyzed into glycine-HCl (50 mM glycine, adjusted to pH 2.6) or universal buffer (20 mM HEPES, 20 mM MES, 20 mM sodium acetate, 150 mM NaCl, 5 mM $CaCl_2$) adjusted to a pH of 4.0, 5.5, 6.0, or 7.4, respectively. All pH treated VLP samples were analyzed by SDS-PAGE and negative-stain EM prior to 3D structure determination.

Cryo-EM and Data Collection. HBoV1 VLPs (~1 mg/ml) were applied to C-flat holey carbon grids (Protochips, Inc.) while HBoV2 VLPs were applied to a thin carbon coated over holey

QUANTIFOIL® grids to overcome aggregation. The samples were vitrified using a Vitrobot™ Mark IV (FEI Co.) and screened on a 16-megapixel CCD camera (Gatan, Inc.) in a Tecnai (FEI Co.) G2 F20-TWIN Transmission Electron Microscope (200 kV, ~ $e^-/Å^2$) prior to data collection. For high resolution data collection of HBoV2 at pH 7.4 micrographs were collected by using a Titan Krios electron microscope (FEI Co.) operating at 300 kV with a DE20 (Direct Electron) direct electron detector. This data set was collected as part of the NIH Southeastern Center for Microscopy of MacroMolecular Machines (SECM4) project. For the HBoV VLPs at low pH conditions data was collected using a Titan Krios electron microscope on a K2 Summit (Gatan) direct electron detector at the UMASS Cryo-EM core. Images were recorded as 'movies' consisting of multiple frames and their parameters are summarized in Table 1. Subsequently, the frames of the micrographs were aligned for motion correction and to enhance the signal-to-noise ratio by using the DE_process_frames software (Direct Electron) with corresponding dark and bright reference images without radiation dose damage compensation for the data collected on the DE20 direct electron detector (59). The frames collected on the K2 Summit direct electron detector were aligned using MotionCor2 (60).

Structure Determination of HBoV VLPs. For the 3D image reconstruction of HBoV1 and HBoV2 at the different pH conditions the cisTEM software package was utilized (61). Briefly, the aligned micrographs were imported into the program and their contrast transfer function (CTF) parameters estimated. The CTF information was used to eliminate micrographs of poor quality. This was followed by automatic capsid picking using a radius of 125 Å. The selected capsids for each data set were subjected to 2D classification that eliminated ice particles and debris from the automatic picking process. Following 2D classification, the structures were reconstructed using default settings. This included ab initio 3D model generation, auto refinement, and density map sharpening with a pre-cut off (low resolution amplitudes) B-factor value of -90 $Å^2$, and

variable post-cut off (high resolution amplitudes) B-factor values such as 0, 20, and 50 $Å^2$. The sharpened density maps were inspected in the Coot and Chimera applications (62, 63). The -90 $Å^2$ (pre-cut off) and 0 $Å^2$ (post-cut off) sharpened maps were used for assignment of the amino acid main- and side chains for the majority of the capsid. For the more disordered surface loops, VR-I and VR-V (particularly at pH 7.4 and pH 2.6), the -90 $Å^2$ (pre-cut off) and 50 $Å^2$ (post-cut off) sharpened maps were used, which were less noisy and allowed a better assignment of the amino acid main chain in these VP regions. The resolution of the cryo-reconstructed maps was estimated based on a Fourier shell correlation (FSC) of 0.143 (Table 1).

VP3 Model Building and Structure Refinement. A model of the HBoV2 VP3 monomer was generated based on the protein sequence (NCBI accession number: AFW98869.1) on the SWISS-MODEL protein structure homology-modelling server (https://swissmodel.expasy.org/) (64) using the structure of HBoV3 as the template (28, 64). The resulting monomer was utilized to build an icosahedral 60-mer capsid model in VIPERdb (65). The 60-mer capsid model was docked into the HBoV2 cryo-reconstructed density map using the 'fit in map' subroutine in UCSF-Chimera (63). The quality of the fit between the map and the model was evaluated by a correlation coefficient (CC) calculation. The pixel size of the reconstructed map was adjusted to obtain the best fit of the 60-mer model in the reconstructed map. Using the e2proc3d.py subroutine in EMAN2 and the program MAPMAN, a CCP4 format map file with the correct pixel size was generated that also represents a compatible file type for Coot (66-68). In Coot the reference VP3 model was fitted into the density map by adjusting the position of residues through interactive model building and the real-space-refine options (66). For the HBoV capsids at low pH conditions, the pH 7.4 VP3 model was fitted into the maps as described above and structural rearrangements refined in Coot using the real-space-refine option. All VP3 models were further refined using PHENIX real space refinement with the default settings (69). The

resulting model was analyzed in Coot with the density map and side chains were modified if necessary. Finally, a B-factor refinement of the models was conducted in PHENIX.

Structure Alignment of HBoVs. For comparative analysis, the VP3 structures of HBoV1-4 or of the HBoV1 and HBoV2 at the different pH conditions, were superimposed with each other by using the secondary structure matching (SSM) program in Coot (70). The program also calculated root mean square deviations (RMSDs) for the superposed structures and the distances between the aligned Cα positions. The cartoon representations of the VP3 structures and side chain density images were generated in Chimera (63).

Fluorescent Labeling of VLPs. 300-500 µl of purified HBoV1, HBoV2, AAV2, and AAV5 VLPs (0.5-0.8 mg/ml) were labeled using the DyLight 488 antibody labeling kit (Thermo Fisher) as described previously (71). Unbound dye was removed by repeated dialysis. The success of capsid labeling was confirmed by SDS-PAGE and analysis of the gel on a UV transilluminator. Labeled VLPs were aliquoted and stored until usage at -80°C.

Cell Lines and Cell Binding Assay. The CHO cell lines Pro5, Lec2, and Lec8 were cultured as monolayers in MEM-α with 10% FBS (fetal bovine serum) and 1% Antibiotic-Antimycotic (Gibco) in a 5% CO_2 37°C incubator. For cell binding assays, the cells were diluted to 5×10^5 cells/ml, pre-chilled for 30 min at 4°C and aliquoted in 500 µl fractions. Each tube of cells was then incubated with the fluorescently labeled VLPs at a MOI of 10^6 under constant rotation for 3~4 h at 4°C. Following the incubation, the cells were pelleted at 2000 rpm for 10 min and the supernatant discarded. Unbound VLPs were removed by washing the cells with 300 µl ice-cold 1×PBS, followed by another centrifugation. Pellets were resuspended in 300 µl 1×PBS and

analyzed by fluorescence-activating cell sorting (FACs) utilizing a FACS Calibur (Becton & Dickinson).

Heparin Binding Assay. Microspin columns (BioRad) were washed with 200 µl TNTM buffer followed by 1 ml 1×TD buffer (1×PBS with 1 mM $MgCl_2$, 2.5 mM KCl). Then 50 µl of heparin-conjugated agarose type I resin (Sigma) was loaded into the columns. The affinity columns were equilibrated with 1 ml of 1×TD buffer and charged by washing with 500 µl of 1×TD/1 M NaCl buffer followed by three sequential washes with 1 ml of 1×TD buffer. Subsequently, 10 µg of HBoV1, HBoV2, AAV5, and AAV2 VLPs were diluted in 60 µl 1×TD buffer respectively and 30 µl of the samples loaded onto the columns followed by sequential collection of flow through, five column washes with 1×TD buffer (30 µl each), and five elution fractions with 1×TD/1 M NaCl buffer (30 µl each). All fractions were analyzed by SDS-PAGE.

Differential Scanning Fluorescent (DSF) Stability Assay. Purified HBoV and AAV5 VLPs (72) were diluted to ~0.2 mg/ml in 1× PBS buffer and then dialyzed in universal buffer to pHs of 4.0, 5.5, 6.0, or 7.4 or glycine-HCl at pH 2.6, respectively. For the DSF assay 2.5 µl of 1% SYPRO-Orange dye (Molecular Probes, Invitrogen) was added to 22.5 µl of dialyzed sample. The assay was conducted in a thermocycler (BioRad CFX Connect) with the temperature ramped from 30 to 99 °C, increasing by 0.1 degrees every 6 s. The melting temperature (T_M) for each sample was defined as the vertex of the first derivative (dF/dT) of relative fluorescence unit (RFU) values.

Structure accession numbers

The HBoV1 and HBoV2 cryo-EM reconstructed density maps and models built for their capsids were deposited in the Electron Microscopy Data Bank (EMDB) with accession numbers EMD-XXXXX/PDB ID XXXX (HBoV1 pH5.5), EMD-XXXXX/PDB ID XXXX (HBoV1 pH2.6), EMD-XXXXX/PDB ID XXXX (HBoV2 pH7.4), EMD-XXXXX/PDB ID XXXX (HBoV2 pH5.5), and EMD-XXXXX/PDB ID XXXX (HBoV2 pH2.6), respectively.

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Author contributions

M.L. was responsible for VLP production, purification, and dialysis to the desired pH condition, fluorescent labeling of VLPs, the execution of the cell and heparin binding assays, DSF assay, initial structure determinations using reconstruction by cryo-EM, and wrote a first draft of the manuscript. M.M. was responsible for cell binding assay analysis, cryo-reconstruction, structure refinement and analysis, model building and refinement, and manuscript preparation. P.C. vitrified sample, frozen, and screened cryo-EM grids. C.X., J.S. and D.S. collected cryo-EM data. R.M. and M.S-V. contributed to interpretation of the results and manuscript preparation. M.A-M. conceived and designed the project, analyzed all results, and contributed to manuscript preparation. All authors have read and agreed to the published version of the manuscript.

Competing interests

560 The authors declare no conflict of interest.

561

562 **Figure Legends:**

563 **Fig. 1:** The HBoV2 capsid structure. **A)** The reconstructed capsid surface map colored
564 according to radial distance from the capsid center (blue) to outermost regions (red) as
565 indicated by the scale bar. **B)** Cross-sectional view of the capsid. Locations of the 5-fold channel
566 (5f), 3-fold (3f), 2-fold (2-f) axes, and 2/5-fold wall are indicated by arrowheads. Density coloring
567 is as in (A). **C)** Example of fitted amino acid residues, W94 - N98¸ in βC strand. The density map
568 depicted as a black mesh. The VP model is shown in stick representation and the atoms are
569 colored: C: yellow; O: red, N: blue. **D)** Closeup of a cross-sectional view of "the basket" under
570 the 5-fold channel (refer to panel B). The ribbon diagram of VP3 is shown within the
571 semitransparent density. The first ordered residue G33 is labeled. This figure was generated
572 using UCSF-Chimera (63).

573 **Fig. 2:** HBoV2 VP structure. **A)** Ribbon diagram of HBoV2 VP structure. The conserved β-barrel
574 core motif (βB-I), βA strand, αA and αB helices are indicated. The N- and C-terminal are
575 labeled. The loops inserted between these secondary structure elements also contain β-strand
576 regions, as indicated. **B)** Structural superposition of HBoV1 (blue), HBoV2 (orange), HBoV3
577 (green), and HBoV4 (red), with the location of variable loops VR-I to IX labeled. The
578 approximate icosahedral 2-, 3-, and 5-fold axes are indicated as a filled oval, triangle, and
579 pentagon, respectively. The images were generated with PyMOL (73).

580 **Fig. 3:** Glycan binding analysis of HBoV1 and HBoV2. **A)** SDS-PAGE of heparin binding assay
581 fractions for AAV2, AAV5, HBoV1, and HBoV2 VLPs. FT: flow-through, W1-5: wash fraction;
582 E1-5: elution fractions. **B)** SDS-PAGE of labeled HBoV1, HBoV2, AAV2, and AAV5 VLPs. Gels
583 (left) stained for proteins shows the presence of VP3 of HBoV1 and HBoV2 (~60kDa) as well as

584 VP1 (~87 kDa), VP2 (~72 kDa) and VP3 (~62 kDa) of AAV2 and AAV5 (AAV5 VP1 is low
585 concentration). Gels (right) imaged under UV light to confirm successful fluorescent capsid
586 labeling. **C)** Cell binding assay analysis of fluorescently-labeled VLPs incubated with the
587 indicated cell lines. Results show the percentage of cells bound by the fluorescently-labeled
588 capsids and displayed as mean ± standard deviation (n=3).

589 **Fig. 4:** HBoV capsids under different pH conditions. Negative-stain EM analysis of HBoV1 **(A)**
590 HBoV2 **(B)** capsids dialyzed into indicated pH. Sample labeled 2.6>7.4 was dialyzed to pH 2.6
591 then subsequently re-dialyzed to pH 7.4. **C)** Capsid stability analysis using differential scanning
592 fluorimetry. The melting temperature (T_M) of HBoV1, HBoV2, and AAV5 capsids were
593 determined at pH 2.6, 4.0, 5.5, 6.0, and 7.4. Results are displayed as mean ± standard
594 deviation (n=3).

595 **Fig. 5:** Capsid density maps of HBoV1 and HBoV2. HBoV1 structures at pH 7.4, 5.5, and 2.6
596 are shown on the left-hand side. Similar pH structures for HBoV2 are shown on the right-hand
597 side. The reconstructed maps are colored according to radial distance (blue to red), as indicated
598 by the scale bar below. Shown are surface and cross-sectional views of HBoV1 and 2 at the
599 different pH conditions indicated. The images were generated with UCSF-Chimera (63).

600 **Fig. 6:** VP structure of HBoV1 and HBoV2 at varying pHs. **A)** Conformation of VR-I in HBoV1 at
601 pH 7.4 (blue), 5.5 (yellow-orange) and 2.6. (red) shown as coil diagrams. Amino acid side
602 chains are shown within the corresponding density map for each pH condition. **B)** Conformation
603 of VR-I in HBoV2 shown as in (A). **C)** Conformation of VR-V in HBoV1 and **D)** HBoV2 presented
604 as in (A). **E)** Superposition of the HBoV1 and **F)** HBoV2 VP structures at the different pH
605 conditions. The positions of VR-I to VR-IX, the N-, and C-terminus are labeled. The images
606 were generated with UCSF-Chimera (63).

607 **Fig. 7:** The structures of the N-termini of HBoV1 and HBoV2 at varying pHs. **A)** Cross-sectional
608 views of the HBoV1 density maps and fitted models shown as ribbon diagrams for each pH
609 condition (pH 7.4 – blue; pH 5.5 – yellow-orange; and pH 2.6 – red) studied. The 5-fold
610 symmetry axis, glycine 33, and β-strand G are indicated. **B)** The amino acids of the HBoV1 N-
611 terminus and β-strand G are shown in stick representation inside the pH 2.6 mesh density map.
612 **C)** Cross-sectional views of HBoV2 as in (A) and **D)** the amino acids of the HBoV2 N-terminus
613 and β-strand G inside the pH 2.6 mesh density map as in (B). **E)** Representation of the interior
614 surface of the HBoV1 or **F)** HBoV2 capsid from a 9-mer at a pH of 2.6. At this condition, the N-
615 terminus is extending away from the 5-fold channel. The N-terminal residue, G33, are colored
616 red and the last N-terminal residue ordered, 24, are colored blue. The asymmetric unit, the 2-,
617 and 3-fold symmetry axes is indicated. Panel A-D were generated with UCSF-Chimera (63) and
618 E-F with PyMOL (73).

619 **Fig. 8:** Modification of cysteines at low pH conditions. **A)** HBoV1 and HBoV2 model and density
620 map (black mesh) are shown at pH7.4, 5.5 and 2.6. Additional density relative to pH 7.4 is
621 indicated as a red mesh. Density meshes are contoured at a σ-level threshold of 2.0. The
622 additional cysteine density for Cys159 causes F243/239 to flip at pH 5.5 and 2.6, indicated by a
623 red arrow. **B)** Similar depiction (as in A) for cysteines with additional densities exclusively at pH
624 2.6. In HBoV2 C393 causes extra density at the center of the 3-fold symmetry axis. The view is
625 rotated by 90^{o} without density mesh for the VP3 amino acids. The images were generated with
626 UCSF-Chimera (63). **(C and D)** Position of cysteines on the exterior and interior surface of
627 HBoV1 and HBoV2 capsids. Cysteines labeled red indicate that these possess additional
628 densities whereas cysteines in blue display no additional densities. The images were generated
629 with PyMOL (73).

630 **Fig. 9:** Modification of histidines at low pH conditions. **A)** HBoV1 and HBoV2 paired-histidines
631 and density (black mesh) are shown at pH7.4, 5.5 and 2.6. Additional density relative to pH 7.4

632 is indicated as a red density mesh. Density meshes are shown at a σ-level threshold of 2.0. The
633 movement of H230 indicated by a red arrow. **B)** Similar depiction (as in A) for “single” histidines.
634 The conformation change to Y462 at pH2.6 is indicated by a red arrow. The images were
635 generated with UCSF-Chimera (63). **(C and D)** Position of histidines on the exterior and interior
636 surface of HBoV1 or HBoV2 capsids. Histidines labeled red indicate that these possess extra
637 densities, in orange the paired H163 with H443/439 and in blue histidines that displayed no
638 additional densities. The images were generated with PyMOL (73).

Table 1: Summary of data collection, image-processing parameters, and refinement statistics

	HBoV1		HBoV2		
	pH 2.6	**pH 5.5**	**pH 2.6**	**pH 5.5**	**pH 7.4**
Total number of micrographs	785	711	691	927	2,921
Defocus range (μm)	0.84 - 4.03	0.82 - 3.74	0.83 - 4.02	0.82 - 4.03	0.78-4.39
Electron dose (e^- / Å^2)	32	32	32	32	64
Frames / micrograph	50	50	50	50	36
Pixel size (Å / pixel)	0.90	1.04	0.90	0.91	0.96
Particles used for final map	50,342	66,471	19,648	30,308	188,857
Resolution of final map (Å)	2.54	2.74	2.51	2.74	2.71
PHENIX model refinement statistics					
Map CC	0.81	0.80	0.84	0.87	0.83
RMSD [bonds] (Å)	0.01	0.01	0.01	0.01	0.01
RMSD [angles] (Å)	0.85	0.84	0.88	0.84	0.81
All-atom clash score	11.88	10.55	9.66	8.17	10.17
Ramachandran plot					
Favored (%)	96.7	95.9	96.9	97.2	96.8
Allowed (%)	3.3	4.1	3.1	2.6	3.2
Outliers (%)	0	0	0	0.2	0
Rotamer outliers (%)	0.5	0.2	0.2	0.2	0.2
C-β deviations	0	0	0	0	0

Table 2: Summary of observed modification of side chain densities

HBoV1	extra density			comment	HBoV2	extra density			comment
	pH 7.4	pH 5.5	pH 2.6			pH 7.4	pH 5.5	pH 2.6	
C89	no	no	yes		C89	no	yes	yes	causes flip of K262
C104	no	no	yes	density to C477, M177	S104	N/A	N/A	N/A	
C159	no	yes	yes	causes flip of F243	C159	no	yes	yes	causes flip of F239
C247	no	yes	yes		C243	no	yes	yes	
C322	no	no	yes		V318	N/A	N/A	N/A	
C347	no	no	yes		S343	N/A	N/A	N/A	
V397	N/A	N/A	N/A		C393	no	no	yes	density at 3-fold
C421	no	no	yes	density towards H170	C417	no	weak	yes	density towards H170
C471	no	no	no		C467	no	no	no	
C477	no	no	yes	density to C104, M177	C473	no	no	no	
C531	no	no	no		C527	no	no	no	
H48	no	no	no		Y48	N/A	N/A	N/A	
H70	no	no	yes		H70	no	no	yes	flip of K450 / Y462 nearby
H105	yes	yes	yes	coordinated ion?	H105	yes	yes	yes	coordinated ion?
Q127	N/A	N/A	N/A		H127	no	no	yes	shift of K129 nearby
H156	no	no	yes	paired with H230	H156	no	yes	yes	paired with H226
H163	no	no	yes	paired with H443	H163	yes	yes	yes	paired with H439
H170	no	weak	yes	density towards C421	H170	no	weak	yes	density towards C417
N197	N/A	N/A	N/A		H197	no	no	no	
H230	no	no	yes	paired with H156	H226	yes	yes	yes	paired with H156
N278	N/A	N/A	N/A		H274	no	no	†	
H329	†	no	†		A325	N/A	N/A	N/A	
H429	no	no	yes		N425	N/A	N/A	N/A	
H443	no	no	yes	paired with H163	H439	yes	yes	yes	paired with H163
H497	no	no	yes		H493	no	no	yes	
H515	no	no	no		H511	no	no	no	

† local resolution not sufficient to determine whether extra density is present

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