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Conformational lability in the class II MHC 3\(_{10}\) helix and adjacent extended strand dictate HLA-DM susceptibility and peptide exchange

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HLA-DM (DM) facilitates peptide exchange on class II MHC (MHC II) proteins, and is required for efficient peptide loading in vivo (1). MHC II molecules assemble in the endoplasmic reticulum with the class II-associated invariant chain chaperone, which is subsequently cleaved by endosomal proteases leaving a short fragment known as CLIP bound in the MHC peptide-binding groove (2). DM facilitates the exchange of CLIP for peptides generated by digestion of endogenous and exogenous proteins, resulting in a library of peptide antigens bound to MHC II proteins that are transported to the cell surface (3, 4). In vitro experiments have corroborated the roles for DM as a peptide-exchange factor (5, 6) and as a molecular chaperone that prevents peptide-free MHC II molecules from becoming inactive and from forming aggregates (7, 8).

The mechanism by which DM mediates these effects has received much attention (7–17). Peptides are released by DM with different rates (18), with DM susceptibility a major factor in whether or not a particular peptide is recognized by the cellular immune system (19–21). Understanding the mechanism of DM-mediated peptide release would promote efforts to predict immunogenicity of known and emerging pathogens. Moreover, the mechanism involves catalysis of a protein conformational change (16), and its elucidation would have implications for our understanding of protein-folding processes. However, despite intensive investigation and crystal structures of both MHC II and DM proteins, the mechanism of DM-facilitated MHC-peptide binding and exchange is not understood. Part of the difficulty in developing an understanding of the interaction of DM and MHC II may be because of the presence of multiple conformers of DM (22), as well as MHC II (10, 23). Previous studies using directed screens (11, 14) and tethering approaches (24, 25) have identified residues from both DM and MHC II that are critical for the functional interaction. These residues include a cluster of acidic residues on DM (14) and several residues in the vicinity of the P1 pocket of MHC II (9, 11, 15). Models for the DM–MHC II complex that place these regions in apposition have been proposed (11, 26). However, structural characterization of the DM–MHC II complex, dynamic changes that occur as a result of the interaction, and how formation of the complex facilitates peptide exchange have not been described.

In part because of the paucity of structural information, models for the interaction of DM with MHC II have been controversial. Disruption of the MHC-peptide H-bond network was put forth as an attractive target for DM because it allows for a peptide sequence-independent exchange mechanism (6). In this type of model, DM-induced disruption of one or more of the ~16 conserved H-bonds between the peptide backbone and conserved MHC II main-chain and side-chain atoms results in catalyzed peptide exchange (13, 15). However, mutagenesis of the MHC II side chains involved in these interactions have shown for the most part that disruption of individual H-bonds does not diminish DM catalysis (15). Other models include roles for conformational heterogeneity and involvement of peptide side-chain interactions. One study suggests that DM recognizes a flexible conformation of MHC II with alterations of the P1 pocket (10), and another recent study has suggested that spontaneous release of the peptide from the P1 pocket is a prerequisite to DM interaction (9). In contrast, a study of many MHC II and peptide variants suggests that interactions along the entire peptide-binding groove contribute to the susceptibility of DM (18), and it has also been suggested that DM functions by reducing the cooperativity of peptide side-chain interactions and mediating peptide exchange by way of a “push-off” mechanism (27).

In this work, we used a newly developed mass spectrometry (MS) approach to establish the relative strength of the individual H-bonds along the backbone of MHC II-bound peptide. Through targeted mutagenesis of MHC II residues in the vicinity of the peptide N terminus and P1 pocket, we identified a point substitution that results in alterations within the H-bonding network, increased sustainability to DM-mediated peptide exchange, and greatly increased DM binding affinity. Structural characterization of the protein revealed an altered MHC II conformation in a molecule contacted at the presumptive DM interaction site, with alterations in the α-subunit 3\(_{10}\) helical region (α45–50) and flanking extended strand region (α51–54). These changes appear to be key structural determinants for DM interaction and peptide exchange.

**Results**

**Measurement of MHC-Peptide H-Bond Strengths Using Hydrogen-Deuterium Exchange MS.** The MHC II-peptide H-bonds are believed to be of fundamental importance to the MHC II-peptide


Author contributions: C.A.P. and L.J.S. designed research; C.A.P., M.P.N., and Z.Z-R. performed research; K.A.K. and J.E.E. contributed new reagents/analytic tools; C.A.P. and L.J.S. analyzed data; and C.A.P. and L.J.S. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 3QXA and 3QXD).

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interaction, but their role in DM-mediated catalysis has been controversial (12, 13, 17, 18), in part because of a lack of methods to examine the strength of these bonds directly. We devised an experimental protocol that uses amide hydrogen-deuterium exchange (HDx) and MS to examine the strengths of the MHC II-peptide H-bonds in native, unlabeled MHC II-peptide complexes. Amide hydrogens exchange with solvent on a time scale of millisecond to hours, with the exchange rate depending on the strength of the amide H-bond (28). As outlined in Fig. 1A, we stripped the peptide from the MHC II under conditions that preserve the pattern of amide H/D present in the MHC-peptide complex, and used high-resolution linear trap quadrupole HPLC/MS to evaluate HDx in MHC II-peptide amide bonds by direct examination of the released peptide.

For the free HA peptide in H2O solution, the average mass (1,503.8) and the stable isotope ion distribution pattern (Fig. 1B, Left) correspond with that expected for the HA peptide sequence and the known isotope abundances. For the free HA peptide transferred to 90% D2O solution, the average mass increases to 1,511.9, with changes in the mass spectrum (Fig. 1B, Center), corresponding to addition of 12 D, after accounting for back-exchange during the analysis (SI Appendix, Fig. S1), as expected for the 12 amide bonds in the HA peptide. For the MHC II-HA complex immediately after transfer to 90% D2O, the average mass is 1,504.6 (Fig. 1B, Right). Thus, interaction with the MHC II protein protects ~90% of the peptide amides from exchange.

With increasing time in D2O, additional H/D exchange is observed, with multiphasic kinetics (Fig. 1C, black line). The time-dependent H/D exchange kinetic is fit well by an exponential model (19) that describes a fast phase (~1/2 < 1 min), a fast phase with half-time of a few minutes, and a much longer slow phase with a half-time of ~18 h (SI Appendix, Table S1). The slow phase is still substantially faster than the rate of peptide release (Fig. 1D). Overall, this analysis reveals that the bound peptide’s amide NH bonds exchange at dramatically different rates.

We were able to assign several individual peptide amides to particular kinetic phases using multidimensional electron transfer dissociation (ETD) MS (29). In ETD, peptides are fragmented between NH and CO groups (Fig. 2A), yielding c-series and z-series ions (Fig. 2B), analogous to the b-series and y-series of conventional collision-induced dissociation (29). Unlike collision-induced dissociation, however, ETD does not result in H/D scrambling (30), so that the ion fragments retain the pattern of deuterium incorporation present in the eluted peptides. Peptide fragments exhibit different kinetics of deuterium incorporation, depending on the protection of the particular amide NH atoms present in that fragment. For example, the c3 fragment has very little deuterium incorporation, even at 512 min, whereas the c6 fragment is substantially exchanged by 2 min (Fig. 2C). We were able to measure deuterium incorporation rates for six c-series ions (c2, c3, c5, c6, c8, and c9) and seven z-series ions (z4, z5, z7, z9, z10, z11, and z12), although with weaker signals for the z-series (complete analysis in SI Appendix, Table S2). By subtracting the relative differences (∆MW) of adjacent c-series (or z-series) ions, the contributions of particular peptide NH atoms (or pairs of NH atoms) can be obtained. Fig. 2D shows such a ∆∆MW analysis, with the extracted H/D exchange of each peptide NH atom (or pairs of NH atoms) at various times shown in open bars and the fully exchanged mass difference shown in black bars. Corresponding z-series data are shown in SI Appendix, Fig. S2A, with an overall summary of both c-series and z-series data in SI Appendix, Fig. S3. It is readily apparent that the V4 position is the most strongly protected of all of the peptide NH, indicating that this residue participates in the strongest H-bonding interaction. The next strongest H-bonds emanate from the Y3 and N7 positions. The V4 and Y3 positions flank the p1 side-chain binding pocket. Thus, it would be highly unlikely for the p1 side chain to spontaneously escape the P1 pocket, as recently proposed (9), and so we sought other explanations for the key role of this region in DM-mediated peptide exchange catalysis.

Substitution of αF54 Dramatically Increases Susceptibility of MHC II to DM-Catalyzed Peptide Dissociation. To determine the relevance of the region proximal to the P1 pocket for DM catalyzed peptide dissociation, we engineered mutations in the extended strand region of the α-subunit at residues αF51, αS53, and αF54, and surrounding the P1 pocket, at residues αL45, αF48, βF89, and βW153 (Fig. 3A, and SI Appendix, Fig. S4 and Table S5). As a control, we also mutated αQ57, located on the α-subunit helix one turn beyond the extended strand region. We monitored the intrinsic rate of peptide dissociation from the mutant proteins using Alexa488-labeled CLIP peptide in a fluorescence polarization assay (Fig. 3B–G, blue triangles, and SI Appendix, Fig. S4 B–E). Dissociation time courses fit well to single exponential decays, with t1/2 and α values shown in SI Appendix, Table S1, ranging from a 9.5-fold increase in dissociation rate for αF54C to a 1.9-fold decrease for αS53A.

We tested the susceptibility of the MHC II mutant proteins to DM in a functional assay (Fig. 3B–G and SI Appendix, Fig. S4 B–E). In the range tested, peptide dissociation rate constants increased linearly with increasing DM concentration for each of the mutant proteins (Fig. 3H and SI Appendix, Fig. S5), with the slopes reflecting the susceptibility of each mutant protein to DM-mediated peptide dissociation. As previously observed in the context of another MHC II protein (11), substitution of αF51 resulted in a protein that was resistant to DM (Fig. 3E). The DM-susceptibility of the control mutant αQ57C and P1 pocket mutants αL45A, αF48A, βF89A, and βW153A were similar to WT, whereas αS53A was approximately sevenfold more sensitive to DM than WT (Fig. 3B–G and SI Appendix, Fig. S4 B–E). In contrast, αF54C and
αF54A were exquisitely sensitive to DM, with concentrations as low as 30 nM DM, inducing large increases in peptide dissociation rates (Fig. 3 D and G). Values for the slopes of the DM-dependent rate profiles (i.e., the DM susceptibility) were 302- and 378-times greater for αF54C and αF54A, respectively, compared with WT (Fig. 3H and SI Appendix, Table S3).

Previous studies have used the specific rate enhancement (i.e., the slope of the DM susceptibility curve divided by the intrinsic dissociation rate constant) as a measure of DM’s catalytic efficacy toward a particular substrate (6, 15). Even after this normalization, the αF54 mutations still are extreme outliers, with specific rate enhancements over 400 μM⁻¹ for αF54A, almost 90-times greater than for WT (Fig. 3F, open bars, and J, and SI Appendix, Fig. S6).

Substitution of αF54 Weakens MHC-Peptide H-Bonding Interactions. We determined the effect of the αF54C mutation on the overall H-bond network by HDx MS/MS. The amplitudes of the unresolved initial HDx phases are the same for both WT and αF54C (Fig. 1C). The fast-phase amplitudes also are similar for WT and αF54C, as they were also observed for the HA peptide (Fig. 3F, open bars, and J, and SI Appendix, Fig. S6).

Figs. S2, S3, and S7). For the WT-HA complex, these H-bonds represent the most stable of the interactions, and would be expected to correspond to those most important in determining the overall peptide-MHC lifetime. Our results suggest that the αF54C mutation disrupts these key H-bonds in the N-terminal half of the peptide-bonding groove.

Substitution of αF54 Dramatically Increases Binding to DM. To determine whether the increased DM susceptibility observed upon substitution of αF54 was associated with increased binding affinity for DM, we used a surface plasmon resonance binding assay. Specific, saturable, dose-dependent binding was detected for both αF54 variants but not for WT, αS53A, αF51A, or any of the other mutants (Fig. 4 A–F and SI Appendix, Fig. S4 F–I). Equilibrium binding analysis revealed values of 0.5 μM and 0.7 μM for αF54A and αF54C, respectively (Fig. 4G). We performed several experiments to verify the specificity of the tight binding observed for αF54A and αF54C to DM. First, we observed substantially reduced binding of DM to immobilized αF54C at pH 7.0 relative to pH 5.6 (Fig. 4H), consistent with the known pH dependence of the interaction (31). Second, binding was abrogated by preincubation with a monoclonal antibody (LB3.1) specific for a conformational epitope on MHC II (32) near the presumptive DM binding interface (11) (SI Appendix, Fig. S8 A–D). Finally, DM binding was observed also for CLIP peptide complexes of αF54 but not WT (SI Appendix, Fig. S8 B–D). These results indicate that αF54 substitutions, which increase sensitivity to DM-mediated peptide release, also increase the binding affinity for DM.
Crystal Structure of αF54C-CLIP Shows a Conformational Change in the Vicinity of a Critical DM Contact Residue. To investigate the structural basis for the altered H-bond strengths, increased DM susceptibility, and increased DM binding activity of αF54C, we determined its 2.3 Å crystal structure in complex with the CLIP peptide, and for comparison the 2.7 Å crystal structure of WT bound to CLIP, in the same unit cell (SI Appendix, Table S4). Both crystal structures have two molecules in the asymmetric unit. For WT-CLIP structure, the molecules (blue and green in Fig. 5 and SI Appendix, Fig. S9 A–E) overlay with no major deviations (except for an exposed loop near βP108 that shows large rmsd between most MHC II structures solved to date). The structure of αF54C (purple and red in Fig. 5 and SI Appendix, Fig. S9 A–E) overall was very similar to WT except for a region proximal to the αF54C mutation (indicated by carets in SI Appendix, Fig. S9 B and F), for which large differences were observed specifically in one of the two molecules in the asymmetric unit (SI Appendix, Fig. S9 F–I).

Fig. 3. Substitution of αF54 results in a dramatic increase in the rate of DM catalyzed peptide exchange. (A) Location of mutated residues in MHC peptide-binding groove (additional mutations shown in SI Appendix, Fig S4). (B–G) Peptide release kinetics in the presence of various concentrations of DM. (H) The k_{on} values plotted against the concentration of DM. (Inset) Shows WT, αQ57C, and αS53A with expanded y axis. (I) DM-mediated rate enhancements were calculated for each mutant by dividing the slope of the k_{on} vs. [DM] plots by the intrinsic dissociation rates. Closed bars, MHC II-CLIP. Open bars, MHC II-HA. (J) HA k_{on} values plotted against the concentration of DM.

Fig. 4. Surface plasmon resonance analysis of αF54CαF54A binding to DM. (A–F) Various concentrations of DM were flowed over immobilized WT and mutant MHC II. (G) Equilibrium analysis of DM binding to immobilized αF54C (circles) and αF54A (triangles), with Scatchard plot shown as an inset. (H and I) pH Dependence, showing DM (10 μM) injected over immobilized αF54C (H) or WT (I) at pH 7.0, solid line, or at pH 5.6, dotted line.

The conformational changes, which involve a loop between two strands of the β-sheet platform, the short 3₁₀ helix encompassing residues αL45 to αF50 at the edge of the binding site, and the extended strand region αF51 to αF54, can be seen in Fig. 5. The conformational change results in a ~20° reorientation of the short 3₁₀ helix, together with a partial unwinding toward a more canonical α-helical pitch (compare Fig. 5 B and C). These changes are accompanied by a concerted set of rotamer changes in residues that surround the P1 pocket. Large changes are observed for αF48, which positions its side chain on the side of the P1 pocket, displacing βF89, which also undergoes a rotamer change, and for αL45, which moves to contact βW153, replacing the contact normally made by the αF48 side chain. The rearrangements of αL45, αF48, and βF89 resulted in changes in the shape and volume of the P1 pocket (SI Appendix, Fig. S10).

The conformational changes in the 3₁₀ helix and adjacent strand region appear to be induced by contact at the αF51 position in molecule 2 of the asymmetric unit with another molecule in the crystal (SI Appendix, Fig. S11). The contact is observed for both WT and αF54C, but only in the mutant is the conformational change induced. These changes result in lowered B-factors at the contact site relative to the remainder of the αβ1 peptide-binding domain, but increased B-factors for the bound peptide (SI Appendix, Fig. S12). The αF51 position is known to be a critical determinant for DM binding and activity (11), and contact with another molecule at the αF51 position may simulate the effect of a contact at this position with DM.

Discussion

The mechanism of DM-mediated catalysis of peptide exchange on MHC II proteins and the nature of the DM-MHC interaction has been the subject of intensive investigation (6, 9–18, 24, 27). In this work, we evaluated the contribution of MHC II structural elements at the N-terminal side of the peptide binding site, using HDx MS to measure MHC-peptide H-bonding strengths, site-specific mutagenesis to identify MHC II side chains with important contributions to DM binding and facilitated peptide exchange, and crystal structure analysis of the HLA-DR1 mutant αF54C. Substitution of αF54 in the MHC II extended strand region of the peptide binding site results in a protein with greatly increased susceptibility to DM-mediated peptide release, increased DM binding affinity, and increased MHC II-peptide dynamics, particularly in the MHC II-peptide H-bonding interactions that surround the P1 pocket. The crystal structure of αF54C reveals conformational alterations near the N terminus of the bound peptide involving the reorientation of the 3₁₀ helical region (α45–
50) and changes in the flanking extended strand regions (α39–44 and α51–54); adoption of the altered conformation is dependent on contact of αF51, a known DM-MHC II contact site (11). The extended strand region α51–54 is usually anchored by interaction of the αF54 side chain with a hydrophobic cluster on the side of the binding groove. Replacement of the αF54 side chain by cysteine or alanine would weaken this interaction, and facilitate adoption of an alternate conformation that favors DM binding.

Several factors indicate that the conformation observed in the αF54C mutant can be adopted by the WT and is relevant to DM-mediated peptide exchange. Modeling studies of MHC II suggest that the N-terminal side of the peptide binding site undergoes conformational alteration concurrent with peptide release (32–34). In a molecular dynamics simulation of peptide-free MHC II (32), we observed changes strikingly similar to those observed in the αF54C structure determined here, in particular, a change in pitch and partial unwinding of the 3_10 helix and concerted rotamer changes of αF51, αF45, αF48, and βF89 (SI Appendix, Fig. S13). The P1 pocket, a locus of conformational change in molecule 2 of the αF54C crystal structure, is known as a key determinant of MHC II-peptide interaction (35) and has long been proposed by numerous groups to be involved in DM catalyzed peptide exchange (9, 10, 13, 14, 25, 26, 36). Finally, the α39–44 strand, which also rearranges in molecule 2 of the αF54C structure, carries two other residues in addition to αF51 implicated in DM-MHC II contact: αE39, which was identified in a random screen as a residue important in mediating DM’s functional effect (11); and αW43, which was identified by mutagenesis as important in DM-DR binding (9). Thus, the altered conformation observed in the crystal structure of molecule 2 of αF54C provides a structural explanation for the involvement of the regions previously implicated in DM-mediated catalysis.

A model for how the altered conformation observed for αF54C might function as an intermediate state in the DM-mediated peptide release pathway is shown in Fig. 6. In this model, interaction of the MHC II-peptide complex stabilizes a conformation with decreased MHC II-peptide H-bonding and rearrangements in the crucial P1 pocket (Fig. 6, red). These structural alterations lead to reduced MHC II-peptide binding affinity and facilitated peptide release. DM interaction with this intermediate form persists until binding by a peptide with affinity sufficient to shift the equilibrium back toward the original MHC II-peptide conformation with release of DM (Fig. 6, blue). If an exchange peptide were not available, DM dissociation would lead to formation of the peptide-refractive empty conformation (Fig. 6, orange). Computational studies suggest that the conformation of the peptide-free protein has alterations near the P1 pocket as well as elsewhere in the binding groove (32–34), with the α-subunit extended strand region occupying part of the canonical peptide binding site (32), creating a peptide refractive state. In the model shown in Fig. 6, association with DM would reverse these interactions, providing a structural explanation for DM’s chaperone function. It is possible that the F54C mutation has stabilized the peptide-receptive conformation by reducing the interaction of the strand region with the P1 pocket, an interaction proposed to be mediated by F54 engagement in the P1 pocket (32). Recently, a model has been put forward for the DM-mediated peptide release mechanism based on DM binding of HLA-DR2 variants, in which spontaneous release of peptide from the P1 pocket facilitates DM interaction (9). The hydrogen-deuterium experiments reported here argue against such a model, because they demonstrate displacement of the αF54C CLIP complex protected in hanging drops over 12% PEG 4000, 100 mM sodium acetate (pH 5.6), 100 mM NaCl, 50 mM NaAc, 0.1% octyl glucoside, 5 mM EDTA, and 1 mM PMFS. Intrinsically and DM-mediated dissociation was monitored in triplicate by fluorescence polarization (488-nm excitation, 520-nm emission) and addition of 100-fold excess unlabeled HA peptide. Dissociation rate constants were determined by fitting to a single phase exponential decay. The rate enhancement by DM was calculated as the slope of the linear rate vs. DM concentration plot, and the DM-enhancement factor calculated as this slope divided by the intrinsic (i.e., no DM) rate constant (6). Similar values were obtained regardless of whether intrinsic dissociation rates were determined by experiments performed in the absence of DM or by extrapolation of DM-dependent rate profiles.

Surface Plasmon Resonance. Surface plasmon resonance experiments were performed in the absence of DM or by extrapolation of DM-dependent rate profiles. Protein Production. The extracellular domains of wild-type and mutant HLA-DR1 α- and β-chains were individually expressed in Escherichia coli and folded in vitro as previously described (37). Soluble DM molecules secreted from stably transfected Drosophila S2 cells were isolated by immunoaffinity chromatography. HLA-DR1 and DM were further purified by size exclusion chromatography before use (Superdex 200).

Polarization Assays. N-Terminal acetylated HA (Ac-PFKREVRNLTDLAT-OH) and CLIP (Ac-VSKRMRATPML IQ-OH) peptides (21st Century Biochemicals) were labeled with Alexa-488 tetrafluorophenyl ester (Molecular Probes) at position K2 (HA) or K3 (CLIP). Peptide complexes were prepared by incubation of WT or mutant MHC II (150 nM) with 25 nM labeled peptide for 3 d at 37 °C in 96-well low protein-binding black polystyrene assay plates (Corning) in 200 μL binding buffer (100 mM Na acetate, 50 mM NaCl, 0.1% octyl/glycoside, 5 mM EDTA, and 1 mM PMSF). Intrinsically and DM-mediated dissociation was monitored in triplicate by fluorescence polarization (488-nm excitation, 520-nm emission) and addition of 100-fold excess unlabeled HA peptide. Dissociation rate constants were determined by fitting to a single phase exponential decay. The rate enhancement by DM was calculated as the slope of the linear rate vs. DM concentration plot, and the DM-enhancement factor calculated as this slope divided by the intrinsic (i.e., no DM) rate constant (6). Similar values were obtained regardless of whether intrinsic dissociation rates were determined by experiments performed in the absence of DM or by extrapolation of DM-dependent rate profiles.
crystals (WT: 0.1 x 0.05 x 0.05, F54C: 0.6 x 0.07 x 0.07 mm) using an ADSQ Q315 detector were processed with HKL2000. Initial phasing was obtained by molecular replacement using Phaser (38) with coordinates from another HLA-DR1 structure (PDB code: 1PWY) as a search model. Multiple rounds of refinement and building were carried out using Phenix (39) and COOT (40). PDB ID codes are 3QXD (F54C-CLIP) and 3QXA (WT-CLIP).

H/D Exchange. WT and ΔF54C loaded with HA peptide were transferred to PBS pH 7.2 and concentrated to 55 μM. To initiate H/D exchange, MHC II-peptide complexes or HA peptide alone were diluted 1:10 into PBS in D2O, followed by incubation at 25 °C. At each time interval we quenched the reaction by diluting the exchange mixture 1:10 into 1 M glycine pH 2.7 at 4 °C and flash-freezing at −40 °C. All components of the LCMS instrument (Thermo Scientific) in contact with the sample were chilled to 4 °C before data collection. Samples were diluted twofold in ice cold buffer A (0.1% TFA in 2% ACN) immediately before injection (50 μL/min) onto 1 x 15-mm C4 PepMap300 and C18 PepMap100 columns in the presence of HPLC. With transfer to 25% ACN the MHC II is retained and the peptide is released for infusion into the mass spectrometer, using capillary temperature 275 °C and source voltage 4.5 kV. Spectra were acquired from m/z 450–1,600 with a time resolution of 1 s. Average m/z values were calculated by summing each peak in the isotope distribution weighted by its relative abundance, and converted to fractional H/D exchange by reference to the values for unexchanged free peptide in the same experiment. Fractional H/D exchange versus time curves were fit to a two-phase exponential equation.

The expected isotope distribution for HA peptide was calculated from the atomic formula (C$_{60}$H$_{111}$N$_{24}$O$_{17}$S). For calculation of the expected mass distribution under various conditions, the probability of H/D exchange at each of the 12 peptide main chain amide hydrogens was estimated using a binomial equation, which assumes equal probability of exchange at each position:

$$P(k) = \frac{n!}{k!(n-k)!} \times P^k \times (1-P)^{(n-k)}$$

where $n = 12$ for the 12 amide positions, $p = 0.9$ for exchange into 90% D$_2$O, and $k$ is number of hydrogens exchanged.

**Electron Transfer-Induced Dissociation Mass.** Peptides eluted as described above were introduced into an amaZon-ETD quadrupole ion-trap instrument (Bruker Daltonics) operating in positive ion mode and subjected to ETD fragmentation. ETD spectra were processed and assigned to c- and z-series ions using Bruker Daltonics Data Analysis and BioTools software. Average peptide fragment masses observed at various times in D$_2$O were compared with H$_2$O values to determine HDX-ΔMW. Sequential c-series or z-series ions were subtracted to give ΔΔMW values, which represent the amount of deuterium incorporation per amide (or pairs of amides) at each time point.

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