Capsid structure and dynamics of a human rhinovirus probed by hydrogen exchange mass spectrometry

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Abstract

Viral capsids are dynamic protein assemblies surrounding viral genomes. Despite the high-resolution structures determined by X-ray crystallography and cryo-electron microscopy, their in-solution structure and dynamics can be probed by hydrogen exchange. We report here using hydrogen exchange combined with protein enzymatic fragmentation and mass spectrometry to determine the capsid structure and dynamics of a human rhinovirus, HRV14. Capsid proteins (VP1–4) were labeled with deuterium by incubating intact virus in D₂O buffer at neutral pH. The labeled proteins were digested by immobilized pepsin to give peptides analyzed by capillary reverse-phase HPLC coupled with nano-electrospray mass spectrometry. Deuterium levels incorporated at amide linkages in peptic fragments were measured for different exchange times from 12 sec to 30 h to assess the amide hydrogen exchange rates along each of the four protein backbones. Exchange results generally agree with the crystal structure of VP1–4, with extended, flexible terminal and surface-loop regions in fast exchange and folded helical and sheet structures in slow exchange. In addition, three α -helices, one from each of VP1–3, exhibited very slow exchange, indicating high stability of the protomeric interface. The β -strands at VP3 N terminus also had very slow exchange, suggesting stable pentamer contacts. It was noted, however, that the interface around the fivefold axis had fast and intermediate exchange, indicating relatively more flexibility. Even faster exchange rates were found in the N terminus of VP1 and most segments of VP4, suggesting high flexibilities, which may correspond to their potential roles in virus uncoating.

Keywords: human rhinovirus; viral capsid; protein structure and dynamics; hydrogen exchange; mass spectrometry

Viral capsids are highly organized, noncovalent assemblies of hundreds of protein subunits, which play a substantial role in many stages of the virus life cycle, including cell attachment, cell entry, and release of the

viral genome (Rossmann 1994). The functions of viral capsid require dynamic structure. On the one hand, the capsid needs to be stable enough to protect the interior genome when virions move from cell to cell. On the other hand, it must have the ability to change its conformation as it disassembles to release the genome. Characterizing the structure and dynamics of viral capsids is essential to understanding and controlling the virus life cycle.

A member of the family of picornavirus, human rhinovirus (HRV) has > 100 serotypes and causes common cold infections in humans (Rueckert 1996). These non-enveloped viruses consist of one single-stranded, positive-sense RNA (~7200 bases) and an icosahedral capsid. As illustrated in Figure 1A, the HRV capsid

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Abbreviations: CID, collision induced dissociation; ESIMS, electrospray ionization mass spectrometry; GuHCl, guanidine hydrogen chloride; HRV, human rhinovirus; HXMS, hydrogen exchange mass spectrometry; NI_m, neutralizing immunogenic; TFA, trifluoroacetic acid.

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Figure 1. A schematic view of HRV14 icosahedral capsid (A) and partial capsid surface bound with an antiviral drug (B). The thick lines in A outline five copies of protomers forming a capsid pentamer. The thick curved lines in B indicate the canyon surface, and the dotted lines represent the drug binding pocket. The icosahedral two-, three-, and fivefold axes are shown as ovals, triangles, and pentagons, respectively. This figure is adapted from literature (Arnold and Rossmann 1990; Hadfield et al. 1995).

contains 60 protomers, each composed of four proteins. Three larger proteins (VP1–3, ~30 kDa each) are located on the external surface of the virus and one smaller protein (VP4, ~7 kDa) lines the inner surface, interfacing with VP1–3 and RNA. Crystallographic studies of HRV14 showed that VP1–3 adopts eight-stranded, antiparallel β -barrel folds and that VP4 is an extended polypeptide (Rossmann et al. 1985). Loops from proteins VP1–3 make up capsid surface protrusions that host neutralizing immunogenic (NIm) sites. The surface canyons illustrated by the shaded circles in Figure 1B serve as the cellular receptor binding sites (Rossmann et al. 1994).

A number of studies of interaction of HRV with antiviral agents (Smith et al. 1986; Badger et al. 1988, 1989; Chapman et al. 1991; Kim et al. 1993; Oren et al. 1996), neutralizing antibody fragments (Smith et al. 1996) and cellular receptor molecules (Kolatkhar et al. 1999; Xing et al. 2003; Hewat and Blaas 2004) suggest that capsid protein dynamics may play a role in viral functions. For example, a series of antiviral agents were found to bind with HRV14 capsid and to inhibit viral replication. Crystallographic data showed such binding occurred mostly in the hydrophobic pockets of the β -barrel fold of VP1 located right below the canyon floor where cellular receptors bind (Fig. 1B). It was

suggested that binding induced conformational changes that might interfere with binding of cellular receptors (Pevear et al. 1989). In other HRVs of minor receptor group such as HRV1A, however, the binding of antiviral agents does not seem to cause major conformational changes and there is no blocking of receptor attachment (Kim et al. 1989, 1993). In addition, the overall stability of HRV14 capsid increases upon binding with antiviral agents, which may block the virus from uncoating and thereby inhibit the virus from replication (Vaidehi and Goddard 1997; Lewis et al. 1998; Speelman et al. 2001; Reisdorph et al. 2003).

Although capsid structure and dynamics in solution can be inferred from the high-resolution structure of HRV viral capsids determined by X-ray crystallography or cryo-electron microscopy, experimental approaches that are capable of probing directly the in-solution structure of viral capsids may provide complementary information. For instance, limited proteolysis combined with peptide mass mapping has suggested remarkable plasticity of the HRV14 capsid that was not evident in the crystal structure (Lewis et al. 1998; Reisdorph et al. 2003). Amide hydrogen exchange (HX) has been another useful approach to elucidate protein structure and dynamics in solution for decades (Hvidt and Nielsen 1966; Woodward et al. 1982; Englander and Kallenbach 1984). Based on the fact that the HX rates at protein amide linkages are highly dependent on the solvent accessibility of amide hydrogens and their participation in hydrogen-bonding networks, this methodology has the advantage of following the structure and dynamics of the whole protein backbone. Recent methods using a combination of hydrogen/deuterium exchange and mass spectrometry (HXMS) are well suited for studies of large proteins and protein assemblies (Robinson et al. 1996; Zhang et al. 1996; Chen and Smith 2000; Baerga-Ortiz et al. 2002; Yamada et al. 2002). These methods have been recently applied to detection of structural changes in viral capsids (Tuma et al. 2001; Wang et al. 2001; Lanman et al. 2004). Although HXMS can be used to measure deuterium levels in intact proteins to reveal information on the cooperativity of structural changes, it is most useful when the spatial resolution has been improved by fragmenting the labeled protein with an acid protease (Englander et al. 1985; Zhang and Smith 1993; Kim et al. 2001).

In this study, amide HX combined with enzymatic fragmentation of labeled proteins was used to study the structure and dynamics of the HRV14 capsid in solution under physiological conditions. A high-sensitivity HXMS approach using capillary HPLC coupled with nano-electrospray mass spectrometry (Wang and Smith 2003) was adopted to greatly reduce sample consumption. Relatively comprehensive local exchange profiles of HRV14 capsid were determined, thereby facilitating

characterization of the capsid structure and protein dynamics at capsid protein–protein interfaces.

Results

HX and protein dynamics

The relationship of amide HX and protein structure is indicated by the simple model illustrated by Equation 1 (Engen and Smith 2001):

$$k_{\text{ex}} = (K_{\text{u}} + \beta) k_{\text{int}} \quad (1)$$

where k_{ex} and k_{int} are the phenomenological and intrinsic exchange rate constants for individual amide linkages, respectively; K_{u} is the equilibrium constant for partial or total unfolding of the protein; and β is the probability for exchange from the folded protein. The sum of $(K_{\text{u}} + \beta)$ directly reflects protein conformation. In a totally unfolded polypeptide, k_{ex} equals to k_{int} . In a folded protein, however, exchange rates for amide hydrogens participating in hydrogen-bond networks or trapped in hydrophobic cores may be dramatically reduced. This reduction in exchange rates is attributed to a reduction in $(K_{\text{u}} + \beta)$. In general, the exchange half-lives at amide linkages in folded proteins can be from <1 sec to days or months, depending on the local structural environment of those amide hydrogens. For example, the exchange rates in folded aldolase spanned a range greater than four orders of magnitude (Zhang et al. 1996). Although the intrinsic rates vary at different amide linkages due to side-chain effects, such differences generally span a range of only 10-fold.

Identification of different structures by their HX properties requires detection of a wide range of exchange rates in the HRV14 capsid. Experiments were designed to label native HRV14 in D₂O at physiological conditions (pH ~7, room temperature) using a large range of exposure times from 12 sec to 30 h. Following pepsin digestion, deuterium levels in peptic fragments were measured for each time point to obtain time-course exchange profiles for various segments of HRV14 coat proteins. These profiles were further grouped by their exchange kinetics to facilitate detection of different structural and dynamic features in the coat proteins and protein–protein interfaces.

Deuterium levels in the peptic fragments of HRV coat proteins

To measure HX rates in HRV capsid, intact HRV14 was first labeled in D₂O and then digested with an immobilized pepsin column under minimum exchange conditions (pH 2.5, 0°C). To increase the pepsin digestion efficiency, guanidine hydrochloride was mixed with

the labeled HRV sample before passing through the pepsin column. Digestion was found complete in 40 sec, the residence time of the sample inside the column. Peptides were trapped and desalted online before analysis. The use of a pepsin column along with a peptide trap in the experimental system facilitated rapid loading and digestion of labeled HRV, therefore reducing the time for back exchange (Wang and Smith 2003).

Digestion of HRV capsid proteins with pepsin generated a large number of peptides. While pepsin tends to cut before or after hydrophobic residues, the cleavage sites cannot be predicted accurately. Analyses of digested HRV14 containing no deuterium by LC/MS and LC/MS/MS were performed to identify peptic fragments of the four HRV14 coat proteins prior to HXMS experiments. A total of 190 peptides were identified covering ~95% of the four protein sequences. To minimize deuterium loss during HPLC, a steep acetonitrile gradient was used. The elution time of most peptides was <6 min. The large number of peptides in the digest and the short elution time led to coelution of many peptides. Although most of the unlabeled peptides had different molecular weights, only about half of the identified peptides could be used in HX experiments because partially deuterated peptides have broad envelopes of isotope peaks. As a result, a subset of ~90 peptides was used in the HXMS study, which covers an average of 80% of capsid protein sequence.

Typical mass spectra of partially deuterated peptides of HRV14 capsid are presented in Figure 2, which shows three VP1 peptides, including residues 1–6, 53–60, and 110–118 for labeling times of 12 sec, 20 min, and 30 h, respectively. Mass spectra for the 0% reference (no deuterium exchange) and 100% references (fully exchanged) are also included. The isotope pattern for 0% reference reflected the natural abundance of heavy isotopes, while the 100% reference was used to evaluate deuterium loss in the digestion and LCMS analysis (Smith et al. 1997). The nondeuterated form (0% reference) of VP1 peptide, including residues 1–6, had an average mass of 603.69. Exposure of HRV to D₂O for 12 sec led to an average mass of 606.80. After adjustment for artifactual deuterium loss, which was 31% for this peptide, there was an average of 4.5 deuteriums incorporated in this segment of VP1. Adjusted deuterium levels in segment 1–6 for exchange times of 12 sec, 20 min, and 30 h were 4.5, 4.7, and 4.7, respectively. The typical experimental error for these measurements was 0.2 deuterium, estimated by repeated analysis of replicate samples. The 4.5–4.7 deuterium in segment 1–6 with five amide linkages corresponded to a relative deuterium level of 90%–94%. In contrast, the deuterium level in segment 53–60 of VP1 was only 2.8 deuterium at 12 sec, corresponding to a relative deuterium level of 40 ± 3%. This deuterium level increased to 59 ± 3% in 20 min and 65 ± 3% in 30 h, showing that

Figure 2. Mass spectra of three VP1 peptic fragments containing residues 1–6, 53–60, and 110–118 after labeling intact HRV14 in D₂O for 12 sec, 20 min, and 30 h. Spectra of the three peptides derived from the 0% and 100% reference samples are also included. Deuterium levels given for each peptide at various exchange times were corrected for artifactual deuterium losses.

the average exchange rate in this segment is slower than in segment 1–6. The average exchange rate was extremely slow in segment 110–118, with only $10 \pm 3\%$ deuterium found in the longest exposure time of 30 h.

From the plots of deuterium levels in three VP1 segments (Fig. 3), it is evident that different regions of VP1 exhibit different exchange kinetics. Segment 1–6 had fast exchange rates at its amide linkages, which suggested that this segment had little structural protection. In contrast, finding $<10\%$ deuterium in segment 110–118 of VP1 at all exchange times showed that most amide linkages were well protected from exchange, suggesting that this segment is located in a stable structural unit where the amide hydrogens may not be highly accessible to the bulk solvent. The gradual increase in deuterium level in segment 53–60 indicated an intermediate average exchange rate for the amide linkages in this region,

Figure 3. Plots of deuterium levels vs. exchange time for three VP1 peptic fragments containing residues 1–6, 53–60, and 110–118.

consistent with its location in a VP1 region where the structure is not as rigid as in segment 110–118.

The deuterium levels found in 90 peptic fragments of VP1–4 at five exchange times (12 sec, 180 sec, 20 min, 2.5 h, and 30 h) are presented in Figure 4. In each fragment, the deuterium level has been normalized to the total number of amide hydrogens and adjusted for the deuterium loss from each fragment. Deuterium losses for these peptides spanned a range of 10%–60%. The internal consistency of these exchange data was indicated by the similar exchange patterns of a number of peptides with partially overlapping sequences. The deuterium level differences of these overlapping peptides could be used to improve the spatial resolution of HXMS (Zhang and Smith 1993). These results clearly show that very different exchange kinetics existed in different regions of the HRV14 capsid proteins. For instance, fast exchange was observed in both the N- and C-terminal peptides of VP1 and VP2. Meanwhile, some segments of VP1–3 had $<10\%$ – 20% , even after 30 h of exchange, indicating very slow exchange. In contrast to the wide range of exchange rates found in VP1–3, all peptides in VP4 exhibited relatively fast exchange, as indicated by deuterium levels of $>90\%$ in most peptides after 30-h exposure time. The results presented in Figure 4 provide a detailed fingerprint of the HX for all four capsid proteins of HRV14, in which significant differences in exchange kinetics were observed at various different regions of the proteins. As will be discussed later, structural and dynamic features in each capsid protein and in the protein–protein interfaces of the whole capsid can be investigated by looking at these differences in exchange rates.

Figure 4. Deuterium levels found in 90 peptic fragments of HRV14 capsid proteins following incubation of intact HRV14 in D₂O at various exchange times from 12 sec to 30 h: VP1 (A), VP2 (B), VP3 (C), and VP4 (D). The bar heights show deuterium levels expressed as the percentage of the total number of amide hydrogens in each fragment. The shading of the bars illustrates exchange times. Experimental errors of deuterium levels are indicated as the error bar for each fragment.

Discussion

Correlation of HX with crystal structures of HRV14 capsid proteins

Correlation of HX with capsid protein structure may be illustrated by Figure 5, where the amino acid sequences

of VP1–4 are presented along with their secondary structures. The rectangles represent identified peptic fragments, with the color codes indicating their HX rates. Fragments with deuterium levels > 80% in 12 sec are colored red, indicating very fast exchange; those in the remaining fragments with > 70% deuterium

Figure 5. Hydrogen exchange kinetics along the backbones of HRV14 capsid protein VP1 (A), VP2 (B), VP3 (C), and VP4 (D). The rectangles under the protein sequences represent peptic fragments with length corresponding to the peptide size, and color indicating exchange kinetics (qualitative exchange rates): red for very fast, pink for fast, yellow for intermediate, green for slow, and blue for very slow. Secondary structural elements of each protein are aligned on the top of their sequences. NIm sites are labeled with *.

in 180 sec are colored pink, indicating fast exchange. Slow exchange regions, colored green, are defined as regions with <30% deuterium in 2.5 h. Very slow exchange regions, colored blue, are those with <20% deuterium in 30 h. Peptides that did not fit into any of the above categories were colored yellow, indicating exchange with intermediate rates.

HX results for VP1 (Fig. 5A) show clearly that highly dynamic regions undergo rapid exchange. For instance, exchange in peptide segments 1–6, 9–12, 82–98, 142–146, and 277–289 was rapid, indicating a relatively large degree of structural flexibility, consistent with the crystal structure of HRV14 capsid showing that these peptides are mostly located in the extended loops. In particular, one fast exchanging peptide segment including residues 82–98 is composed of the BC loop located in the protrusion areas of the exterior surface of the capsid and contains immunogenic sites, suggesting

structural flexibility of antibody binding sites. In addition, the N-terminal residues 1–6 that were not observed in the crystal structure were found exchanging with fast rates, consistent with high disorder. Limited proteolysis of HRV14 with trypsin showed fast cleavage of VP1 peptides 1–8 and 1–13, suggesting transient exposure of the N terminus to the exterior surface of virus (Lewis et al. 1998). This highly dynamic nature of the VP1 N terminus was clearly consistent with our HX results. The C terminus of VP1 (segment 277–289) was also found with fast exchange rates, suggesting structural freedom, which agreed with the crystal structure model, indicating that this segment was a flexible loop exposed to the exterior surface of the virus.

In contrast with the fast exchange regions, VP1 segments of 110–124 and 163–177 (Fig. 5A, blue) were found with <20% deuterium, even after 30 h, indicating very slow HX. Two α -helices, α A and α B, were located in

these regions, which essentially had little deuterium exchanged in. The slow exchange nature suggests that the two α -helices are stable, rigid structures in the capsid. Slow exchange was also found in the segments including residues 99–102, 178–200, and 219–225, most of which are β -strands (CFGH), consistent with general stability of the β -barrel fold in VP1. Among these strands, β D, β F, and β G were found having slower exchange rates than β C, β E, and β H, indicating some structural or dynamic difference of these β -strands. The former strands (DFG) make up the bottom of the β -barrel pocket, where the antiviral drugs bind, while the latter β -strands (CEH) are located close to the pocket entrance. The exchange results suggested relatively more flexibility for the β -strands at the entrance of the pocket than those at the bottom. The higher exchange rates may also result from more solvent exposure of the outside strands. This structural feature allows various antiviral agents to enter the same binding pocket with less structural hindrance. Intermediate exchange was mostly found in the regions that were composed primarily of the loops 13–60 and 71–81 and the loops between the β -strands that form the β -barrel fold. Our exchange results indicate partial flexibility in the VP1 β -barrel, which is important for HRV capsid to meet the need of structural rearrangement upon binding with antiviral agents or pocket factors (Smith et al. 1986; Arnold and Rossmann 1990).

The general features of the VP2 exchange kinetics (Fig. 5B) were similar with those of VP1. The disordered N terminus (residues 1–8, shown in pink) was found to exchange rapidly. Other fast-exchange segments included the C-terminal segment 252–262, which is on the exterior surface of the capsid. About 85% of amide linkages in the segment 156–168 were completely deuterated in the shortest time period (12 sec), indicating very fast exchange (red) and much structural flexibility. This region is located in a long exterior puff loop of VP2 containing immunogenic sites of NIm-II. The majority of the β -barrel fold in VP2 (residues 80–92, 98–112, 199–211, and 221–238) was found to have slow exchange (in green). Slow exchange regions also included residues 16–27 that form two β -strands (A1 and A2), consistent with their role in stabilizing the capsid. Strands β -A1 and -A2 run antiparallel to form the protein–protein interface of capsid pentamers. As was found for the two helices in VP1, the helix α B exhibited extremely slow exchange, indicating high stability for this region.

As shown in Figure 5C, VP3 was observed having fast exchange rates at exposed loop regions, including residues 53–66, 74–84, and 222–236, at the C terminus (in red and pink). Although some regions of the β -barrel were not detected (uncolored regions), it is largely a stable fold with slow exchange, particularly in the segment 117–129 and 211–221 (in green or blue), similar to VP1 and VP2.

Exchange in residues 97–103 in the helix α A was very slow, a common feature of all three VPs. However, compared with VP1 and VP2, the N terminus of VP3 (segment 1–7, in yellow) had much slower exchange, indicating more structural order. This result is consistent with the crystal structure of HRV14 capsid, which shows a β -strand at the N terminus of VP3 (residues 1–7), as well as five strands from five subunits of VP3 forming a unique β -cylinder structure to stabilize the capsid around the fivefold axis (Arnold and Rossmann 1990). This β -cylindrical structure is conserved in many rhinoviruses and plays a critical role in viral disassembly.

VP4 of HRV14 was shown by X-ray diffraction to be an extended polypeptide lining the interior surface of the capsid (Rossmann et al. 1985). With little secondary structure, VP4 is expected to have fast exchange rates at most of its solvent accessible amide linkages, assuming that there is no significant hindrance for D₂O to penetrate inside the virus. The present HXMS results for VP4 support this prediction. As shown clearly in Figures 4D and 5D, most fragments of VP4, including the N and C termini, had deuterium levels of >70%–90% in only 2.5 min, which is an indication of fast exchange. The overall fast exchange nature of internal protein VP4 indicates that the interior of the virus is solvent accessible, possibly due to the dynamic nature of the viral capsid. A recent finding that alkylating agents can permeate the protein capsid of a naked virus without affecting the particle integrity also supports this idea (Broo et al. 2001).

In contrast with fast exchange in most segments of VP4, deuterium exchange was found slower in two regions. One is segment 46–61, indicated by the gradually increased deuterium level from 27% in 12 sec to 88% in 30 h (Fig. 4D). This slower exchange may be explained by the presence of a short α -helix (residues 49–54) in this segment and hydrogen bonds formed between VP4 residues 53, 55, and 57, and residues from VP1 and VP2 (Arnold and Rossmann 1990). Slower exchange was also found in segment 27–31, which may indicate presence of unreported interchain hydrogen bonds or other unknown factors that decrease the exchange rates. The general consistency of our HX results with the fine structural features of HRV14 capsid proteins found in crystallographic studies demonstrates the sensitivity of HX rates as a probe for protein structure and dynamics.

Correlation of HX with B-factors and solvent accessibility

Thermal parameters, or B-factors, of individual atoms in protein determined by crystallography reflect atomic vibration or disorder and may be used to assess protein dynamics in solution. Since hydrogen atoms are usually invisible in protein crystal structures, deuterium levels in

Figure 6. Deuterium levels in the peptic fragments of VP1 at an exposure time of 12 sec (*A*) and plots of solvent accessible areas ($\text{\AA}^2$) and B-factors ($\text{\AA}^2$) of backbone amide nitrogens vs. residue numbers (*B,C*). Rectangles in *A* represent peptic fragments, with height indicating deuterium levels and length the peptide size. Secondary structures are aligned on the *top* with loops, helices, and strands illustrated as straight lines, ovals, and rectangles, respectively.

the HRV14 capsid are plotted with B-factors of amide nitrogens as well as with their solvent accessible areas to see whether there is general agreement between HX and crystallographic models of HRV14 capsid (Fig. 6). Results presented in Figure 6, A–C, show general correlation between deuterium levels in VP1 and the solvent accessibility of the amide nitrogens and their B-factors. It was found that higher deuterium levels generally corresponded with higher solvent accessible areas and B-factors. For instance, the amide nitrogens in VP1 segment 82–98 that contain the BC loop have an average B-factor of $28.9 \text{ \AA}^2$ and an average solvent accessible area of $3.8 \text{ \AA}^2$, which are higher than those in most other fragments. Accordingly, a high deuterium level ($\sim 72\%$ in 12 sec) was found in this segment, consistent with its higher structural flexibility and solvent accessibility. Meanwhile, the fragment 110–118 containing the αA helix has much lower average B-factors ($11.7 \text{ \AA}^2$) and smaller average solvent accessible areas ($0.6 \text{ \AA}^2$) for amide nitrogens, consistent with very slow exchange ($\sim 6\%$ in 12 sec). Note that a few regions seem not to have good correlation, such as N-terminal fragments including residues 35–43, 44–50, 53–60, and 71–81. These segments have relatively low B-factors ($10\text{--}15 \text{ \AA}^2$) of amide nitrogens (Fig. 6). However, $\sim 40\%$ of their amide linkages were found deuterated in 12 sec, indicating intermediate exchange rates. This rather high level of exchange may be due to the high solvent accessibility of some of these amide linkages.

Protein dynamics at HRV14 capsid protein–protein interfaces

The HRV14 capsid is composed of 240 protein subunits associated together by extensive protein–protein

interactions. Examining HX rates at protein–protein interfaces in the viral capsid led to information on the structure and dynamics in these regions, which are of critical importance in understanding capsid assembly and disassembly.

A cartoon diagram of the crystal structure of a protomer of HRV14 composed of VP1–4 is presented in Figure 7, A and B. Note that different color schemes are used in these figures. In Figure 7A, VP1–4 are colored blue, green, yellow, and red, respectively, illustrating the different locations of the four capsid proteins in the protomer. The color coding in Figure 7B indicates different exchange rates in various regions of the capsid protomer. Three α -helices, αA from VP1, αB from VP2, and αA from VP3, were found clustering in the protomer interface to form a triangle, with each helix at an apex of the triangle. As shown in Figure 7B, the three helices, colored blue, had very slow exchange rates (with $< 15\%$ deuterium level in 30 h, as discussed previously), indicating high stability of the protomeric protein–protein interface. It is proposed that these stable helical structures may play an important role in the formation of the 6S protomer, an early product of HRV assembly.

A view of the interior surface of the viral capsid pentamer is presented in Figure 7, C and D. Similarly, different color codes are used indicating different locations of VP1–4 (Fig. 7C) and exchange kinetics in different regions of viral capsid (Fig. 7D). As shown in Figure 7C, five copies of VP1 are located in the center of pentamer circling around the fivefold axis. The N-terminal arm of VP1 extends out under VP3, with the N terminus winding back toward the center of the pentamer. The N-terminal residues 1–16 are disordered and not shown. Next to VP1 are VP2 and VP3, which form the five edges of the pentamer. Note that the N-terminal

Figure 7. Ribbon diagrams of HRV14 capsid protomer (*A,B*) and pentamer (*C,D*) and an enlarged view of pentamer interface around the fivefold axis (*E*). In *A* and *C*, VP1–4 are colored blue, green, yellow, and red, respectively. In *B*, *D*, and *E*, the color codes represent qualitative hydrogen exchange rates in various segments of capsid proteins (same coloring scheme as in Fig. 5). Gray-colored regions indicate segments where hydrogen exchange rates were not available. The three α -helices from VP1–3 and some β -sheets in VP1 were labeled as letters (*A,B*). The diagrams are arranged as viewed from the interior of the virus. The protein models were created using Molscript 2.1 (*A,B*) and Rasmol 2.6 (*C–E*).

arms of VP3 curve through VP1 and cluster around the fivefold axis forming a β -tube structure. As the least folded polypeptide among the four proteins, VP4 extends under VP1–3 with its C terminus heading away from the fivefold axis. The N terminus of VP4, including residues 1–28, is disordered in the crystal structure of HRV14 and not shown.

It is clearly shown in Figure 7D that the capsid pentamer has quite different HX rates in different regions, suggesting unique capsid dynamics in these regions. Slow exchange regions (deuterium level < 30% in 2.5 h, colored green) included the fringes of the pentamer, where VP2 subunits are located, indicating that these regions have generally stable conformations. Very slow exchange

regions were identified mostly in the protomer interface (in blue), as observed in Figure 7B, where residues form stable and rigid structures (the three α -helices) to stabilize the whole capsid. Very slow exchange was also observed in a few β -strands in VP2 at the edges of the pentamer where the pentamer contacts another pentamer, indicating the highly stable nature of such contacts.

The pentamer interface around the fivefold axis (Fig. 7E) is composed of five copies of the N terminus of VP3 (in yellow), VP1 loops (flexible, in yellow and pink), and the VP4 N terminus (flexible or disordered, in pink; residues 1–28 missing from the crystal structure). These regions exhibited intermediate and fast exchange, indicated by their color codes. Although the pentamer interface is stabilized by the β -cylinder structure, finding intermediate exchange rates indicates that this region is partially flexible. In addition, the N terminus and surface loops of VP1, and the N-terminal arms of VP4 exhibited much faster exchange (in pink and red), suggesting more flexibility than the β -cylinder structure. These results are consistent with the proposed uncoating events occurring at this interface (Kolatkhar et al. 1999), in which cellular receptor binding with VP1 triggers a hinge-type movement of VP1 away from the fivefold axis, thus opening a channel for the release of VP4 and RNA release. The partial flexibility at the protein–protein interface around the fivefold axis allows such channel opening. More recent studies of HRV uncoating by cryoelectron microscopy suggested different dynamics for the N terminus of VP3 (Hewat et al. 2002; Xing et al. 2003; Hewat and Blaas 2004). An unconcerted movement of the VP3 N terminus was proposed for HRV14 to allow at least one β -cylinder channel to open at a time for RNA release (Hewat and Blaas 2004). Comparing exchange rates in the N-terminal region of VP3 in different HRVs and monitoring the changes of exchange profiles during uncoating may provide additional information to understand the role of capsid dynamics in HRV uncoating.

In conclusion, HXMS was shown as a powerful technique in characterizing the structure and dynamics of HRV14 capsid. The viral capsid was labeled intact with deuterium at neutral pH for a large range of exposure times, which allows for detection of a wide range of amide HX rates in the capsid. Local exchange profiles for each of the four capsid proteins were established through protease fragmentation of HRV14 capsid at minimal exchange conditions. The exchange results obtained from the in-solution experiments were generally consistent with the crystal structure of HRV14 capsid and could be correlated with structural parameters such as B-factors and solvent accessibility. In addition, dynamics in the protein–protein interfaces of the capsid protomer and pentamer were probed based on exchange

results. The three α -helices of VP1–3 clustering at the protomeric interface were found in extremely slow exchange, suggesting high stability of this interface. Strong pentamer–pentamer interactions were indicated by slow exchange in the regions of pentamer edges. In addition, it was noted that the interface around the fivefold axis exhibited partial flexibility, consistent with its role in viral disassembly. As the first HX study of HRV, these results give additional information regarding viral capsid dynamics and may provide further insights for the relationship between the structure and function of the HRV14 capsid. Our results also suggest the potential applications of HXMS in studying structural changes in the HRV capsid induced by a wide range of events, such as binding with cellular receptors or antiviral agents. In such cases, HX rates in the native capsid would be compared with exchange rates in the capsid bound with receptors or drug molecules.

Materials and methods

HRV14 preparation

The initial stock of HRV14 (3 mg/mL in 20 mM Tris buffer [pH 7.2]) was prepared in the laboratory of Prof. M.G. Rossmann at Purdue University, following procedures described previously (Rossmann et al. 1985).

Identification of peptic fragments of HRV14 capsid proteins

A column (2 mm ID $\times$ 50 mm/L) packed with immobilized pepsin prepared as described (Wang et al. 2002) was used for online digestion of HRV14 coat proteins. The pepsin column was incubated in ice and arranged in-line with a small peptide trap (C18, 1 $\times$ 8 mm, Michrom BioResources) to trap and desalt peptides. To increase the digestion rate, HRV14 was mixed with 1.5 mM GuHCl and 10 mM phosphate buffer (pH 2.4) before loading to the pepsin column. A solution containing 5 μ g of HRV14 was loaded on to the pepsin column by a carrier flow of 0.05% TFA at 200 μ L/min. The digestion time, or the flow-through time of the virus, was $\sim$ 50 sec. After desalting, the trapped peptides were separated in a capillary LC Packings HPLC peptide mapping column (0.3 $\times$ 150 mm, C18, flow rate 5 μ L/min) with a gradient of 2%–65% acetonitrile (0.05% TFA) in 65 min. A Finnigan LCQ ion trap mass spectrometer operated in the triple play mode (each triple play cycle contains a full mass scan, a zoom scan, and an MS/MS scan) was used to analyze the peptides. Peptides were identified by MS and CID MS/MS. Peptide identities were further confirmed by accurate mass measurement (accuracy $\sim$ 10 ppm) using a Waters QTOF mass spectrometer with a resolution of $\sim$ 7000.

Hydrogen deuterium exchange of HRV14

HRV14 was labeled as intact virus by diluting 14 μ L of stock solution (280 pmol of capsid proteins) 20-fold into D₂O buffer

(10 mM phosphate [pD 7.0]) followed by incubation for 12 sec, 3 min, 20 min, 2.5 h, and 30 h. At each time point, a solution of 20 μ L was taken out and 10 μ L DCl (30 mM, precooled to 0°C) was added to decrease pH to $\sim$ 2.4 followed by immediate freezing in dry ice and storage at -70°C . By doing this, the hydrogen deuterium exchange in the HRV sample was essentially quenched before digestion and analysis.

Pepsin digestion and LCMS analysis of labeled HRV14 capsid

An apparatus similar to the one previously described (Wang and Smith 2003) was used to digest HRV14 capsid proteins followed by LCMS analysis. Briefly, the apparatus consisted of a small pepsin column with 20 μ L bed volume in line with a capillary peptide trap (C18, LC Packings) for fast digestion and desalting. A solution of 7–8 μ L of labeled HRV14 sample containing $\sim$ 5 pmol of capsid proteins was mixed with 4 μ L of 3 M GuDHCl and injected into the pepsin column. Peptides were trapped and desalted by flowing 0.05% TFA through the pepsin column and peptide trap at 30 μ L/min for 2.5 min. A switching valve was used to switch the peptide trap in line with a capillary C18 column (0.1 $\times$ 50 mm, Michrom BioResources). Peptides were eluted from the trap and separated on the C18 column using a gradient of 10%–45% ACN (0.05% TFA) in 6 min at a flow rate of 0.9 μ L/min generated by a Shimadzu micro HPLC system and a precolumn flow splitter (LC Packings). The total time for digestion and analysis of each deuterated sample was $\sim$ 13–14 min including a 3- to 4-min delay caused by the dead volume of the system.

A Waters QTOF orthogonal electrospray time-of-flight mass spectrometer equipped with a nano-electrospray probe and coupled with capillary HPLC was used in the analysis of labeled peptides. The mass spectrometer was calibrated using CID-fragments of Glu¹-Fibrinopeptide B (Sigma). Deuterium levels were obtained by subtracting the average mass of nondeuterated peptides from the average mass of their deuterated counterparts and adjusting for artificial deuterium loss following procedures described (Wang et al. 2001). Deuterium loss in pepsin digestion and LC/MS analysis was evaluated by analyzing fully exchanged reference samples under the same conditions. This reference was prepared by labeling HRV14 in D₂O buffer (pD 2.4) at 37°C for 40 h. The labeling buffer contained 3 M GuDCl to denature capsid proteins for complete deuteration.

Calculation of solvent accessibility and construction of the atomic-resolution structure of a partial HRV14 capsid

HRV14 crystal structure deposited in the Protein Data Bank (PDB code 4RHV) was used to obtain the B-factors and solvent accessible areas of amide nitrogens in VP1–4. Solvent accessibility was calculated using the WHAT_IF software package (<http://www.cmbi.kun.nl/gv/servers/>) with the option of calculating accessible molecular surface and giving output per atom. To include effects of the bordering set of atoms to the solvent exposure of the reference protomer, a structure model of a partial HRV14 capsid was built, which contained one reference protomer surrounded with seven neighboring protomers. The PDB files for the above model and the capsid pentamer model in Figure 7 were constructed according to the icosahedral symmetry of the capsid using Swiss PDB Viewer (<http://www.expasy.ch/spdbv/>).

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References

- Arnold, E. and Rossmann, M.G. 1990. Analysis of the structure of a common cold virus, human rhinovirus 14, refined at a resolution of 3.0 Å. *J. Mol. Biol.* **211**: 763–801.
- Badger, J., Minor, I., Kremer, M.J., Oliveira, M.A., Smith, T.J., Griffith, J.P., Guerin, D.M., Krishnaswamy, S., Luo, M., and Rossmann, M.G. 1988. Structural analysis of a series of antiviral agents complexed with human rhinovirus 14. *Proc. Natl. Acad. Sci.* **85**: 3304–3308.
- Badger, J., Minor, I., Oliveira, M.A., Smith, T.J., and Rossmann, M.G. 1989. Structural analysis of antiviral agents that interact with the capsid of human rhinoviruses. *Proteins* **6**: 1–19.
- Baerga-Ortiz, A., Hughes, C.A., Mandell, J.G., and Komives, E.A. 2002. Epitope mapping of a monoclonal antibody against human thrombin by H/D-exchange mass spectrometry reveals selection of a diverse sequence in a highly conserved protein. *Protein Sci.* **11**: 1300–1308.
- Broo, K., Wei, J., Marshall, D., Brown, F., Smith, T.J., Johnson, J.E., Schneemann, A., and Siuzdak, G. 2001. Viral capsid mobility: A dynamic conduit for inactivation. *Proc. Natl. Acad. Sci.* **98**: 2274–2277.
- Chapman, M.S., Minor, I., Rossmann, M.G., Diana, G.D., and Andries, K. 1991. Human rhinovirus 14 complexed with antiviral compound R 61837. *J. Mol. Biol.* **217**: 455–463.
- Chen, J. and Smith, D.L. 2000. Unfolding and disassembly of the chaperonin GroEL occurs via a tetradecameric intermediate with a folded equatorial domain. *Biochemistry* **39**: 4250–4258.
- Engen, J.R. and Smith, D.L. 2001. Investigating protein structure and dynamics by hydrogen exchange MS. *Anal. Chem.* **73**: 256A–265A.
- Englander, S.W. and Kallenbach, N.R. 1984. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* **16**: 521–655.
- Englander, J.J., Rogero, J.R., and Englander, S.W. 1985. Protein hydrogen exchange studied by the fragment separation method. *Anal. Biochem.* **147**: 234–244.
- Hadfield, A.T., Oliveira, M.A., Kim, K.H., Minor, I., Kremer, M.J., Heinz, B.A., Shepard, D., Pevear, D.C., Rueckert, R.R., and Rossmann, M.G. 1995. Structural studies on human rhinovirus 14 drug-resistant compensation mutants. *J. Mol. Biol.* **253**: 61–73.
- Hewat, E.A. and Blaas, D. 2004. Cryoelectron microscopy analysis of the structural changes associated with human rhinovirus type 14 uncoating. *J. Virol.* **78**: 2935–2942.
- Hewat, E.A., Neumann, E., and Blaas, D. 2002. The concerted conformational changes during human rhinovirus 2 uncoating. *Mol. Cell* **10**: 317–326.
- Hvidt, A. and Nielsen, S.O. 1966. Hydrogen exchange in proteins. *Adv. Protein Chem.* **21**: 287–385.
- Kim, S.S., Smith, T.J., Chapman, M.S., Rossmann, M.C., Pevear, D.C., Dutko, F.J., Felock, P.J., Diana, G.D., and McKinlay, M.A. 1989. Crystal structure of human rhinovirus serotype 1A (HRV1A). *J. Mol. Biol.* **210**: 91–111.
- Kim, K.H., Willingmann, P., Gong, Z.X., Kremer, M.J., Chapman, M.S., Minor, I., Oliveira, M.A., Rossmann, M.G., Andries, K., Diana, G.D., et al. 1993. A comparison of the anti-rhinoviral drug binding pocket in HRV14 and HRV1A. *J. Mol. Biol.* **230**: 206–227.
- Kim, M.Y., Maier, C.S., Reed, D.J., and Deinzer, M.L. 2001. Site-specific amide hydrogen/deuterium exchange in *E. coli* thioredoxins measured by electrospray ionization mass spectrometry. *J. Am. Chem. Soc.* **123**: 9860–9866.
- Kolatkhar, P.R., Bella, J., Olson, N.H., Bator, C.M., Baker, T.S., and Rossmann, M.G. 1999. Structural studies of two rhinovirus serotypes complexed with fragments of their cellular receptor. *EMBO J.* **18**: 6249–6259.
- Lanman, J., Lam, T.T., Emmett, M.R., Marshall, A.G., Sakalian, M., and Prevelige Jr., P.E. 2004. Key interactions in HIV-1 maturation identified by hydrogen-deuterium exchange. *Nat. Struct. Mol. Biol.* **11**: 676–677.
- Lewis, J.K., Bothner, B., Smith, T.J., and Siuzdak, G. 1998. Antiviral agent blocks breathing of common cold virus. *Proc. Natl. Acad. Sci.* **95**: 6774–6778.

- Oren, D.A., Zhang, A., Nesvadba, H., Rosenwirth, B., and Arnold, E. 1996. Synthesis and activity of piperazine-containing antirhinoviral agents and crystal structure of SDZ 880-061 bound to human rhinovirus 14. *J. Mol. Biol.* **259**: 120–134.
- Pevear, D.C., Fancher, M.J., Felock, P.J., Rossmann, M.G., Miller, M.S., Diana, G., Treasurywala, A.M., McKinlay, M.A., and Dutko, F.J. 1989. Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors. *J. Virol.* **63**: 2002–2007.
- Reisdorph, N., Thomas, J.J., Katpally, U., Chase, E., Harris, K., Siuzdak, G., and Smith, T.J. 2003. Human rhinovirus capsid dynamics is controlled by canyon flexibility. *Virology* **314**: 34–44.
- Robinson, C.V., Chung, E.W., Kragelund, B.B., Knudsen, J., Aplin, R.T., Poulsen, F.M., and Dobson, C.M. 1996. Probing the nature of non-covalent interactions by mass spectrometry. A study of protein-CoA ligand binding and assembly. *J. Am. Chem. Soc.* **118**: 8646–8653.
- Rossmann, M.G. 1994. Viral cell recognition and entry. *Protein Sci.* **3**: 1712–1725.
- Rossmann, M.G., Arnold, E., Erickson, J.W., Frankenberger, E.A., Griffith, J.P., Hecht, H.J., Johnson, J.E., Kamer, G., Luo, M., Mosser, A.G., et al. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature* **317**: 145–153.
- Rossmann, M.G., Olson, N.H., Kolatkar, P.R., Oliveira, M.A., Cheng, R.H., Greve, J.M., McClelland, A., and Baker, T.S. 1994. Crystallographic and cryo EM analysis of virion-receptor interactions. *Arch. Virol. Suppl.* **9**: 531–541.
- Rueckert, R.R. 1996. Picornaviridae: The viruses and their replication. In *Fields virology* (eds. B.N. Fields et al.), pp. 609–654. Lippincott-Raven Publishers, Philadelphia, PA.
- Smith, T.J., Kremer, M.J., Luo, M., Vriend, G., Arnold, E., Kamer, G., Rossmann, M.G., McKinlay, M.A., Diana, G.D., and Otto, M.J. 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. *Science* **233**: 1286–1293.
- Smith, T.J., Chase, E.S., Schmidt, T.J., Olson, N.H., and Baker, T.S. 1996. Neutralizing antibody to human rhinovirus 14 penetrates the receptor-binding canyon. *Nature* **383**: 350–354.
- Smith, D.L., Deng, Y., and Zhang, Z. 1997. Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. *J. Mass Spectrom.* **32**: 135–146.
- Speelman, B., Brooks, B.R., and Post, C.B. 2001. Molecular dynamics simulations of human rhinovirus and an antiviral compound. *Biophys. J.* **80**: 121–129.
- Tuma, R., Coward, L.U., Kirk, M.C., Barnes, S., and Prevelige Jr., P.E. 2001. Hydrogen-deuterium exchange as a probe of folding and assembly in viral capsids. *J. Mol. Biol.* **306**: 389–396.
- Vaidehi, N. and Goddard, W.A. 1997. The pentamer channel stiffening model for drug action on human rhinovirus HRV-1A. *Proc. Natl. Acad. Sci.* **94**: 2466–2471.
- Wang, L. and Smith, D.L. 2003. Downsizing improves sensitivity 100-fold for hydrogen exchange-mass spectrometry. *Anal. Biochem.* **314**: 46–53.
- Wang, L., Lane, L.C., and Smith, D.L. 2001. Detecting structural changes in viral capsids by hydrogen exchange and mass spectrometry. *Protein Sci.* **10**: 1234–1243.
- Wang, L., Pan, H., and Smith, D.L. 2002. Hydrogen exchange-mass spectrometry: Optimization of digestion conditions. *Mol. Cell Proteomics* **1**: 132–138.
- Woodward, C., Simon, I., and Tuchsén, E. 1982. Hydrogen exchange and the dynamic structure of proteins. *Mol. Cell. Biochem.* **48**: 135–160.
- Xing, L., Casasnovas, J.M., and Cheng, R.H. 2003. Structural analysis of human rhinovirus complexed with ICAM-1 reveals the dynamics of receptor-mediated virus uncoating. *J. Virol.* **77**: 6101–6107.
- Yamada, N., Suzuki, E., and Hirayama, K. 2002. Identification of the interface of a large protein-protein complex using H/D exchange and Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun. Mass. Spectrom.* **16**: 293–299.
- Zhang, Z. and Smith, D.L. 1993. Determination of amide hydrogen exchange by mass spectrometry: A new tool for protein structure elucidation. *Protein Sci.* **2**: 522–531.
- Zhang, Z., Post, C.B., and Smith, D.L. 1996. Amide hydrogen exchange determined by mass spectrometry: Application to rabbit muscle aldolase. *Biochemistry* **35**: 779–791.