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Identification of Two Distinct Structural Transitions in the Dissociation of Peptides from Class II Proteins of the Major Histocompatibility Complex

Benjamin J. McFarland,[†] Andrea J. Sant,[‡] and Craig Beeson*,[†]

Department of Chemistry, University of Washington Seattle, Washington 98112
Department of Pathology, University of Chicago Chicago, Illinois 60637

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Major histocompatibility complex (MHC) Class II proteins initiate an immune response by binding a broad range of antigenic peptides that are subsequently presented to T cells.[1] Two kinds of interactions secure the peptides in long-lived complexes: (1) specific interactions between several MHC binding pockets and peptide side chains, and (2) 10−15 hydrogen bonds between MHC side chains and the peptide backbone (Figure 1).[2] The structural complexity of these interactions seems incongruent with the observation that peptides usually dissociate from MHC proteins with monophasic exponential kinetics.[3] The simple kinetics are likely to mask a complex, cooperative process, making it difficult to discern the relative importance of the different binding interactions. Here we report a study of dissociation kinetics for six different peptides that bind to the MHC molecule I-A^d with a range of stabilities. The effects of changing pH and mutation of a single solvent-exposed hydrogen-bond donor on the MHC molecule are compared. Two independent structural transitions during peptide dissociation can be discerned, suggesting that peptide dissociation is minimally described with a preequilibrium kinetic model. These results suggest that even those peptides that dissociate with simple monophasic kinetics do so via at least one kinetic intermediate.

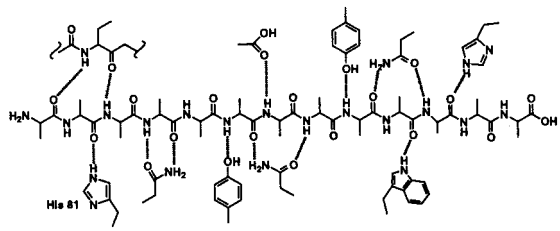

Figure 1. Schematic of the peptide−backbone hydrogen bonds for a peptide bound to I-A^d MHC protein based on the crystal coordinates.[2] The I-A^d His81 residue is labeled. In H81N, this residue is replaced with an Asn, which is too short to form a stable hydrogen bond.

The apparent first-order dissociation rate constants for six peptides with little sequence similarity[4] known to bind I-A^d were determined. Detergent-soluble native I-A^d protein (WT) was purified from transfected L cells.[5] Fluorescein-labeled peptide was incubated at 37 °C with I-A^d until binding was almost maximal and then excess free peptide was removed. The complexes were then incubated at 37 °C and, over time, the amount of bound peptide was measured by a high-performance size-exclusion column connected to a fluorescence detector.[5] Apparent first-order rate constants were obtained from single exponential functions fit to the dissociation data. Peptide dissociation rates were measured at extracellular pH 7.4 and endosomal pH 5.3, representative of the two environments the MHC−peptide complex encounters during antigen processing and presentation. Most peptides dissociate more slowly at pH 7.4 than at pH 5.3, but the magnitude of sensitivity to the pH change is different for each peptide (Table 1).

Disruption of many of the MHC side chains that form a hydrogen bond with the peptide backbone has little or no effect on peptide binding.[6] A notable exception is the mutation of I-A^d β-chain residues that form a hydrogen bond to the peptide's N-terminal region.[7] For example, replacement of histidine-81 of the I-A^d MHC β-chain (His81) with asparagine (H81N) disrupts intracellular MHC trafficking and diminishes peptide stability at the endosomal pH 5.3.[7,8] In X-ray crystal structures of MHC−peptide complexes,[2] the His81 side chain is positioned to form a hydrogen bond with the carbonyl next to the P1 residue (Figure 1). Molecular modeling of peptide−MHC complexes predicts that an Asn at position 81 must adopt a strained, energetically unfavorable rotamer to hydrogen bond to the carbonyl.[7] The effect of disrupting this hydrogen bond in H81N is particularly dramatic given that mutation of the pseudosymmetric histidine-68 of the α-chain of I-A^k has no effect on peptide binding.[2,6] We measured the dissociation kinetics for each of the above peptides using the H81N I-A^d protein at pH 7.4. It was found that the apparent dissociation rate constants were also enhanced for the H81N protein relative to native I-A^d (Table 1). Although the effect of the loss of the His81 hydrogen bond is different for each peptide, for any one peptide, the rate enhancement for the H81N protein relative to native I-A^d is the same at both pH 5.3 and 7.4 (Figure 2).

[†] University of Washington.
[‡] University of Chicago.

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(3) For example, see: Liang, M. N.; Beeson, C.; Mason, K.; McConnell, H. M. *Int. Immunol.* **1995**, *7*, 1397−1404.

(4) Peptide sequences: Cystatin-C 40-55 (CysC), DAYHSRAIQVVRARK.; Influenza Hemagglutinin 126-138 (Ha), HNTNGVTAASSHE.; I-E^k Alpha Chain 52−67 (Ea), ASFEAQGALANIAVDK.; Ovalbumin 323-335 A2 (Ova-W), ISAAVHAAHAEIN; Mouse Invariant Chain 85-99 (mIi), KPVSQMRMATPLLMR; Ovalbumin 326-336 (Ova-A), AVHAAHAEINE. Anchor residues, where known, are underlined. The two Ova motifs represent minimal, independent registers that give stable, monophasic dissociation kinetics.

(5) The purification scheme is described in full in ref 3. Briefly, L cells were transfected with an I-A^d construct to express either native or H81N protein. Protein was purified from cells lysed in detergent, first by affinity chromatography using lentil lectin, then by affinity chromatography using an A^d-specific antibody column (MKD6 or M5114-PGS). Protein was eluted from the antibody at pH 11.5 and immediately neutralized to pH 7.0, then dialyzed into a phosphate-buffered saline solution containing 0.2 mM dodecyl maltoside. Purity was checked with SDS-PAGE. The dissociation kinetics experiments are described in full in ref 3. Briefly, a 50-fold molar excess of fluoresceinated peptide was bound to 100−200 nM MHC at 37 °C for 1−20 h and free peptide removed with a small Sephadex G-50 column, resulting in a dilute sample of the protein−peptide complex (~1 nM). The sample was incubated at 37 °C, and dissociation time points were taken using a 30-cm TosoHaas TSK3000SW$_{XL}$ size exclusion column connected to a fluorimeter set to detect fluorescein with excitation at 495 nm, emission at 525 nm. Fluorescence associated with the MHC protein was measured at a retention volume corresponding to ~60 kDa.

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(9) The structural integrity of the H81N protein has been demonstrated. For instance, the binding of I-A^d-specific antibodies sensitive to peptide−MHC conformation was not affected by H81N. Additionally, mutation of mIi residue 95 from Pro to Ala stabilized peptide dissociation by a similar factor for both I-A^d and H81N. Finally, the observation that the effect of pH on peptide dissociation was not affected by H81N (Table 1) also indicates that the H81N I-A^d maintains its native structure.

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Table 1. Apparent First-Order Rate Constants for Peptide Dissociation from WT and H81N at pH 7.4 and 5.3

peptide	pH	WT k ($\times 10^6$ s^{-1})	H81N k ($\times 10^6$ s^{-1})	factor (k_{H81N}/k_{WT})
CysC	7.4	0.42 ± 0.05	6.2 ± 0.6	15 ± 2
	5.3	1.0 ± 0.2	14 ± 1	14 ± 3
Ha	7.4	1.0 ± 0.2	69 ± 5	70 ± 10
	5.3	4.3 ± 0.2	340 ± 6	79 ± 4
Ea	7.4	1.5 ± 0.2	71± 5	47 ± 6
	5.3	1.6 ± 0.1	100 ± 20	60 ± 10
Ova-W	7.4	2.3 ± 0.3	20 ± 0.6	9 ± 1
	5.3	10 ± 2	69 ± 10	7 ± 1
mIi	7.4	2.5 ± 0.3	490 ± 10	200 ± 20
	5.3	15 ± 2	2500 ± 60	170 ± 20
Ova-A	7.4	8.0 ± 0.3	360 ± 10	45 ± 2
	5.3	8.0 ± 0.1	330 ± 20	41 ± 2

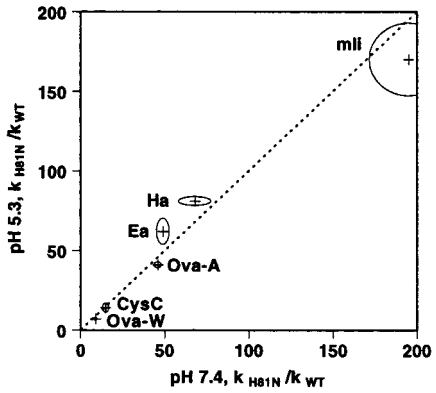

Figure 2. Comparison of the kinetic effect of H81N at pH 7.4 to the effect at pH 5.3. The kinetic effect is obtained by dividing the apparent first-order dissociation rate constants for H81N by the rate constants for WT protein, resulting in the factor by which dissociation is enhanced when the His81 hydrogen bond is disrupted. Individual peptides are labeled. The line has a slope of unity and is provided to guide the eye. The circles define the standard errors based on at least three independent rate measurements.

Since the effects of pH and of the H81N mutation on the rate constants are independent of each other, they must describe separate kinetic steps in the dissociation of peptide. The simplest model of peptide dissociation consistent with these results involves two steps, one sensitive to pH and the other to the His81 hydrogen bond, both occurring at or before the rate-limiting step. This behavior can be accounted for most simply with a preequilibrium model

$$\mathrm{MP} \overset{K}{\rightleftharpoons} (\mathrm{M\ P})_i \overset{k}{\rightarrow} \mathrm{M} + \mathrm{P} \qquad (1)$$

where K is the preequilibrium constant and k is the rate constant for the rate-limiting step. It is assumed here that rebinding of peptide does not occur, as is the case in our experiments.[10] This model has been used previously to describe the reactions of peptides that dissociate from MHC proteins with biphasic kinetics.[11] Here, the preequilibrium model is found to also describe the reactions of peptides that dissociate with monophasic kinetics, implying that most if not all peptides dissociate through at least one kinetic intermediate. This is the simplest possible scenario; another likely scenario is one in which several kinetic intermediates precede the rate-limiting step.

In the simplest case, the apparent rate constant k_{app} is the product of the equilibrium constant K and the rate constant k. The apparent first-order rate constants observed here are readily reproduced with this simple mechanism if the effects of pH and the His81 hydrogen bond are each allowed to affect only one step in the mechanism.[12] The observation that pH and H81N effects are different for each peptide confirms that both structural transitions involved in peptide dissociation are sensitive to peptide sequence (i.e., both K and k are peptide sequence dependent). This argues against previous suggestions that describe the pH-sensitive step as independent of peptide sequence.[13] The observation that both steps are sequence dependent reinforces the concept that the sequential disruption of binding contacts as the peptide exits the binding groove of an MHC protein is similar to a protein unfolding process where each peptide−MHC complex is a unique protein.[14]

The pronounced effect of H81N localizes an important structural event in dissociation to the breaking of the His81 hydrogen bond. The observation that the effect of H81N is independent of the pH effect demonstrates that the structural transition affected by pH is distinct from that involving the His81 hydrogen bond. The fact that both of these structural transitions depend on peptide sequence has biological implications for antigen processing. The assembly of peptide−MHC complexes within cells involves a protein named DM that catalyzes peptide exchange within acidic endosomal compartments.[15] The DM protein binds peptide−MHC complexes and selectively increases the dissociation rate for low-affinity peptides, allowing more stable peptides to occupy the binding groove.[16] It has been suggested that DM either causes a conformational change in the MHC protein or that it stabilizes a transition state in peptide dissociation. The observation that each kinetic step in the dissociation reaction is peptide sequence dependent provides the basis for a mechanistic framework that could describe DM selectivity. A more comprehensive kinetic study of the effects of disrupting other peptide−MHC hydrogen bonds and pocket interactions is in progress. These results should provide a more detailed molecular map of the interrelation of specific structural transitions in peptide dissociation that will ultimately enhance our view of antigen presentation, DM function and, perhaps, protein folding.

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(12) Consider a model in which K is pH dependent and k is His81 dependent and, for illustrative purposes, let $K = 0.10$ at pH 7.4. The observed pH 7.4 dissociation rate constant for the CysC peptide, 4.2×10^{-7} s^{-1}, gives $k = 4.2 \times 10^{-6}$ s^{-1}. Using this value and the observed pH 5.3 dissociation rate for CysC gives $K = 0.24$ at pH 5.3. For the same value of K at pH 7.4, the dissociation of CysC from H81N at pH 7.4 gives $k = 6.3 \times 10^{-5}$ s^{-1} for the intrinsic dissociation rate constant in the absence of the His81 hydrogen bond. Using this value for k and the previously determined $K = 0.24$, one calculates an apparent H81N rate constant of 1.5×10^{-5} s^{-1} for the CysC peptide at pH 5.3, a value that agrees with the experimentally observed rate constant 1.4×10^{-5} s^{-1}.

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