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Kinetics of Protein Complex Dissociation Studied by Hydrogen/Deuterium Exchange and Mass Spectrometry

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Abstract

The growing importance of protein aggregation diseases requires the development of new methods to elucidate the molecular features that are responsible for the incipient protein-protein interactions. Kinetic information from protein-protein association/dissociation reactions is particularly valuable for revealing mechanistic insight, but robust tools that can provide this information are somewhat lacking. In this work, we describe a hydrogen/deuterium exchange (HDX)-based method that provides rate constant information for protein oligomer dissociation, using the well-studied β -lactoglobulin (β LG) dimer as a model system to validate our approach. By measuring the rate of exchange at different regions of the protein using top-down tandem mass spectrometry and fitting the resulting data to an appropriate mathematical model, we are able to extract the dimer's dissociation rate constant. We exploit the fact that regions of the protein that are part of the protein-protein interface have exchange patterns that are distinct from non-interfacial regions. This observation indicates that the HDX/MS method not only provides kinetic information but could also provide structural insight about the interface at the same time, which would be very valuable for previously uncharacterized protein-protein complexes.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

Figures illustrating our data analysis procedure, the extent of dimer re-formation, and example ECD spectra can be found in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Hydrogen/deuterium exchange (HDX) coupled with mass spectrometry (MS) has proven to be a valuable tool for characterizing protein structure and dynamics by measuring exchange along the protein backbone. The method has been applied to study protein structure, folding mechanisms, quality control, and protein interactions with ligands and other proteins.^{1–5} The extent of deuterium incorporation (or deuterium loss) over time provides this information, and site-specific structural insight is often obtained after quenching the exchange reaction, digesting the protein with an acid-stable enzyme, and counting the added (or lost) deuteriums in the resulting proteolytic fragments by MS.^{6–9} An emerging, but less commonly used, approach for site-specific structural information relies on top-down sequencing of the labeled protein via electron capture dissociation (ECD) or electron transfer dissociation (ETD).^{3,10} In principle, the top-down strategy is experimentally simpler to perform, less prone to back exchange, and provides better time resolution, but in practice, the precision of the measurement typically is lower than the more common bottom-up approach.

In addition to structural information, HDX/MS has been used effectively to obtain thermodynamic and kinetic information about protein interactions and protein folding. For example, protein-ligand affinities are measurable using techniques such as PLIMSTEX and SUPREX.^{11,12} The structure and dynamics of oligomer/monomer equilibria can also be obtained from HDX/MS studies.^{13,14} Other thermodynamic parameters, such as protein energy landscapes¹⁵ and binding free energies,¹⁶ can be determined by HDX. Protein folding kinetics have also been studied by HDX/MS, often using pulsed labeling to gain insight into folding pathways and intermediates.^{17–20} HDX/MS has been used much less often, though, to study other aspects of protein kinetics such as association and dissociation rates. One notable exception is work by Jørgensen and co-workers in their studies of urokinase receptor interactions with a peptide antagonist.²¹ From HDX/MS measurements, they demonstrated that peptide dissociation from the receptor was associated with correlated exchange of amide hydrogens on the peptide, allowing the dissociation rate constant to be determined. In principle, such dissociation rate constant information should be accessible for other protein-protein complexes when HDX is performed under appropriate conditions, but to our knowledge the technique has not been used much in this way.

This lack of HDX/MS work to study protein association/dissociation kinetics is somewhat surprising given the ever growing importance of protein aggregation diseases such as Alzheimer's disease and type 2 diabetes.^{22,23} To more fully understand the molecular progression of protein aggregation in these diseases, there is a need to measure protein association/dissociation rates so as to reveal the factors that influence aggregation. Generally speaking, a variety of methods can be used for such purposes. Light scattering is a straightforward method, but it is limited in that it requires large changes in molecular size upon association/dissociation of the protein complexes of interest to detect rates of change.²⁴ Analytical ultracentrifugation (AUC) is also a relatively simple method that can monitor protein oligomer transitions in a controlled centrifugal field; however, monitoring kinetics involving lower molecular species (< 10 kDa) is challenging.²⁵ Like AUC, size-exclusion chromatography (SEC) has been used to monitor protein oligomer kinetics, but just like light

scattering and AUC, SEC is limited to proteins that undergo relatively slow association or dissociation reactions. Fluorescence techniques, such as intermolecular FRET experiments, allow faster rates to be measured directly, but necessary chemical labeling of the proteins can potentially perturb protein structure.²⁶ In addition, information about the sites of interaction are typically required so that the locations of the FRET pairs can be chosen appropriately. Surface plasmon resonance (SPR) is an appealing technique as it can provide direct kinetic information, but the requirement to immobilize one of the interacting proteins can complicate the experiment. Moreover, discriminating between specific and non-specific interactions can sometimes be challenging.²⁷

Given the underutilized potential of HDX/MS to measure protein-protein association/dissociation rates, we have begun to explore the capability of this method to obtain this kinetic information from protein oligomer transitions. Here, we describe our initial work using the β -lactoglobulin (β LG) dimer as a well-studied model system.^{28–30} We perform the deuterium labeling reaction as the dimer dissociates into monomer. The resulting exchange patterns for specific protein regions are then measured and fit to a kinetic model to obtain rate information. In addition to providing rate information, the pattern of exchange is distinct for interfacial residues, and thus HDX/MS could be used to provide both rate and structural information simultaneously, even for impure samples.

EXPERIMENTAL SECTION

Materials

β LG-A from bovine milk, citric acid, tris(2-carboxyethyl)phosphine (TCEP) and deuterium oxide (99.9% ^2H) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate, acetonitrile (HPLC grade) and formic acid were purchased from Fisher Scientific (Waltham, MA, USA).

Hydrogen/Deuterium Exchange

HDX and dimer dissociation were initiated by diluting a 1 mM β LG in H_2O (pH 6.0, all pH values in this paper are uncorrected values that were measured directly from pH meter) 200 times into a citrate (1 mM) or formate (1 mM) buffer in D_2O at 21 °C that was at a pH of 3.5 or 4.0, respectively. Only the β LG dimer is present in the initial 1 mM solution at pH 6.0 as confirmed by dynamic light scattering (see Figure S1 in the Supporting Information (SI) for data and dynamic light scattering measurement details). One of two methods was then used to quench the reaction before performing MS to determine the sites of deuteration. In method 1, HDX reactions were quenched 1:8 (vol/vol) by a cold solution of 60:40 (vol/vol) H_2O : acetonitrile whose pH was adjusted to 2.6 with formic acid. In method 2, the HDX reactions were quenched 1:1 (vol/vol) by cold acetonitrile with 1 mM TCEP and formic acid to adjust pH to 2.6. The samples that were quenched via method 1 were immediately analyzed by ESI-MS and top-down MS/MS, while the samples quenched via method 2 remained in an ice bath for 8 minutes to allow disulfide bond reduction before MS analysis. For control experiments, a fully deuterated protein was prepared by dissolving lyophilized β LG in 99.9% D_2O with 0.1% formic acid and incubating the solution at 37 °C for 2 h. After 2 h, the sample was then lyophilized and re-dissolved again in D_2O with 0.1% formic acid

and incubated for another 2 h. This process was repeated 2 times to produce a fully deuterated protein. To validate our top-down approach (see below) for measuring the extent of deuteration, the β LG dimer at pH 6.0 was diluted 20 times in a deuterated ammonium acetate solution (1 mM, pH 7.0) and then allowed to exchange for various time points. The exchange was quenched by addition of the solution (1:19 (vol/vol)) used in method 1.

Mass Spectrometry

The quenched protein solution was directly sprayed without desalting from a standard ESI source and measured on a 7.0 T Solarix (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The sample, syringe (825RN, Hamilton Co., Reno, NV) and tubing that were connected to the ESI source were kept cold by ice packs to minimize back exchange during the ESI-MS analysis. The syringe that was used has a reinforced plunger to provide an air-tight seal at low temperature, minimizing exposure to any atmospheric water.

For the top-down MS/MS experiments, the protein ion charge states of interest were isolated in the front-end quadrupole mass filter and then dissociated by ECD in the ICR cell. For method 1, the +14 charge state was chosen for ECD, whereas for method 2, the +17 charge state was used instead because its intensity was much higher after disulfide bond reduction. The reduction yield was estimated to be around 25% complete based on a shift to higher mass caused by reduction of the two disulfide bonds. Typically, 500 scans were accumulated for each ECD spectrum to obtain mass spectra with good signal-to-noise ratios. All other instrument parameters were tuned for mild ion desolvation and isolation to minimize scrambling as reported in literature.^{31,32} The following instrument settings were typical: capillary exit: 170 V; capillary temperature: 150 °C; collision voltage: -1.5 V; collision RF amplitude: 350 V; isolation width: 20 m/z units.

Data Analysis

The extent of deuterium incorporation into the ECD product ions was determined from the difference between the measured m/z ratio of the deuterated ions and the measured m/z ratio of the undeuterated ion. One of two methods was used to account for the spread of isotopes after deuteration. For ions with masses below 2000 Da, which have fewer isotopic peaks, the deuterium incorporation was determined by an intensity-based weighted averaging approach. For ions larger than 2000 Da, the deuterium incorporation was determined by fitting the isotopic distribution to a Gaussian to determine the apex.³³ The deuterium incorporation for individual amide sites was calculated by subtracting the determined m/z of two fragment ions that differed by the residue of interest. In some cases pairs of product ions that differed by only residue were not observed. In such cases the deuterium incorporation at individual amide sites was obtained by subtracting the determined m/z of two product ion pairs and then averaging the resulting value using the difference in the number of residues between each product ion. The back exchange was measured to be approximately 30% by analyzing fully deuterated β LG using the same setup. This extent of back exchange was used to adjust the deuterium incorporation calculations. A pictorial representation of how the mass spectral data were analyzed is shown in Figure S2 in the SI.

RESULTS AND DISCUSSION

Theory

To use HDX/MS for determining the dimer dissociation rate, we needed to first establish a set of expressions that could be fit with the experimentally measured HDX data. These expressions were obtained in the following manner. Equation 1 is the kinetic expression for the dimer's dissociation into monomer with k_{off} representing the dimer dissociation rate constant, which is of interest, and $[M_2]$ is the concentration of the dimer.

$$d[M_2]/dt = -k_{\text{off}}[M_2] \quad (1)$$

Equation 2 represents the kinetic expression for the reverse reaction in which the monomer associates back into a dimer; k_{on} is the rate constant for the dimer formation reaction and $[M]$ is the monomer concentration.

$$d[M]/dt = -k_{\text{on}}[M]^2 \quad (2)$$

Equation 3 describes how the extent of deuterium incorporation changes over time, and this change is influenced by the presence of both the dimer and monomer. This equation considers the amount of β LG that is deuterated at a specific amide ($[m^{D,i}]$) based on the concentrations of that specific undeuterated amide in the dimer and monomer at time t , $[M_2^H]_t$ and $[M^H]_t$, respectively. The reasonable assumption is that the concentration of this amide in the dimer and the monomer will be equal to the concentrations of the full dimer and monomer in solution at time t . The HDX rate constants of this given amide residue in the dimer (k_{dimer}) and the monomer (k_{mono}) will then influence the extent of exchange at this site. This expression was used to fit deuterium incorporation at individual amides as measured after top-down ECD of the deuterated protein at increasing time points.

$$\frac{d[m^{D,i}]}{dt} = [M_2^H]_t k_{\text{dimer}} + [M^H]_t k_{\text{mono}} \quad (3)$$

While these three expressions could be solved analytically using Matlab, we surmised that the extent of dimer re-formation is very low under our experimental conditions as long as the HDX reaction times are shorter than 100 s. We arrived at this conclusion based on the known half-life for the β LG monomer to re-form as a dimer, which is approximately 370 s under our initial experimental conditions (see Figure S3). By ignoring the extent of dimer re-formation, these expressions could be fit with one less variable to consider, thereby improving the reliability of the fits. It should be noted that all three expression can be used when enough data points are included to achieve reliable fits.

Predicted Behavior for Interfacial versus Non-Interfacial Residues

After these expressions are considered, the expected HDX behavior of amides near the dimer interface (i.e. interfacial regions) and distant from the dimer interface (i.e. non-

interfacial regions) can be predicted. HDX at non-interfacial regions should adopt a negative exponential curve like that normally observed in typical HDX experiments (Figure 1 green curve). In contrast, HDX at interfacial regions should adopt a positive exponential curve at relatively short deuteration times because as the dimer dissociates the previously buried backbone sites become exposed, and presumably more dynamic, upon release of the monomers (Figure 1 red curve). At very long deuteration times, the extent of deuterium incorporation at the interfacial regions should again level off (as shown in Figure 1). It should be noted that allosteric effects could cause non-interfacial sites to undergo a different exchange behavior in the monomer than in the dimer, thereby slightly changing the green curve in Figure 1. When the dimer interface is known, however, these allosteric effects would not affect determination of the dimer dissociation rate. Indeed, these allosteric effects would be evident in the measured exchange patterns at the appropriate sites and would be uniquely revealed by the HDX/MS measurements. Another issue of note is the rare case in which interfacial residues are more dynamic in protein-protein complexes.³⁴ The interfacial residues in such complexes would not be expected to have the exchange pattern shown in red in Figure 1, so the method described here would not be applicable to such systems. It should be re-emphasized, though, that this behavior is very atypical of most protein complex interfaces.

Method Validation by β LG at pH 7.0

To facilitate short reaction time measurements of individual amides present at both interfacial and non-interfacial residues, we employed a top-down ECD approach in a manner similar to that described by others.^{31,35–36} To validate this top-down approach so that we could confidently use it later to study β LG dimer dissociation, we measured the HDX of β LG at pH 7.0 and compared the resulting data to the known secondary structure of this protein.³⁷ As is evident in Figure 2, the amide hydrogens near the N-terminus have less deuterium incorporation in regions with secondary structure or at the dimer interface than in unstructured regions of the protein. For example, deuterium incorporation on the first 10 amide hydrogens is higher than the exchange measured at amides 20 to 26, which are part of a short β strand. It should be noted that ECD of the protein under the method 1 conditions described in the experimental section produced mostly c ions, and so the most reliable exchange information comes from the N-terminal region of the protein that includes one of the β LG interfaces. As will be seen below, information about the C-terminal end of the protein can be obtained with a slight change of solution conditions (i.e. method 2).

HDX of Non-Interfacial Regions at pH 3.5

Having established the reliability of our top-down ECD approach, we then measured HDX exchange of β LG as the dimer dissociated into monomer. Dimer dissociation is known to occur at lower protein concentrations and at pH values below 7. The extent of deuterium incorporation in different regions of the protein was determined from the appropriate product ions as described in experimental section. As examples, Figure 3 shows the deuterium incorporation over time for two non-interfacial regions. In Figure 3a, the average amide deuterium incorporation for residues Gln5, Thr6, and Met7 is relatively fast, which is consistent with the unstructured nature of this protein region. In Figure 3b, the average deuterium incorporation for residues Lys14, Val15, Ala16, and Gly17 is much slower due to

the partially structured α -helix involving Lys14 and Val15. The plots in Figure 3 show the expected exponential curves observed in typical HDX experiments (i.e. green curve in Figure 1).

H/D exchange rates can be obtained by fitting data in Figure 3. From such fits we find that the average exchange at residues Gln5-Met7 (Figure 3a) is $3.2 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$, while the exchange at residues Lys14-Gln17 (Figure 3b) is $1.9 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$. These values are within the range of exchange rates for other similarly structured regions¹⁵ and are also within a factor of 3 of the calculated values of $10.4 \times 10^{-3} \text{ s}^{-1}$ and $7.9 \times 10^{-3} \text{ s}^{-1}$ at pH 3.5.³⁸ A summary of the measured exchange rates for other sites in the N-terminal region of the protein are shown in Table 1. In all cases, the measured exchange rates for other non-interfacial regions are consistent with their structural features. One peculiarity about the data in Table 1 is that Glu45-Glu51 region does not undergo an increase in labeling rate in going from pH 3.5 to 4.0, as occurs with all the other regions and as would be expected based on the increase in the intrinsic rate of exchange at the higher pH. The reason for this lack of increase is unclear, but it could reflect a pH-dependent decrease in the dynamics of the β sheet that is present in this region of the protein.

HDX of Interfacial Regions at pH 3.5

Having shown that non-interfacial regions follow the expected exchange behavior as the dimer dissociates, we next considered the exchange behavior of interfacial regions. The N-terminal interface of the β LG dimer is formed by hydrogen bonds between identical residues (i.e. Asp33, Ala34, and Arg40) in the two monomers [PDB ID: 1BEB]. We predicted that amides near these residues would undergo slower amide exchange in the dimer than in the monomer, and this should be reflected in a positive exponential at short exchange times (i.e. red curve in Figure 1), during which the dimer begins to dissociate. The c_{35} and c_{32} product ions were used to calculate deuterium incorporation at amide sites near this interface. When the data from these product ions are plotted as a function of exchange time, the predicted positive exponential curve is observed (Figure 4). When this data is fit using equations 1, 2, and 3, a dissociation rate of $0.016 \pm 0.004 \text{ s}^{-1}$ is obtained. This value is close to the value of 0.007 s^{-1} that was obtained previously by analytical ultracentrifugation (AUC).²⁸ Our value is slightly higher than the previously reported value, but this reflects the lower ionic strength used in our experiments. Under low pH conditions, the β LG dimer is well known to be destabilized and to dissociate more rapidly at low ionic strengths.^{28–30} The higher dissociation rate constant at low ionic strengths is due to the weaker electrostatic shielding of Asp33 and Arg40 at the interface.

C-terminal Interface

The β LG dimer is also mediated by interactions near the C-terminus that involve His146, Arg148, and Ser150; however, our initial ECD conditions did not yield C-terminal product ions and so were unable to provide information about exchange at this interface. To produce more abundant z product ions that could report on this region, we investigated disulfide bond reduction with TCEP just after quenching the HDX reaction and prior to ESI-MS analysis. This approach is referred to as method 2 in the experimental section and Table 1. Disulfide bond reduction is well-known to improve sequence coverage in top-down sequencing,³¹ and

indeed new C-terminal product ions emerge upon disulfide reduction (see Figure S4 in the SI). Because during method 2 the protein sits for 8 min at pH 2.6 before ESI-MS analysis, we needed to confirm that the protein did not undergo altered amide exchange under these conditions. To do this, we compared the HDX rates for the non-interfacial residues obtained from the first method (i.e. method 1), as described earlier, with method 2, and we find good agreement between the two methods (see Table 1). In addition, we compared the deuterium incorporation at the newly measurable C-terminal residues with the known structural features of the C-terminal region of β LG and also find good agreement (Figure 5). For instance, the extent of deuterium incorporation is lower in the regions containing the α -helical (residues 153–156) and β -strand (147–150) structural features than in unstructured regions. Together these two sets of data indicate that method 2 is a reliable means of measuring deuterium incorporation.

Because method 2 appears to give reliable information about C-terminal residues, we used it to measure exchange at the amides near the C-terminal interface of the dimer. The z_{16} and z_{15} product ions were used to calculate deuterium incorporation at this interface, and upon fitting the data, we obtain a dissociation rate of 0.010 ± 0.003 (Figure 6), which is consistent with both the literature value and the value obtained for the N-terminal region via method 1.

β LG Dimer Dissociation at pH 4.0

To further validate this HDX strategy for measuring dissociation rates, β LG dimer dissociation was studied at pH 4.0 using both methods 1 and 2. Dimer dissociation occurs faster at this pH, but an accurate value at this condition has been difficult to obtain by other methods.²⁸ The increased dissociation rate with such a small change in pH is thought to occur due to differences in the unique electrostatic shielding that occurs in the β LG dimer.²⁸ At lower pH values, anions present in solution better shield the positively-charged sites, effectively bridging otherwise repulsive interactions at the interface. At higher pH values that are closer to the protein's isoelectric point, where the protein's overall charge is smaller, electrostatic calculations reveal that anionic shielding is much less extensive, which effectively accelerates dissociation.

We again measured the exchange at both non-interfacial and interfacial residues using methods 1 and 2. For the non-interfacial residues, the expected negative exponential curves were again observed (i.e. green curve in Figure 1). The exchange rates at each of the previously measured sites were also consistent with the protein's known structural features (Table 1). Moreover, the HDX rates were generally higher than at pH 3.5, which reflects the faster intrinsic exchange that occurs at higher pH values. By fitting the deuterium incorporation at the interfacial residues, which again show the predicted behavior (i.e. red curve in Figure 1), we obtain dissociation rates of $0.52 \pm 0.03 \text{ s}^{-1}$ and $0.37 \pm 0.03 \text{ s}^{-1}$ for the N- and C-terminal interfaces, respectively, upon dissociation at pH 4.0. While previous studies were unable to provide an accurate β LG dissociation rate constant at this pH, the value was estimated to be larger than 0.1 s^{-1} , which is consistent with our measured values, especially considering the lower ionic strengths used in our experiments. Interestingly, differences in the dissociation rates for the two interfaces may reflect the fact that the protein dissociates in an asymmetric manner with the N-terminus dissociating faster than the C-

terminus. A similar result was observed at pH 3.5. Even though we cannot independently confirm such an asymmetric dissociation process at this point, this information may be a unique feature of HDX/MS measurements.

It is useful to think about the range of dissociation rates that could be accessed by this HDX strategy, and the new information that could be obtained by this method. On the upper end, our method should be able to measure dissociation rates around 1 s^{-1} , especially when a commercial HDX apparatus and a top-down approach are used. We estimate that the lower limit of our method would be around $1 \times 10^{-4} \text{ s}^{-1}$. Slower dissociation rates would necessitate longer HDX times that would result in complete deuteration of most protein structural elements, thereby limiting the distinctive exchange characteristics of the interfacial regions. Because we can obtain site-specific information about exchange rates, our approach could provide mechanistic insight into how a protein dissociates. For example, if one part of a protein interface dissociates before another, this would be revealed in the HDX rates of the two different sites. Moreover, the MS-based readout should allow this method to be used in complex mixtures.

CONCLUSIONS

We have developed an HDX-based method to determine dimer dissociation kinetics. The method exploits the differential rate of exchange undergone by interfacial residues in the dimer and monomer of a given protein. To test this method, we studied the dissociation of the β LG dimer, which is known to undergo a pH-dependent change in its dissociation kinetics. Our results are in good agreement with the AUC-measured dissociation kinetics of the β LG dimer at pH 3.5. Moreover, our method is able to provide a more accurate measure of the dimer dissociation kinetics at pH 4.0, a pH at which the dissociation rates are too fast to measure by AUC. This latter point highlights one of the key advantages of our approach; a wide range of dissociation rates can be studied by this HDX/MS method. We speculate that our method should be able to study complexes with dissociation rates that range from $1 \times 10^{-4} \text{ s}^{-1}$ to $\sim 1 \text{ s}^{-1}$. Another advantage of this HDX/MS approach is that it has the potential to characterize protein oligomer structure while at the same time providing kinetic information. Interfacial residues are predicted to undergo a different pattern of exchange than non-interfacial residues, and thus finding protein regions with the appropriate exchange signatures could elucidate binding sites. While we have demonstrated this approach for a protein-protein complex, the same method should be suitable for protein-ligand complexes, even those with stoichiometries that are different than 1 to 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Predicted behavior for HDX of interfacial and non-interfacial regions

Figure 2. βLG HDX pattern at pH 7.0. Secondary structure features are shown as an arrow for β strands, as a cylinder for α-helices, and a straight line for unstructured regions. The deuterium incorporation of each residue was normalized to the residue that gave the greatest extent of exchange after 60 sec. The orange lines represent the location of N-terminal interface of the βLG dimer.

Figure 3.

Deuterium incorporation at selected non-interfacial regions. a) Average amide deuteration of residues Gln5, Thr6, and Met7 as obtained from the difference in deuterium incorporation between the c_7 and c_4 product ions. b) Average amide deuteration of Lys14, Val15, Ala16, and Gly17 as obtained from the difference in deuterium incorporation between the c_{17}^{3+} and c_{13}^{2+} product ions. Figure S2 in the SI illustrates how the average deuterium content per residue is determined from the product ions.

Figure 4. Deuterium incorporation at the N-terminal interface. The average amide deuteration of residues Asp33, Ala34, and Gln35 are obtained from the difference in deuterium incorporation between the c_{35}^{3+} and c_{32}^{2+} product ions.

Figure 5. β LG HDX pattern at pH 3.5 using method 2. Secondary structure features are shown as an arrow for β strands, as a cylinder for α -helices, and a straight line for unstructured regions. The deuterium incorporation of each residue was normalized to the residue that gave the greatest extent of exchange after 75 sec. The orange framed β strand represents the location of C-terminal interface of the β LG dimer.

Figure 6. Deuterium incorporation at the C-terminal interface. The amide deuteration of Ile147 was obtained from the difference in deuterium incorporation between the z_{16}^{2+} and z_{15}^{2+} product ions.

Table 1

HDX rates for non-interfacial regions at different pH values.

	Method 1 pH 3.5 ($\times 10^{-3} \text{ s}^{-1}$)	Method 2 pH 3.5 ($\times 10^{-3} \text{ s}^{-1}$)	Method 1 pH 4 ($\times 10^{-3} \text{ s}^{-1}$)	Method 2 pH 4 ($\times 10^{-3} \text{ s}^{-1}$)
Leu1-Thr4	5.4 ± 0.4	3.4 ± 0.2	9.6 ± 1.4	5.1 ± 1.0
Gln5-Met7	3.2 ± 0.3	2.9 ± 0.5	7.4 ± 1.0	3.9 ± 0.5
Lys8-Gln13	5.2 ± 0.6	5.2 ± 0.8	9.3 ± 1.2	10.2 ± 2.2
Lys14-Gly17	1.9 ± 0.2	3.6 ± 0.4	2.3 ± 0.5	5.2 ± 1.3
Thr18-Ala23	0.9 ± 0.1	1.0 ± 0.4	1.6 ± 0.3	2.5 ± 0.8
Met24-Ser27	0.5 ± 0.1	1.7 ± 0.5	1.2 ± 0.2	2.8 ± 0.4
Asp28-Leu32	1.2 ± 0.4	N.D. ^a	5.2 ± 0.6	N.D. ^a
Glu45-Glu51	3.7 ± 0.5	N.D. ^a	3.5 ± 0.8	N.D. ^a

^a N.D. indicates that these product ions have ion intensities that are too low to accurately determine HDX rates.