

# Study of the Binding of MHC Class I Molecule and Antigenic Peptide Complex by H/D Exchange and HPLC MS/MS

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Molecular recognition is fundamental to both humoral and cellular immunity. In an adaptive immune response, an antigen is recognized by two distinct sets of receptor molecules: the antibodies of B cells and the antigen-specific receptors of T cells. T-cell receptors detect the antigen by recognizing the peptide bound in a major histocompatibility complex (MHC). The MHC binds peptide fragments derived from protein antigens and delivers them to the cell surface where the recognition takes place.

There are two classes of MHC molecules, MHC class I and MHC class II. MHC class I molecules bind antigenic peptides from antigens in cytoplasm, and present them to CD8 T cells, leading to ultimate destruction of the infected cells by CD8 T cells. MHC class I molecules consist of two polypeptide chains, a heavy chain and a smaller, non covalently associated chain. A cleft on the surface of the MHC molecule is created in the binding, and that cleft is the site of peptide binding.

Because MHC class I molecule-peptide complexes are important in triggering an immune response, we are interested in probing the solution structure and binding of these complexes. X-ray crystallography has been used to determine the structures of some MHC class I molecule-peptide complexes [1], but these crystal structure don't reflect the dynamics of complexes in solution. Multi-dimensional NMR is useful for solution structure, but it is generally limited to small proteins with M.W. < 20 kDa. The molecular weight of the MHC class I molecule is greater than 40 kDa, explaining why no NMR structure is available. Another method that shows high promise for probing protein structure in solution is backbone amide hydrogen exchange followed by mass spectrometry. We report here the application of H/D exchange as measured by HPLC/MS/MS combined with a new kinetic model to determine the properties of an MHC class I protein-peptide complex.

An MHC class I molecule (kbm8-b2m) and an antigenic peptide (Hsv-8) were chosen for our method development study. Kbm8-b2m and Hsv-8 were incubated in a D<sub>2</sub>O buffer for 3 days to exchange the amides of the complex. After incubation, the solution was concentrated to 2 mL using a 30-kDa cutoff membrane filter to eliminate any non-bound peptides. This 2-mL solution was further purified by using a size exclusion column with 20-mM HEPES, 150-mM NaCl and 0.01% NaAzide/H<sub>2</sub>O buffer. Fractions with molecular weight corresponding to the complexes were concentrated to 1 mL using the 30-kDa cut-off membrane filter to a concentration of approximately 26 μM. 200 μL of this 26-μM complex was filtered through a 10-kDa cut-off ultra-filter, concentrating to approximately 10 μL, to which 190 μL of pH 7.4, 10-mM PBS, 135-mM NaCl, 2.7-mM KCl D<sub>2</sub>O buffer was added to form the final 26-μM complex in D<sub>2</sub>O buffer. 2 μL of the 26-μM complex in D<sub>2</sub>O was introduced to 38 μL of H<sub>2</sub>O buffer at room temperature to measure the extent of back-exchange as function of time. The final concentration of the complex after H/D back exchange was 1.3 μM.

The back exchange was quenched by adding formic acid solution to decrease the pH to 2.6 and by dropping the temperature to 0 °C. Upon quenching, the complex dissociated to proteins and peptide, but leaving intact the pattern of back exchange of the complex. The 40 μL quenched solution was loaded onto an HPLC MS system, where the injector, pre-column and connecting tubes were submerged in an ice bath to keep temperature at 0 °C. Immediately after loading, 500 μL of acidic aqueous solution at pH 2.6 was injected to desalt, and a fast gradient from 40% to 80% B (A: 0.3% formic acid in H<sub>2</sub>O, B: 0.3% formic acid in ACN) was run in 4 min to elute the Hsv-8 peptide. Tandem MS was performed on the doubly charged peptide ions. The tandem mass spectrum was input to a kinetic model to calculate the amide hydrogen exchange rate of each amide hydrogen of the Hsv-8 peptide.

The complete series of y ions of back exchanged Hsv-8 peptide (SSIEFARL) was produced in the MS/MS experiment. Figure 1 shows an example of the tandem spectrum of the deuterated Hsv-8 peptide. The complete series of y ions are labeled as  $y_1, y_2 \dots$  to  $y_7$ . The C13 contribution to each peak was subtracted to yield the distribution of deuteriums on the peptide. Our kinetic model (to be described elsewhere) incorporates the difference of H/D back exchange between adjoining members of the complete series of y ions starting with  $y_1$ . The difference shows the state of deuteration of the additional amide in going from one member of the series to the next. This kinetic model also reveals the difference of the H/D back exchange for the same y ion at different times (see Figure 2 for an example of  $y_5$ ). We used the kinetic model to calculate the H/D back exchange rate constants for each amide hydrogen on each amino acid on the peptide, assuming there is only one H/D exchangeable site for the starting ion  $y_1$  (Table 1). Experimental results, however, showed that there are at least two, and possibly three exchangeable sites for  $y_1$ . We tested the assumption and found that the model is not sensitive to this assumption).

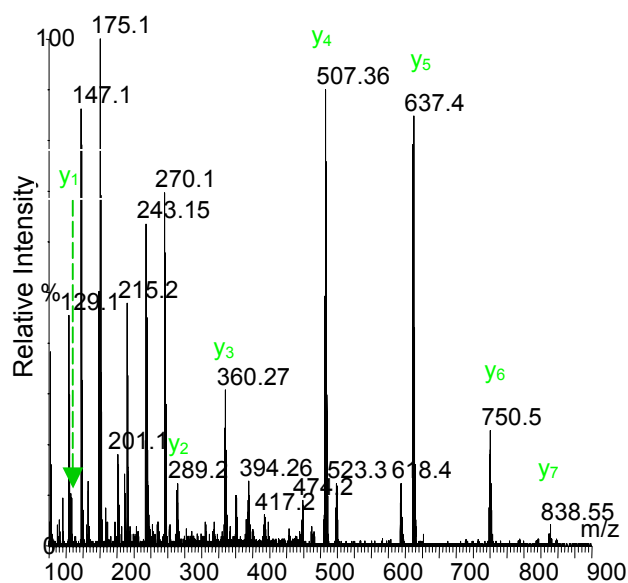


Figure 1. ESI/MS/MS of deuterated peptide

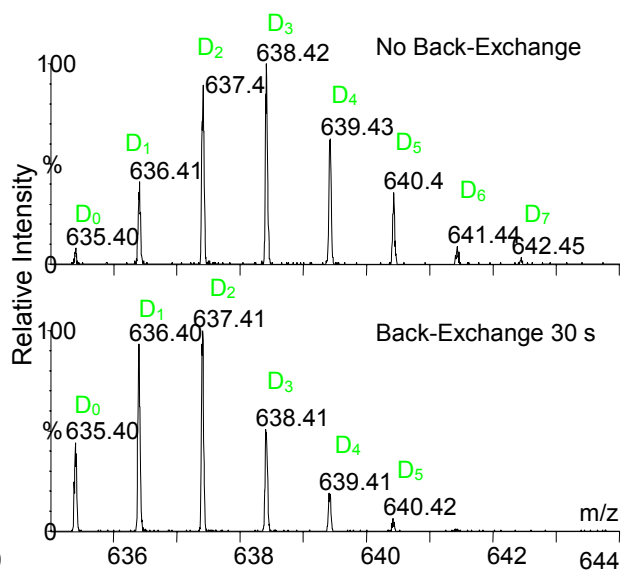


Figure 2. Comparison of  $y_5$  ion at different periods of back exchange

Table 1. The calculated rate constants of H/D exchange on each amino acid of Hsv-8 from kinetic model

	S	I	E	F	A	R	L
Rate Constants	6e-3	1e-1	5e-3	6e-14	8e-2	2e-3	5e-5

The significantly smaller rate constant for H/D back exchange of the phenylalanine amide hydrogen suggests that this amide hydrogen is deeply buried in the binding pocket of the MHC molecule and it is not solvent-accessible compared to the other amides of this peptide. The amide hydrogen on leucine is also protected, but to a lesser extent than that on phenylalanine. We conclude that, in solution, the binding regions of this complex involve principally the amides of phenylalanine and leucine of the peptide. We believe that H/D exchange of the peptide combined with HPLC/MS/MS and kinetic modeling will be a valuable approach to gain insight on the binding of peptide/protein complexes in solution.

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References: .1. Fremont, D.; Matsumura, M.; Stura, E.; Peterson, P., Wilson, I. *Science*. 1992, 257, 919-927.