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Hydrogen-deuterium Exchange as a Probe of Folding and Assembly in Viral Capsids

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The dynamics of proteins within large cellular assemblies are important in the molecular transformations that are required for macromolecular synthesis, transport, and metabolism. The capsid expansion (maturation) accompanying DNA packaging in the dsDNA bacteriophage P22 represents an experimentally accessible case of such a transformation. A novel method, based on hydrogen-deuterium exchange was devised to investigate the dynamics of capsid expansion. Mass spectrometric detection of deuterium incorporation allows for a sensitive and quantitative determination of hydrogen-deuterium exchange dynamics irrespective of the size of the assembly. Partial digestion of the exchanged protein with pepsin allows for region-specific assignment of the exchange. Procapsids and mature capsids were probed under native and slightly denaturing conditions. These experiments revealed regions that exhibit different degrees of flexibility in the procapsid and in the mature capsid. In addition, exchange and deuterium trapping during the process of expansion itself was observed and allowed for the identification of segments of the protein subunit that become buried or stabilized as a result of expansion. This approach may help to identify residues participating in macromolecular transformations and uncover novel patterns and hierarchies of interactions that determine functional movements within molecular machines.

Keywords: hydrogen/deuterium exchange; virus; capsid; protein folding; dynamics

Most of the biochemical reactions within a cell do not occur through the random collision of protein molecules but rather within well defined, large (ten or more protein subunits), macromolecular complexes. These complexes, which form by self-assembly from constituent macromolecules, are efficient molecular machines that undergo many duty cycles per second. During a typical duty cycle, the subunits of the assembly often rearrange and undergo a series of conformational changes.[1] For high processivity, the rearrangements must take place within the intact assembly and therefore require considerable mobility of domains and subunits. Such motions occur *via* the formation of high energy transition state ensembles of intact assemblies, and the formation of these transition states is in turn determined by the strength of local interactions within and between subunits. To understand the function of such an assembly therefore requires an identification of the regions undergoing transformation, elucidation of the pathway of transformation, and characterization of the energetic barriers to transformation. As originally pioneered by Englander and co-workers,[2,3] functional labeling by hydrogen/deuterium exchange (HDX) studies provide a means to obtain the required information. Advances in the use of mass spectrometry to analyze exchange patterns provides the opportunity to utilize this technique to obtain region-specific rather than global information.

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Abbreviations used: HDX, hydrogen deuterium exchange; MALDI, matrix assisted laser desorption ionization; TOF, time of flight.

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Maturation of bacteriophage P22 is one of the best-characterized transitions within large macromolecular complexes.[4–7] P22 is a representative member of the class of dsDNA viruses that assemble in two steps. In the first step, P22 coat protein (gp5, 46,700 Da) assembles with the assistance of approximately 300 molecules of scaffolding protein into a $T = 7$ procapsid (420 subunits, 19.6 MDa) that serves as a precursor for DNA packaging (Figure 1). In the second step, triggered *in vivo* by DNA packaging, the scaffolding protein is released, the procapsid lattice expands, and as a consequence of expansion, the capsid is stabilized.[4] Expansion results in a significant morphological change.[8] Raman spectroscopy, which can provide a global (but not region-specific) measurement of HDX, has revealed that expansion results in a large increase in exchange protection with only a small change in secondary structure.[6] Capsid expansion can be triggered *in vitro* at elevated temperature (55-65 °C) and is governed by large, temperature-dependent, activation energy[4,6] (Figure 1).

It has been proposed that expansion is mediated by domain interchange between neighboring subunits (Figure 1).[6,7,9] Such a large-scale rearrangement requires a transient disruption of interdomain interactions followed by a swiveling motion of whole domains around a flexible hinge. Although the hinge region has been mapped by limited proteolysis to the middle of the protein (residues 180-205)[10] it is not known which residues constitute the invariant interfaces, nor is it known which regions dissociate to allow for the domain movement. The large difference in HDX protection between the procapsid and the mature capsid suggests that the regions involved in the transformation are less protected in the procapsid, and thus that HDX could be used to identify these regions. Additionally, the regions which become transiently exposed to solvent during expansion may be identified by labeling during expansion.

NMR spectroscopy has traditionally been used for high resolution mapping of HDX.[11] However, due to the slow tumbling rates and spectral complexity of large complexes, this technique is limited to relatively small proteins. Recently, mass spectrometry (MS)-based techniques for monitoring site-specific HDX have been developed.[12–15] This

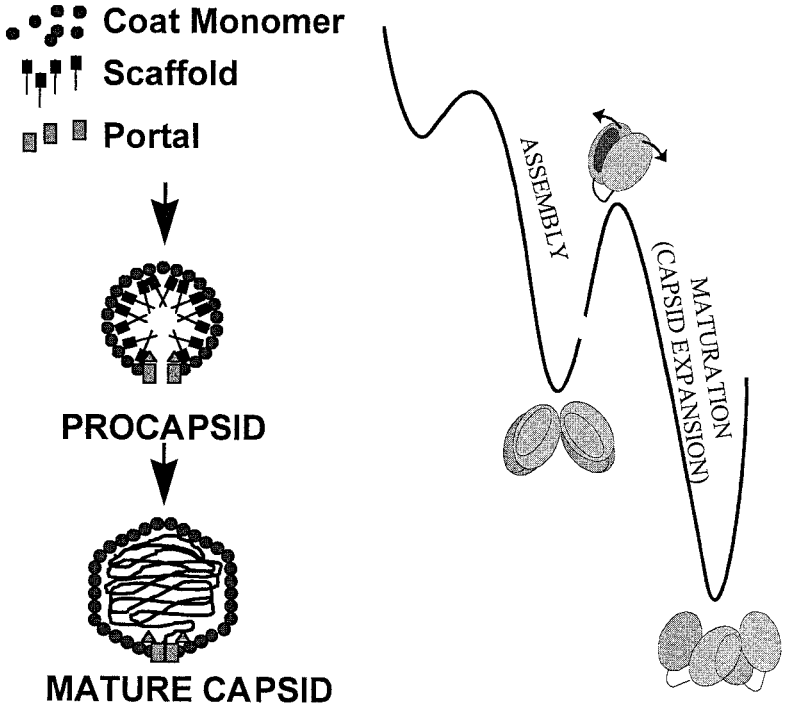

Figure 1. Left: The assembly pathway of bacteriophage P22. Monomeric coat protein assembles with the assistance of scaffolding and portal proteins to form a metastable $T = 7$ procapsid in which the scaffolding protein is contained internally and a portal protein complex is located at one of the icosahedral vertices. Upon DNA packaging through the portal vertex, the scaffolding protein exits, and the lattice expands to become the mature $T = 7$ capsid. The samples used in this study correspond to procapsids from which the scaffolding has been extracted, and expanded shells, analogs of the mature form obtained by heating procapsid like particles.[4] Right: Assembly and maturation of P22 coat protein is superimposed on a hypothetical energy landscape. Domain interchange is proposed as the mechanism of capsid expansion and is illustrated schematically. Domains of the subunit which form internal contacts in the procapsid (buried shaded areas) open up in the transition state (exposed shaded areas) and then form new contacts with their neighbors in the expanded form (buried, swapped, shaded areas). The transition may be mediated by an ensemble of transition states in which the interface between domains has been disrupted allowing for mobility within the assembly.

approach, which is not in principle limited by the size of the assembly, has proven useful for mapping residues constituting subunit interfaces in oligomeric proteins[16–19] as well as to study chaperone-assisted protein folding.[13]

In this study, we demonstrate the utility of HDX and MS for the study of protein dynamics in large assemblies. Deuterium labeling during expansion was employed to identify regions within domain interfaces involved in the transformation from procapsid to capsid. Our results demonstrate that HDX combined with MS can identify regions involved in transformation of large biological complexes.

Measurement of deuterium exchange in a viral capsid

A method, similar to that described,[20] which relies on exchange of the sample under a defined set of conditions, chemical quenching of the back exchange at low pH, rapid digestion of sample under acidic conditions into a reproducible set of short (10-15 amino acid residues) peptides (Figure 2(a)), and determination of the mass distribution by MALDI-TOF (Figure 2(c)), was employed. HDX of amide protons results in a shift of the fragment mass distribution towards higher masses; because side-chain protons back-exchange rapidly during digestion in H_2O they do not contribute to the mass shift. In a single experiment it is possible to obtain mass distribution information for many peptide fragments.

P22 capsids proved to be stable even at pH 2.5, and it was necessary to disrupt the viral capsids prior to digestion by inclusion of a guadinidine hydrochloride (GuHCl) dissociation step. This step was only necessary for efficient digestion of the stable mature shells. For the less stable procapsid shells, identical results were obtained with and without the dissociation step, indicating that the GuHCl treatment did not alter the observed exchange patterns (data not shown). The back-exchange during dissociation and digestion was estimated to be between 20-30 % by comparison of the observed and expected centroids for the fully deuterated control. The results in Figure 2 show that both good signal-to-noise ratio and sufficient resolution can be achieved even in the presence of residual salts (~50 mM total) in the blotting matrix. This makes the MALDI interface preferable over the electrospray interface for the direct analysis of samples. Nine strong peaks in the MALDI were selected for assignment by electrospray MS:MS. These nine fragments cover approximately 28 % of the primary sequence (Figure 2(b)).

The three panels of Figure 2(c) show that region-specific monitoring of HDX is possible in a large macromolecular assembly irrespective of its size, and compare the HDX profiles for folding and assembly intermediates. Although the viral capsid employed in this study is composed primarily of the coat protein (with minor contribution of the portal protein) the same protocol may be used for assemblies containing several protein species. For more complex assemblies, such as ribosomal subunits or the nuclear pore, overlapping fragments may complicate the analysis, and a rapid separation of the peptide digest and the more elaborate electrospray methodology would be preferred over the simpler MALDI protocol.[21]

Detection of subunit-subunit interface rearrangements due to capsid transformation

Changes in the extent and stability of intersubunit interactions during assembly and maturation will result in changes in the degree of HDX protection within the regions involved, and these changes will be evidenced as shifts in the mass distribution of the peptide fragments. Structural elements, which remain relatively constant through assembly, should display unchanged exchange protection. For example, the regions comprising the folding core of the subunit are expected to exhibit similar degrees of protection in the monomer, procapsid, and mature virion forms. A good candidate for a component of the exchange-protected core are the residues 415-430 (Figure 2(c), middle). In comparison with unexchanged and fully exchanged controls, this region shows a high degree of exchange protection in all three forms (monomer, procapsid, and expanded shell). These data are consistent with the observation that the single cysteine residue 415 is buried in both the monomer and procapsid structure.[6,22]

In contrast, regions that comprise interfaces between subunits generated during assembly are expected to show little protection in the monomer and increased protection in the procapsid. Peptides that have their origin in a previously identified flexible hinge region display this behavior (Figure 3, peptides 182-188, 189-195). Recent spectroscopic results have shown that about 20 residues undergo folding during assembly.[22] The present results indicate that the hinge is the likely candidate for the folding region.

The regions that are directly involved in expansion are likely to become more protected in the expanded state. The degree of protection of the assigned peptides was determined for the procapsid and expanded shells under conditions that promote exchange *via* local unfolding (35 °C) (Figures 2 and 3). These conditions are best suited to highlight the differences in protection between the two states.[6] Figure 2(c), left and right, provide examples of peptides (residues 196-208, 156-168) that become protected upon expansion. For the peptide 196-208, the mass distribution for the procapsid is similar to that of the fully exchanged control, suggesting little protection for these species. Peptide156-168 is protected relative to the fully exchanged form, but the protection in the procapsid is similar to that in the monomer. Presumably, this is protection due to folding. In both cases, the mass envelope obtained for the peptide from the expanded shell is signifi-

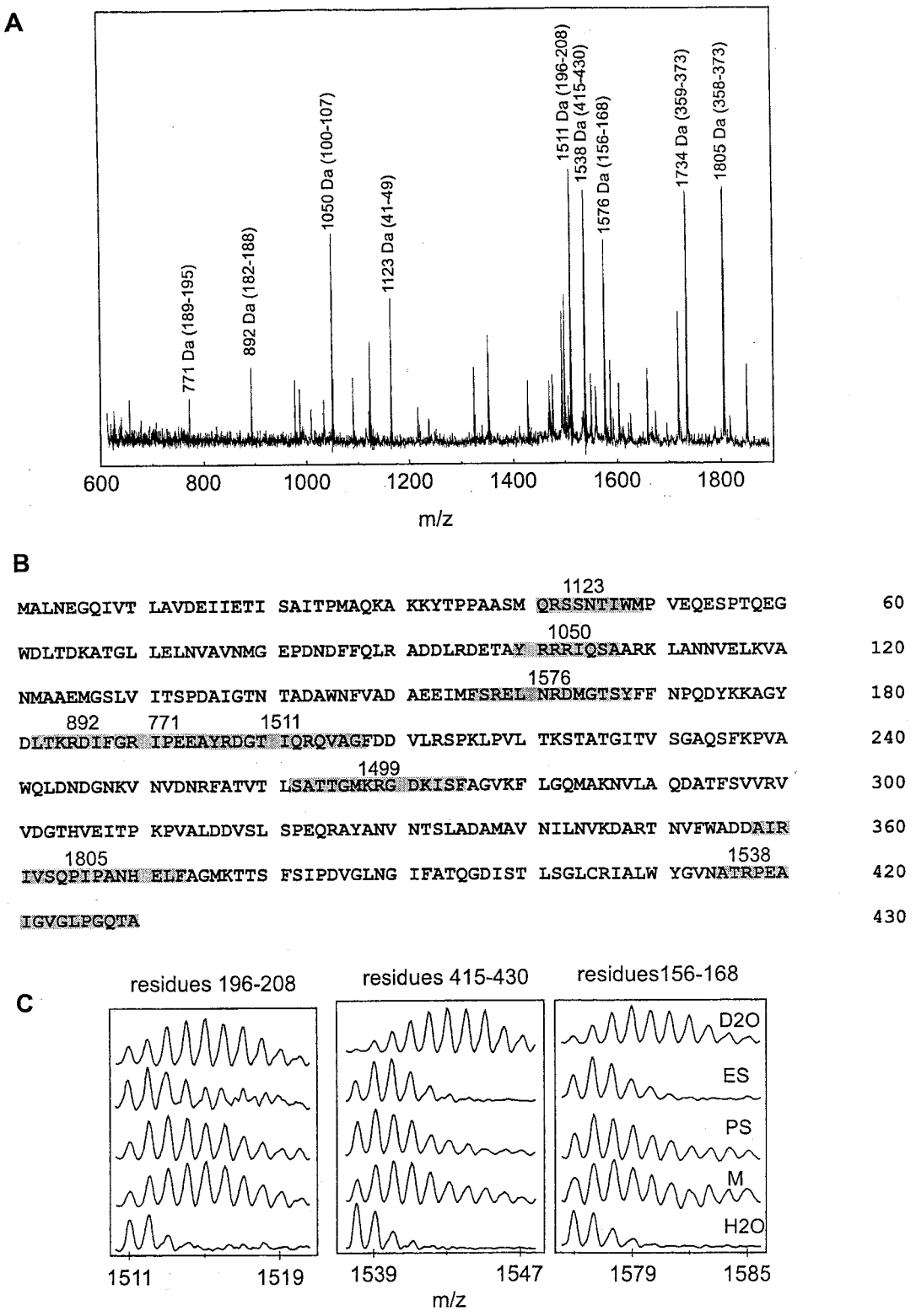

Figure 2 (*legend shown opposite*)

cantly narrower and on average shifted towards lower mass than that in the procapsid. This indicates increased protection as a result of capsid expansion. The observed protection is in accordance with the location of peptide 196-208 within the flexible hinge region that becomes protease-resistant in the expanded shell (residues 180-205).[10] Similarly, other regions within and near the hinge

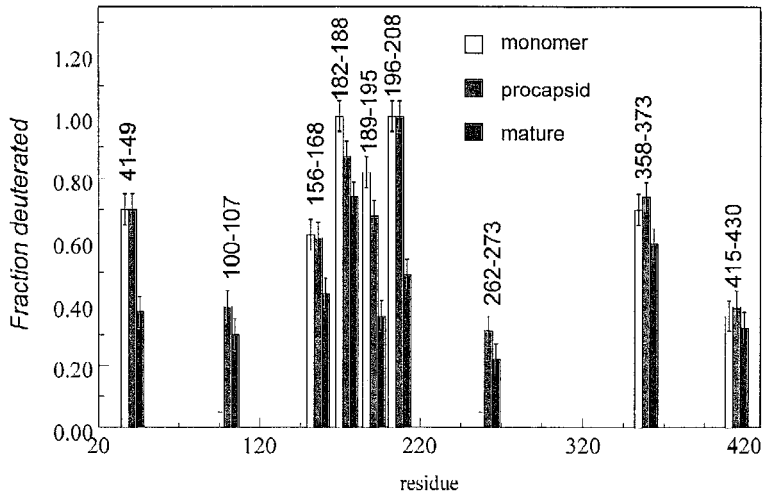

Figure 3. Deuterium exchange of coat protein subunit in different assembly states determined as described in Figure 2. Bar graph above each sequence region indicates fraction of deuterated peptides in the monomer (after 24 hours, 4 °C, yellow), procapsid (after 100 hours, 35 °C, red), expanded shell (after 100 hours, 35 °C, blue). The data represent the average and standard deviations obtained from four independent experiments.

(182-188,189-195) exhibit less protection in the procapsid than in the mature shell (Figure 3). Several regions (41-49, 156-168) become significantly more protected upon expansion (summarized in Figure 3). A slightly increased protection was further observed for residues 100-107, 262-273 and 358-373. The low magnitude of these changes, and the fact that they are distributed throughout the protein, suggests that they arise from the global stabilization which is known to accompany expansion.[4]

Interestingly, we have as yet been unable to identify a single contiguous region that would remain completely unprotected in all three assembly states and thus would represent a surface epitope. This may in part be due to incomplete "coverage" of the coat sequence in the peptide fragments. On the other hand, none of the peptides for which exchange was reported shows 100 % protection. Thus, it is conceivable that a stable viral capsid assumes a polypeptide fold in which short exposed epitopes are interspersed within regions that are stabilized by cooperative interactions. For

Figure 2. (a) MALDI-TOF of peptic digest of P22 coat protein. The amino acid residue numbers defining the fragment are indicated in parenthesis. Identification of the peptides was carried out in separate MS/MS experiments using a PE Sciex (Concorde, Ontario, Canada) API III triple quadrupole mass spectrometer with an electrospray ionization interface. The molecular ions of peptides identified in the MALDI-TOF analysis were subject to collision with Ar/N_2 (90 %:10 %) gas to generate daughter fragment ion spectra. Proteins and assemblies were prepared and purified as described previously.[6,10] $H_2O/^2H_2O$ buffer changes were achieved using BioGel P-6 desalting spin columns which were pre-equilibrated three times with the desired H_2O or ^2H_2O buffer (50 mM sodium phosphate, 25 mM NaCl, pH 7.5). Exchange was initiated by a transfer of the sample into ^2H_2O and incubation at the desired temperature (4 and 35 °C). Because mature shells were stable even at pH 2.5, all assemblies were subject to dissociation in a buffer (10 mM ammonium phosphate, pH 2.5) containing 3 M guanidine hydrochloride. After dilution 1:10 into 10 mM ammonium phosphate (pH 2.5) the dissociated subunits were digested with agarose-immobilized pepsin (0 °C, two minutes, 200 units of Sigma insoluble pepsin). The resulting solution of peptides was diluted 1:10 into matrix solution (0.1 % trifluoroacetic acid in 50 % v/v acetonitrile/water, saturated with α-hydroxycinnamic acid) and spotted onto a chilled gold-coated MALDI plate. Masses of the peptides were determined using a Perseptive Biosystems (Framingham, MA) Voyager Elite MALDI-TOF mass spectrometer in the reflector mode (mass resolution approximately 1:2000) using a pulsed nitrogen laser operating at 337 nm. Each spectrum represented data accumulated from up to 100 laser shots. (b) The amino acid sequence of bacteriophage P22 coat protein. The shaded regions represent peptides resulting from pepsin digestion and assigned by MS:MS. The numbers above the shaded regions correspond to the mass of the peptide as determined in (a). (c) Mass spectra of three peptides corresponding to different coat protein regions. The mass distribution for exchanged forms is shifted towards higher mass due to the replacement of the amide protons by deuterons. In each panel the top and bottom traces represent mass spectrum of fully exchanged and non-exchanged controls, respectively. The two controls were used in quantitative analysis for determination of the fraction of deuterated peptide groups according to: $d = (c - n)/(f - n)$, where c is the centroid of the isotopic distribution of the peptide peak, n and f are the centroids of the fully protonated and the fully deuterated control, respectively. The centroid was calculated as a weighted average of positions of five strongest peaks in the isotopic distribution. The centroid reproducibility was ±0.5 Da between different experiments. The second and third traces from top (ES and PS, respectively) represent endpoint of exchange *via* local unfolding in expanded shells and procapsid shells, respectively (100 hours in ^2H_2O at 35 °C[6]). The second trace from the bottom (M) represents the endpoint of exchange for the native state of coat protein monomer (24 hours, 4 °C).

P22 and other dsDNA bacteriophages this feature is consistent with great stability of the mature capsid shell and resistance to protease cleavage.[4,10,23] Therefore, it may not be possible to use the difference in the protection of the monomeric subunit and the assembled subunit to identify subunit-subunit interfaces directly, as demonstrated for smaller, less stable and reversible, protein oligomers such as cAMP-dependent protein kinase/inhibitor and thrombin/thrombomodulin binding.[19] In order to determine subunit association within large stable assemblies, for which subunit folding and assembly are often coupled, characterization of the energetics of local unfolding may be required.[24]

The use of H/^{2}H exchange to follow dynamic transformations

While structural studies can characterize stable states, often in great detail, and thereby suggest pathways for structural transformations, characterizing the actual pathways of these transformations remains a significant challenge. In principle, HDX techniques should be applicable to this problem in much the same way they have been used to characterize protein folding pathways. As a proof of principle, we have pulse-labeled procapsid shells during the process of expansion and examined the pattern of deuterium retention after back-exchange. We use exchange of the peptide corresponding to the hinge region (residues 196-208) to illustrate dynamic behavior during expansion (Figure 4).

Procapsids were incubated in ^2H_2O at 68 °C for 30 seconds, conditions under which approximately 40 % of the shells expand (rate of expansion at 68 °C is one min^{-1}),[6] and the distribution of deuterium in the peptide corresponding to amino acid residues 196-208 was determined (Figure 4(a), trace II). Pre-expanded shells were incubated in parallel as a control (Figure 4(b), trace II). Within the pro-

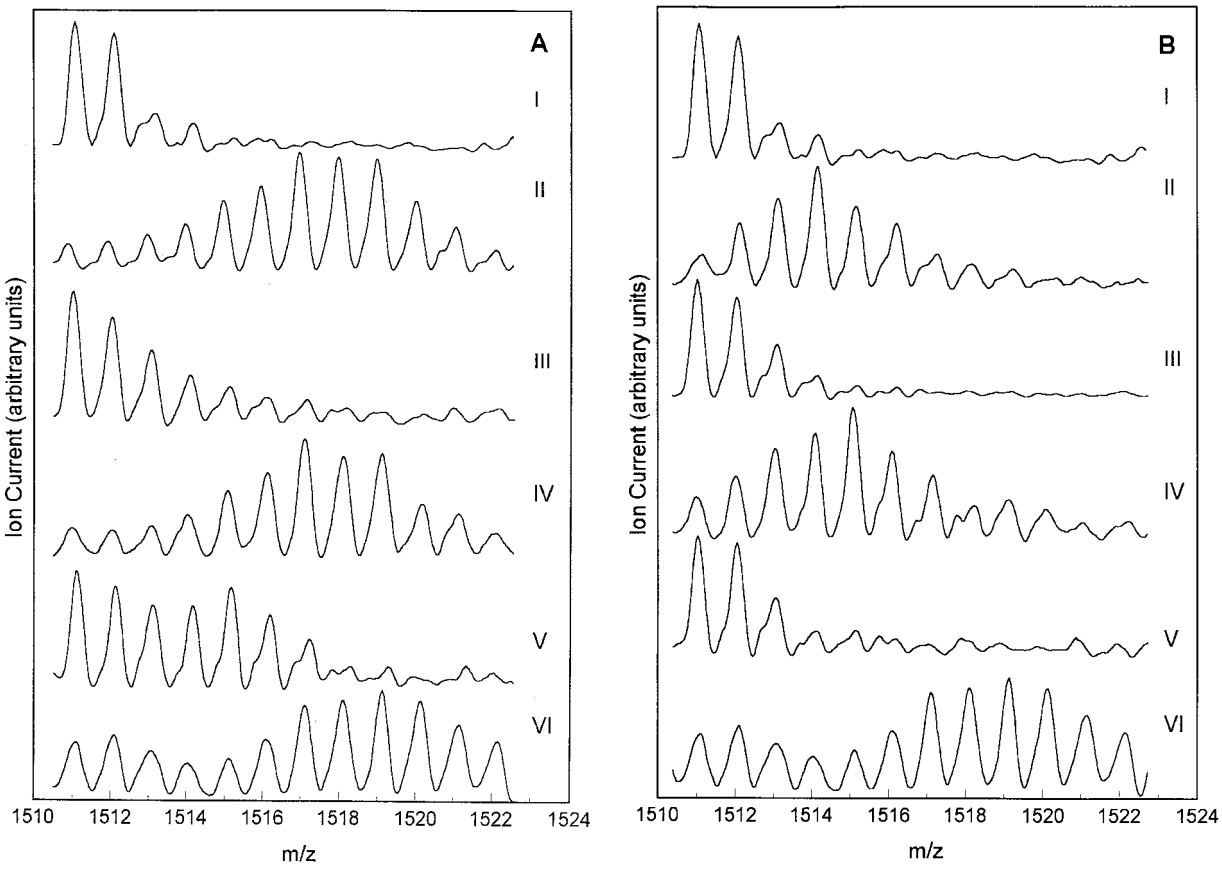

Figure 4. Deuterium labeling during procapsid shell expansion at 68 °C. (a) (I) Mass spectra of protonated control; (II) procapsids labeled at 68 °C for 30 seconds during which 40 % shells expanded (the extent of expansion was assessed by agarose gel electrophoresis);[6] (III) procapsids labeled at 68 °C for 30 seconds and back-exchanged in H_2O for 2.5 hours at 35 °C; (IV) procapsid shells were heated at 68 °C in ^2H_2O for ten minutes during which 70 % shells expanded; (V) procapsid shells were heated at 68 °C in ^2H_2O for ten minutes and back-exchanged in H_2O for ten minutes at 68 °C; (VI) fully deuterated control. (b) The same as (a) but expanded shells were used instead of procapsids. The fully deuterated control spectra for both procapsid and pre-expanded shells were superimposable and were averaged to improve the signal-to-noise ratio, Note that the appearance of bimodality in the fully deuterated sample ((a) and (b), trace VI) is due to the overlap of the deuterated tail of the m/z 1499 peak, This does not affect the conclusions.

capsid, the peptide became almost fully deuterated whereas in the expanded shell only approximately half of the NH sites were exchangeable. The fact that a similar degree of labeling can be achieved in the procapsids at 35 °C (a temperature where there is no expansion[6]) (Figure 3) suggests that the deuterium is likely incorporated before the expansion. To determine if this exchange was reversible, both samples were then back-exchanged at 35 °C for 2.5 hours (at 35 °C no detectable expansion is observed,[6] and data not shown). For the pre-expanded shells, the forward exchange was completely reversible (compare Figure 4(b) traces I and III). For the procapsid-derived samples, the reaction was not fully reversible, some deuterons were retained as evidenced by the peaks observed between m/z 1515-1519 (Figure 4(a), III). In contrast, when the forward exchange was performed at 35 °C (Figure 2(c)) it was fully reversible (not shown). Thus, the retained deuterons were stabilized by the expansion and face a higher activation barrier for back-exchange (Figure 1).

More extensive incubation at 68 °C for ten minutes results in the continued expansion of procapsids until they comprise 70 % of the population. Once again, it is evident that the pre-expanded shells are more resistant to forward exchange than the procapsid-derived samples and upon back-exchange at 68 °C the pre-expanded shells display complete reversibility (compare Figure 4(b) traces I and V). However, for the procapsid-derived samples approximately half the initial amount of deuterium was retained as a second peak centered at around m/z 1515 in the overall envelope (Figure 4(a), trace V). We interpret these data as follows: the 196-208 region is exposed prior to expansion. Upon expansion, approximately one half of the amino acid residues in this fragment become fully protected against exchange and retain deuterium, while the other half continue to exchange freely. This confirms the existence of a large increase in the activation barrier for local exchange upon expansion affecting approximately half of the residues within the hinge. The high activation barrier is a result of higher stability of this region in the mature shell as depicted on the energy landscape in Figure 1. Flexibility within the hinge region is required for expansion.[10] Furthermore when the mobility of the loop was increased by proteolytic cleavage expansion was greatly accelerated.[10] The use of $H/^2H$ exchange permits better mapping of the hinge region dynamics.

Several other regions (residues 41-49 and 156-168) also became labeled during expansion and retained deuterium (data not shown). The retention of deuterium reflects the increased energy barrier for exchange that results from stabilization of the expanded state. On the other hand, as would be expected if the C-terminal region was a component of the constant folding core, none of the C-terminal regions trapped deuterium during expansion.[6]

Implications for dynamics of viruses and other assemblies

A viral capsid undergoes a series of controlled structural transformations during the viral life cycle and as such represents a dynamic protein assembly. This dynamic character is critical to their function, and mutations which alter the stability of the capsid frequently have a deleterious effect on viral replication.[25] Stabilization of the capsid structure, thereby preventing uncoating, is in fact the mechanism of action of one class of antiviral agents.[26] However, investigating the progressive alterations in backbone dynamics as the proteins comprising viral capsids fold, assemble, and function has proven to be a challenge. Proteolytic cleavage and mass spectrometry has been used to detect flexible regions,[10,25] and HDX studies by Raman spectroscopy have been used to gain an overall estimate of exchange kinetics and protection.[6,27,28] The use of HDX and mass spectrometry extends these studies and allows for determination of flexibility throughout the polypeptide backbone. The present method exploited the irreversibility of the procapsid-to-capsid transition to probe the expansion dynamics. Although molecular machines undergo cyclic changes to return to the identical initial state, individual steps in the cycle are usually coupled to an irreversible chemical reaction (e.g. ATP hydrolysis) and thus the complex moves along an energy landscape similar to that of the P22 capsid (Figure 1). Thus, the methodology presented here could be applied to more complex molecular machines.

Acknowledgments

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