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The Thermodynamic Mechanism of Peptide–MHC Class II Complex Formation Is a Determinant of Susceptibility to HLA-DM

Andrea Ferrante,* Megan Templeton,*† Megan Hoffman,‡ and Margaret J. Castellini‡

Peptides bind MHC class II molecules through a thermodynamically nonadditive process consequent to the flexibility of the reactants. Currently, how the specific outcome of this binding process affects the ensuing epitope selection needs resolution. Calorimetric assessment of binding thermodynamics for hemagglutinin 306–319 peptide variants to the human MHC class II HLA-DR1 (DR1) and a mutant DR1 reveals that peptide/DR1 complexes can be formed with different enthalpic and entropic contributions. Complexes formed with a smaller entropic penalty feature circular dichroism spectra consistent with a non–compact form, and molecular dynamics simulation shows a more flexible structure. The opposite binding mode, compact and less flexible, is associated with greater entropic penalty. These structural variations are associated with rearrangements of residues known to be involved in HLA-DR (DM) binding, affinity of DM for the complex, and complex susceptibility to DM-mediated peptide exchange. Thus, the thermodynamic mechanism of peptide binding to DR1 correlates with the structural rigidity of the complex, and DM mediates peptide exchange by “sensing” flexible complexes in which the aforementioned residues are rearranged at a higher frequency than in more rigid ones. *The Journal of Immunology, 2015, 195: 1251–1261.

Major histocompatibility complex class II (MHCII) molecules are transmembrane heterodimeric proteins expressed on the surface of APCs and are fundamental in initiating or propagating an immune response by presenting antigenic peptides to CD4+ T lymphocytes. Newly synthesized MHCII molecules are transported from the endoplasmic reticulum to the MHCII compartments (MIIC) as multimeric complexes with the chaperone protein invariant chain, which stabilizes the nascent MHCII and prevents the binding of other peptides that are present in the endoplasmic reticulum (1). Upon arrival in the MIIC, the invariant chain molecule is cleaved primarily by cathepsin S (and to a lesser extent by cathepsins L, V, F, and K) (2), leaving a peptide fragment termed CLIP in the MHCII binding groove. For most MHCII alleles, CLIP is released by the action of the nonclassical MHCII molecule HLA-DM (DM) to allow antigenic peptides to bind MHCII (3, 4). The role of DM exchange is not limited to CLIP, as it can catalyze the exchange of antigenic peptides to select for a stable peptide/MHCII (pMHCII) repertoire (5).

The crystal structures of peptide-complexed MHCII molecules have shown that peptide binding relies on interactions between pockets lining the class II groove and side chains of the peptide, and on a series of hydrogen bonds between nonpolymorphic MHCII side chains and the peptide backbone (6). The primary pockets are indicated as P1, P4, P6, and P9, with P1 being the pocket located at the N-terminal side of the complex, and the individual interaction is allele specific owing to the size and the hydrophobicity of the pocket. The encapsulation of bulky hydrophobic side chains of the peptide into the P1 pocket of the human MHCII HLA-DR (DR) is considered a requirement for stable peptide binding (7, 8) and is regarded as a major source of binding energy (9, 10).

Owing to its role in the generation of the MHCII-restricted peptide repertoire and in stimulating the presentation of immunodominant epitopes, DM activity has been the focus of intense investigation. DM would function as an enzyme, facilitating the release of the peptide bound to MHCII and accelerating peptide exchange (11). However, the susceptibility to DM action varies among peptides, and significant efforts have been made to identify the features of a pMHCII complex that make it a target for DM. In keeping with a recently published review, we think that significant insights gained particularly in the last decade suggest two possible, non–mutually exclusive factors determining DM susceptibility (12). The first model indicates that the occupancy state of the P1 pocket plays a major role in determining DM susceptibility. For instance, it has been shown that DM specifically binds DR2 variants in which the N-terminal site of the complex was emptied (13), and the crystal structure of a covalent DM-DR1 complex has been resolved in which the Ag was a covalently linked peptide lacking three N-terminal residues, thus leaving the P1 pocket vacant (14). The second model proposes that DM susceptibility correlates with the pMHCII complex undergoing conformational rearrangements. In support of this model are SDS-based studies of complex stability (7, 15) and, more recently, the analysis of α54-
substituted DR1 molecules bound to a high-affinity peptide (16). This latter study showed that these mutants are more susceptible to DM-mediated peptide release than wild-type (wt) DR1, they feature increased affinity for DM and increased peptide vibration, especially in the H-bonding network at the N-terminal site of the complex. The resolved structure of HLA-DO bound to DM points again to the possibility that conformational variation of the MHCII, in particular alterations in the α-subunit 310 helix and adjacent regions, is responsible for tight binding to DM (17). In the same vein, we have shown that MHCII molecules loaded with different peptides sharing a Y at P1 can assume two conformations that are either susceptible or resistant to DM-mediated peptide release. The generation of the susceptible isomer appears to be correlated to the affinity of the bound peptide for DR or can be triggered by adding a second peptide to a reaction containing only the resistant form (18).

The evidence that peptides able to fill the P1 pocket are potential DM targets leaves the question open as to which complex features might be responsible for DM susceptibility, and whether these features are somehow related to the peptide sequence. In this study, we show that DM susceptibility is determined by interactions throughout the peptide binding site, in that the thermodynamic mechanism adopted by a pMHCII dyad for binding, irrespective of P1 occupancy state, is reflected in the overall conformation and residual flexibility postcomplex formation. In turn, these structural features correlate with DM-mediated peptide release. This resolves the question of epitope prediction to one of predicting the structural features of the complex.

Materials and Methods

Peptide synthesis

Peptides derived from the sequence GPKYVKQNLKLAT, representing residues 306-319 of the hemagglutinin (HA) protein from influenza A virus (H3 subtype), are described in Table I. The N-terminal Gly facilitated expression and purification of recombinant soluble DR1 protein. DR-expressing clones were selected and expanded. His-tagged DR1 proteins were purified with a His-trap HP column coupled to the In Tune injection needle tip.

Expression and purification of recombinant soluble DR1 protein

Recombinant soluble empty (peptide-free) DR1 was produced and purified by ioGenetics (Madison, WI) from a stably transfected CHO mammalian cell line with a proprietary retroviral vector transduction system essentially as described for Abs (20). The genes code for proteins of 192 (α) and 198 (β) residues, which terminate just before the beginning of the predicted transmembrane spans (residues 193–197 and 199–203, respectively). The vector was designed to generate a poly-His tag at the C terminus of the expressed protein. DR-expressing clones were selected and expanded. His-tagged DR1 proteins were purified with a His-trap HP column coupled to an AKTA FPLC chromatography system and buffer was exchanged into PBS (7 mM Na+/K+ phosphate, 135 mM NaCl [pH 7.4]) using centrifugal ultrafiltration (Amicon). Soluble HLA-DO bound to DM was isolated from a stably transfected Drosophila S2 cell line as described (21). To avoid contamination with Flag peptide, DM elution from the resin was performed with 0.1 M glycine HCl (pH 3.5). Both DR1 and DM proteins were purified and buffer was exchanged into K/Na phosphate buffer (1.47 mM KH2PO4, 8.1 mM Na2HPO4, 135 mM NaCl, 2.7 mM KCl [pH 7.4]) using centrifugal ultrafiltration (Amicon). Purity (>95%) was confirmed by SDS–PAGE stained with GelCode blue stain reagent (Pierce). DR1 proteins were quantified by measuring the UV absorbance at 280 nm using an E280=45,494 M−1 cm−1 before use as calculated with the ExPASy ProtParam tool (22).

Generation and expression of β81-mutated DR1 molecules

Plasmids encoding truncated forms of the HLA-DRα and -DRβ*0101 genes were the gift of Dr. Lawrence Stern (University of Massachusetts Medical School) (23). Position 81 His of the β-chain was mutated to Asn through the use of the QuikChange site-directed mutagenesis kit (Stratagene) and the primer 5′-CCACCCCGTAGTTGTTCAGGTTAGGTC-3′. The mutation was confirmed by sequencing, and wt α and mutant β plasmids were cotransfected into CHO cells for subsequent production by ioGenetics as indicated above. SDS–PAGE analysis of purified β81-mutated DR1 molecules (β81mut) and DR1 proteins revealed no significant differences in migration or purity.

Isothermal titration calorimetry

Titrination calorimetry was carried out with a Microcal ITC200 (GE Healthcare). Analysis was performed at least in triplicate with peptide in the syringe and DR1 in the calorimeter cell at 25°C, pH 7.4. Starting protein concentration in the calorimeter cell was 5 μM, whereas peptide concentration in the syringe was 50 μM. Isothermal titration calorimetry (ITC) injection volumes were 2 μl, and injections were performed during 10 s at a stirring speed of 500 rpm spaced 180 s apart to allow for a complete return to baseline. Dilution heats were measured by titrating 50 μM peptide from the syringe into the cell containing only buffer. Data were processed and integrated with Origin software. Single data sets were fit to a single site ITC binding model, using a baseline offset parameter to account for heat of dilution. The first data point was excluded from analysis due to dilution across the injection needle tip.

pMHCII complex generation

pMHCII complexes were formed by incubating 1 μM MHCII protein with a 10-fold molar excess of either unlabeled or FAM-labeled peptide (depending on the experiment) in PBS (pH 7.4) and protease inhibitors for 16–18 h at 37°C. pMHCII complexes were then purified from unbound peptide with a Centricon-30 spin filter that had been precirculated with 25 mM MES (pH 6.4). Purified complexes were then quantified by reading the UV absorbance at 280 nm, factoring in an E280=1280 M−1 cm−1 for the Y residue and 10,846 M−1 cm−1 for the fluorescein present in the peptide backbone. The latter measurements would add to the extinction coefficient endogeneous value reported above.

Circular dichroism spectroscopy

For circular dichroism (CD) analysis, empty and peptide-loaded complexes were exchanged into 5 mM sodium phosphate/5 mM sodium acetate buffer, pH adjusted with concentrated stocks of HCl and filtered to a final concentration of 3.5 μM (±0.2 mg/ml). Dichroism measurements were made using a 1-mm path length cuvette on a Jasco J-720 spectrophotometer. Wavelength scans were obtained using 1.5-nm bandwidth, constant 10°C temperature, and 1-nm sampling with a 5 s dwell time per point. All experimental scans were adjusted for background signal by subtracting out the signal from a dialyzing buffer scan.

Thermal denaturation

Thermal stability data were obtained by monitoring the CD signal at 204 nm while the temperature was increased from 10°C to 90°C, using 1°C intervals, 1 min equilibration time, 1 min dwell time at each temperature, and 2-nm bandwidth. For each unfolding transition, the midtransition temperature (Tm) was determined as a peak in the first derivative function of the unfolding curve, and also separately by curve fitting to a seven-parameter function that describes a two-state transition (24, 25):

$$
T_m = \frac{\theta_{U} + m_{U}T_f}{1 + e^{-\frac{T_f - T_m}{\Delta T}}} + \frac{\theta_{D} - \theta_{U} + T_r (m_f - m_u)}{1 + e^{-\frac{T_r - T_m}{\Delta T}}}
$$

where \(\theta_U\) and \(m_U\) describe the slope and \(\theta_D\) the y-intercept of the unfolded state baseline; \(\theta_T\) and \(m_T\) describe the slope and \(\theta_f\) the y-intercept of the folded state baseline; \(T_m\) is the midpoint of the transition (where \(\Delta T = 0\); \(\Delta T\) is the heat capacity change upon unfolding; and \(\Delta H\) is the enthalpy of unfolding at the \(T_m\). The thermodynamic values derived in this analysis are likely to depend on the concentration at which the equilibrium is measured, and therefore only hold for the concentration ranges tested (0.1–1 mg/ml) (25). The relationship of the unfolding transition to an irreversible denaturation that occurs in the same temperature range was investigated by recording the dependence of the midpoint temperature on the rate of the scan for overall scan rates 0.3 to 5°C/min. As described previously for DR1, only a slight dependence was observed over the rates tested, indicating that the two-state approximation can be used at the experimental scan speeds (8, 26).
Molecular dynamics simulation

The molecular dynamics (MD) simulation was performed with the software package NAMD (27) using the CHARMM22 force field with an explicit water model and all simulations were carried out at constant temperature (298 K) and pressure (1 atm). All molecular graphics images were generated using the Visual Molecular Dynamics software (28). The structure of the MHCII molecule in complex with peptide epitope (Protein Data Bank 1DLH) was taken from the Protein Data Bank. The complexes formed by HA-substituted peptides and DR1 or β81mut were prepared by applying the appropriate mutations within the sequence of the wt peptide with the Visual Molecular Dynamics mutator plugin, version 1.3.

The peptide/MHC complex was solvated in a box of transferable intermolecular potential water with at least 10 Å distance between protein and the boundary of the water box. The system was first minimized with 10,000 steps of steepest descent followed by 100,000 steps of conjugate gradient descent. The MD simulation time step was 2 femtoseconds, and trajectory was saved every 1 picosecond. The length of the simulation was determined by monitoring the convergence of various mechanical properties of the system. The simulation was stopped when the value for the root mean square deviation (RMSD) did not fluctuate >3.0 from its average value during 2 nanoseconds (ns). As previously indicated, when the simulation reaches an RMSD that oscillates around a constant value, it can be assumed the system has converged to a stable or a metastable structure. A twin range cutoff of 0.9/1.4 nm for van der Waals interactions was

FIGURE 1. Representative raw ITC data titrating HA-derived peptides into DR1 (A and B) and β81mut (C and D) and the fitted binding curves. Starting protein concentrations in the calorimeter cell was \( \sim 5 \) μM, whereas concentrations in the syringe was \( \sim 50 \) μM. ITC injection volumes were 2 μl, and injections were performed during 10 s spaced 180 s apart to allow for a complete return to baseline. Data were processed and integrated with Origin software. Single data sets were fit to a single site ITC binding model, using a baseline offset parameter to account for heat of dilution. The first data point was excluded from analysis due to dilution across the injection needle tip. Experiments were performed at least in triplicate. Measured thermal parameters are indicated in Table I.
applied, and the particle mesh Ewald method was used to treat long-range electrostatic interactions. Constant temperature was controlled by Langevin dynamics, and pressure was maintained by using Nosé–Hoover Langevin piston pressure control.

**Surface plasmon resonance**

Surface plasmon resonance (SPR) experiments were performed on a Biacore 2000 instrument. Antibody (Ab M2 for DM capture) was immobilized on the CM5 sensor chip using standard amine coupling procedure. FLAG-tagged DM was diluted to 75 μg/ml in 10 mM sodium acetate buffer. Five thousand resonance units DM protein was immobilized on the anti-FLAG-coated chip at a flow rate of 5–10 μl/min at 25°C, and the surface was subsequently blocked with 1 M 2-aminomethyl-sulfate and washed with 50 mM CAPS solution. Affinity experiments were performed by injecting the various pMHCII complexes in 2-fold dilutions and at seven concentrations from 8 μM and run over the DM surface at flow rate of 5 μl/min. The running buffer in all phases was composed of 10 mM sodium citrate (pH 5.5), 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20. PBS buffer (0.05% (v/v) surfactant P20) was used for control experiments at pH 7.4. Regeneration of the DM-coupled surface was carried out by flowing 50 mM CAPS (pH 11.5) for 30 s until a stable baseline was reached. Binding data were fit to a Langmuir binding model using BIAevaluation software.

**Fluorescence polarization dissociation measurements**

Intrinsic and DM-mediated peptide dissociation measurements were performed via fluorescence polarization (FP), which quantifies the ratio between bound and free fluorophore-labeled ligand by measuring the tumbling speed of the fluorophore, as the speed is faster when the ligand is unbound. Purified complexes (100 nM) generated as indicated above were incubated with 100-fold excess of unlabeled HA306–319 peptide and 3-fold excess DM protein. After incubation to a Langmuir binding model using BIAevaluation software. In our past investigation of peptide binding to and release from MHCII, we have observed that we are able to isolate pMHCII complexes with a bound MHCII/total MHCII ratio of 90% and bound peptide/total peptide ≥ 97% (31). Fraction of bound peptide is then plotted against time and fit to a one- or a two-phase exponential function for $t/2$ calculation. Each experiment was performed in triplicate, and the reported dissociation rate reflects the mean ± SD of three independent experiments.

**Results**

**pMHCII dyads form complexes with different thermodynamic mechanisms**

In our past investigation of peptide binding to and release from MHCII we have observed by indirect approaches the occurrence of isothermal entropy–enthalpy compensation (32) and binding cooperativity (32–34), and we interpreted them as the thermodynamic epiphenomena of the system structural flexibility. These experiments were performed with a panel of peptides derived from the sequence of HA306–319 via cycle mutation, and or a mutant DR1 (β81mut), in which formation of the H-bond between the peptide backbone and the nonpolymorphic His at position 81 of the β-chain (β81 H-bond) is inhibited by an H→N mutation. Among those sequences we identified a peptide, HASG, in which the combined effect of the P2V→S and P10A→G mutations resulted in an ~4-fold decrease of $K_D$ for DR1 as assessed in competitive binding assays (31, 34). Although HASG can still be considered a high-affinity binder, its intrinsic kinetic stability for DR1 and β81mut was found to be significantly decreased with respect to the wt sequence. Thus, we reasoned that this sequence would be particularly suitable to investigate the potential association among thermodynamic mechanisms adopted for binding, structural conformation of the resulting complex, and susceptibility to DM action.

The thermodynamic parameters of the peptide binding reactions to DR1 and β81mut were derived by ITC. The calorimetric isotherm of binding of peptide to MHCII illustrates an exothermic binding characteristic at 25°C. The standard enthalpy change ($\Delta H^s$), $K_D$, and stoichiometry (n) and the SE for each variable were derived on the basis of the one-site model fit of the pMHCII isotherm (Fig. 1). The $\Delta H^s$, $K_D$, and n values of peptide binding listed in Table I are the error-weighted mean values and the SEM for each variable from three repeated experiments. The SEs of the mean for standard free energy decease ($\Delta G^s$) and standard entropy change ($\Delta S^s$) were calculated using the SE of the $K_D$ ($\Delta H^s$) by statistical error propagation method. Observed enthalpies derive largely as a consequence of changes in interatomic interactions (e.g., hydrogen bonds, van der Waals interactions, π–π interactions), in which the sign indicates that there is a net favorable (negative $\Delta H$) redistribution of the network of interactions between the reacting species (including solvent). Hydrophobic interactions are related to the relative degrees of disorder in the free and bound systems, and thus these interactions are reflected in the entropy change. The release of “bound” water molecules from the binding groove surface and the peptide to the bulk solvent is a source of favorable entropy (positive $\Delta S$). A reduction in conformational states in either ligand or protein upon binary complex formation is entropically unfavorable (negative $\Delta S$). All binding reactions are enthalpy driven; however, the measured overall entropic contribution differs, indicating that the restriction in conformational mobility occurring upon ligand binding varies as a function of the peptide. Indeed, in the case of HASG, binding to DR1 is associated with a smaller entropic penalty as compared with HA, suggesting a more pronounced residual conformational mobility after complex formation.

The analysis of β81mut binding is consistent with our observations derived with indirect methods (32). In the case of HA, the enthalpic contribution to binding free energy does not change significantly with the β81 H-bond disruption, whereas the entropic penalty is reduced with the consequence that binding to β81mut is

<table>
<thead>
<tr>
<th>Complex</th>
<th>Sequence</th>
<th>n</th>
<th>$K_D$ (nM)</th>
<th>$\Delta G$ (kJ mol⁻¹)</th>
<th>$\Delta H$ (kJ mol⁻¹)</th>
<th>$\Delta S$ (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA/DR1</td>
<td>PKYVKQNLKLTAT</td>
<td>0.93</td>
<td>15.6 ± 1.3</td>
<td>−44.5 ± 1.6</td>
<td>−53.6 ± 0.7</td>
<td>−9.02 ± 0.08</td>
</tr>
<tr>
<td>HA/β81mut</td>
<td>PKYVKQNLKLTAT</td>
<td>0.96</td>
<td>11.2 ± 1.5</td>
<td>−45.4 ± 1.4</td>
<td>−46.9 ± 0.5</td>
<td>−1.50 ± 0.06</td>
</tr>
<tr>
<td>HASG/DR1</td>
<td>PKYVKQNLKLTGT</td>
<td>0.98</td>
<td>82.1 ± 6.3</td>
<td>−40.4 ± 1.4</td>
<td>−40.1 ± 0.4</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>HASG/β81mut</td>
<td>PKYVKQNLKLTGT</td>
<td>0.94</td>
<td>132.5 ± 9.4</td>
<td>−39.2 ± 1.3</td>
<td>−30.5 ± 0.4</td>
<td>8.78 ± 0.11</td>
</tr>
</tbody>
</table>

Mutations applied to the HA peptide are in bold.
favored as compared with DR1. Conversely, the inability to form the β81 H-bond for HASG has a greater impact on binding free energy, in that it cooperatively prevents formation of other interactions, with a significant reduction of the enthalpic component. For this latter complex, the entropic contribution is positive, indicating that reduction in conformational mobility upon binding is limited and it reflects predominantly a favorable desolvation-related entropy change.

These results show that formation of high-affinity (pMHCII) complexes (such as HA/DR1 and HASG/DR1) can be achieved with different enthalpic and entropic contributions, and they confirm that the impact of one specific interaction on binding (such as the β81 H-bond) depends on the overall energetics of the system.

**Thermodynamics of pMHCII complex formation is associated with complex secondary structure**

To investigate whether alterations in MHCII secondary structure accompany the different thermodynamic profile of the various complexes, we used far-UV CD spectroscopy to probe conformational specificities of HA, and HASG bound to DR1 or the β81mut (Fig. 2A). The CD spectrum of empty DR1 and that of empty β81mut were substantially similar to each other but altered relative to that of the HA/DR1 and HA/β81mut complexes, exhibiting decreased intensity in the positive band at short wavelengths as well as in the negative band centered at 210–220 nm. These alterations are consistent with those observed in similar investigations performed by other groups on human and murine MHCII (8, 9, 26, 35, 36). Also, the spectra relative to HASG bound to either DR1 or β81mut appear similar with respect to each other, but different as compared with HA spectra, with values of ellipticity closer to the ones observed for the empty DR.

We then investigated possible differences in protein structure between the various complexes and empty MHCII by thermal denaturation measured as change in ellipticity at 204 nm (Fig. 2B, 2C). Empty DR1 and β81mut featured a thermal denaturation with a midpoint temperature of the thermal unfolding transition $T_m$ of $\sim$68°C. The presence of the peptide increased the stability of the MHCII, although this effect appears to be a function of the bound peptide and the MHCII, in that complexes featuring smaller enthalpic contributions to binding energy undergo denaturation at lower temperature (Table II). The slope of the $\theta$ versus temperature plot indicates a cooperative nature of denaturation. The low cooperativity of denaturation of empty MHCII suggested by the broad curve indicates that the number or strength of intramolecular contacts in this form is limited, resulting in a denaturation enthalpy ($\Delta H_m$) value as low as $\sim$190 kJ mol$^{-1}$. Cooperativity of denaturation for peptide-bound MHCII increased with respect to empty MHCII, as indicated by the steepness of the slope, and also in this case the effect appears to be a function of the peptide. To quantitate this effect we derived $\Delta H_m$ and the difference in heat capacity ($\Delta C_p$) between the folded and unfolded states. The values for $\Delta H_m$ and $\Delta C_p$ (Table II) indicate a global effect of peptide in stabilizing the overall folded MHC structure; however, the lower enthalpy of denaturation for the multiple-substituted complexes would reflect a structurally “loose” conformation (36). These observations indicate that the thermodynamic profile of a pMHCII dyad is correlated with the conformation of the complex and its secondary structure.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_m$ (kJ mol$^{-1}$)</th>
<th>$\Delta C_p$ (kJ mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty DR1</td>
<td>341 (68)</td>
<td>192 ± 8</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Empty β81mut</td>
<td>341 (68)</td>
<td>188 ± 6</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>HA/DR1</td>
<td>352 (79)</td>
<td>298 ± 15</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>HA/β81mut</td>
<td>349 (76)</td>
<td>310 ± 17</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>HASG/DR1</td>
<td>345 (72)</td>
<td>229 ± 12</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>HASG/β81mut</td>
<td>343 (70)</td>
<td>212 ± 15</td>
<td>5.2 ± 0.7</td>
</tr>
</tbody>
</table>
MD simulation reveals greater flexibility in complexes whose formation is associated with smaller entropic penalty

To assess whether differences in entropic contribution to binding energy and variation of secondary structures are correlated to differences in conformational flexibility, we performed large-scale molecular dynamics simulations of unbound MHCII molecules and MHCII bound to the peptides under scrutiny. The binding dynamics of DR1 and β81mut bound to the two peptides differed during the simulation time of 60 ns. We probed RMSD and root mean square fluctuation (RMSF) variation between the different structures. RMSD for all the Cα atoms of the residues forming the binding site from the initial structure were calculated, which was considered as the central criterion to measure the protein system. As shown in Fig. 3A, unbound DR1 and β81mut showed deviations throughout the simulation from their respective starting structures, resulting in backbone RMSD of ∼3.2 Å for DR1 and ∼3.3 Å for β81mut during the simulation. Complexes showed a distinct trend of deviation when compared with the unbound MHCII, in keeping with simulations performed by other groups on the same system (37–39). Interestingly, the overall RMSD fluctuations were very much similar between the HA complex bound to either DR1 (∼1.5 Å) or β81mut (∼1.6 Å). However, DR1 bound and β81mut bound to HASG showed more pronounced fluctuations together with a greater difference between the average RMSD values after the relaxation period (∼2.3 and 2.6 Å, respectively). These results suggest a greater conformational lability of the overall binding site in the case of multiple substituted complexes with altered thermodynamic profiles as compared with the HA/DR1 complex.

With the aim of determining whether the nature of the bound peptide might affect protein dynamic behavior in specific regions of the complex, the RMSF values of MHCII backbone residues were calculated for the different complexes (Fig. 3B). Analysis of fluctuation score revealed the presence of higher degree of flexibility in DR1 or β81mut bound to HASG as compared with complexes containing HA peptide. The presence of higher RMSF values in the former structure suggests that combining the P2V→S and the P10A→G substitutions in the peptide, or breaking the β81 H-bond in the HASG/DR1 complex, reduced constraints in the structural flexibility of the bound protein. The largest difference in conformational mobility can be attributed to the α-helices and to the Ig-like domains of MHCII membrane-proximal region. In
particular, comparison of the average structure of the wt complex with the substituted ones indicate that when structural shifts are visible, these involve the α-chain residues 43–54 (Fig. 3B, arrows) as well as the β-chain residues 63–68 and 79–90 in a peptide and MHCII-dependent fashion (Fig. 3C, arrows). Such variations in flexibility, especially of the α-chain residues 43–54, a stretch of amino acids with possible involvement in DM binding, has already been described (16, 17, 38, 39).

Based on the published structure, 14 H-bonds are established between MHCII helices and the peptide backbone. In particular, residues Pheα51 and Serα53 establish main chain–main chain interactions, whereas side chains of Asnα62, Asnα69, Argα76, Aspβ57, Trpβ61, Argβ71, Hisβ81, and Asnβ82 form H-bonds with the peptide backbone. As shown in Fig. 3D, the analysis of the overall complex H-bonding established by the aforementioned residues shows that the HA-containing complexes form more numerous and more stable H-bonds, whereas the number of H-bonds decreases in the case of complexes formed with HASG. Obviously, Hisβ81 contribution was computed only for complexes formed by DR1, substituted by Arg in the mutated complexes. These observations are further evidence of the increase MHCII structural flexibility in multiple substituted complexes.

The graphic rendering of the MD-based structure of HASG/DR1 in comparison with HA/DR1 is shown as an example of the peptide-dependent conformation assumed by a complex, in particular at the N-terminal region (Fig. 4A, 4B). The most noticeable alterations with respect to the wt complex are relative to αW43, which rotates away from the binding site and increases solvent exposure and a shift of ~4 Å of αF51 toward the groove, resulting in a narrowing of the binding site at that end. Finally, αF54 rotates in the direction of the solvent in the substituted complex as a consequence of the inability to form an H-bond with the backbone of a more fluctuating, loosely tethered peptide. In Fig. 4C and 4D the modifications in the average structures of the substituted complexes in comparison with the HA/DR1 are shown.

Taken together, these results indicate that the complexes of which formation is associated with the smallest entropic penalty exhibit the most flexible behavior, particularly in the region that has been mapped as the DM/MHCII recognition site.

pMHCII affinity for DM and susceptibility to DM-mediated peptide exchange are determined by the thermodynamic and structural correlates of complexation

We have shown that pMHCII dyads with comparable free energy decrease of complexation may rely on different enthalpic and entropic contributions, and a correlation can be observed between thermodynamic profile and structural features of the complex. We hypothesized that these differences in energetics and structure across complexes may impact DM susceptibility, particularly as a consequence of the different availability of residues within the N-terminal region to interact with DM. To test this possibility, we measured the affinity of DM to the various complexes by a SPR

![Figure 4](http://www.jimmunol.org/)
Specific saturable, dose-dependent binding was observed for HASG bound to either MHCII molecules, whereas no DM binding could be measured for the HA peptide bound to either DR1 or β81mut. Equilibrium binding analysis revealed \( K_D \) values \( \sim 1.5 \, \mu M \). To ensure that the binding was specific, we performed these experiments at pH 7.4 and pH 5.4. The significant reduction in binding at pH 7.4 is consistent with the known pH effect on DM activity (Supplemental Fig. 1); moreover, no binding could be observed for empty DR1 and β81mut. These results clearly indicate that the complexes of which formation is correlated to a smaller entropic penalty and potentially featuring an increased structural flexibility are preferential ligands for DM.

Finally, to investigate whether the differences in DM affinity correlate with differences in DM activity, we measured the release of FAM-labeled peptides in the presence and in the absence of 3-fold excess DM from DR1 (Fig. 6A) and β81mut (Fig. 6B) via FP. DM susceptibility was calculated as \( k_{\text{off}} \) fold increase = \( k_{\text{off,DM+}} / k_{\text{off,DM-}} \). We correlated DM susceptibility with the restraint of conformational flexibility associated with complex formation (T\( \Delta S / \Delta G \)). As shown in Fig. 6C, an exponential relationship between residual entropy and DM susceptibility can be determined: complexes with limited conformational flexibility (right side of the plot) are the most stable in the presence of DM, whereas complexes with greater residual entropy are more susceptible to DM activity.

Taken together, these results indicate that the thermodynamic signature of a given pMHCII complex is correlated to the probability for that complex to assume a conformation targetable by DM and, as a consequence, it also determines complex susceptibility to DM activity.

**Discussion**

Recent structural studies have provided important insights into how DM interacts with MHCII to mediate peptide exchange (14, 16, 17). From these structures and the most recent biophysical/biochemical analyses it would appear that DM susceptibility of a pMHCII complex is a function of the frequency with which the P1 pocket is emptied or the probability for the complex to assume a conformation in which critical residues are available for interaction with DM. However, it is still unclear which of these properties is a determinant of DM susceptibility and whether they are related to the sequence of the bound peptide. Conformational analysis for just a few pMHCII complexes have been carried out, limiting our capacity to infer general rules of DM susceptibility on the basis of the nature of the peptide and structural determinants. Moreover, we still do not have a conclusive understanding of DM action on the complex and how it skews the binding of peptides.

In this work we show that the thermodynamic mechanisms adopted by peptides and MHCII molecules to interact may be different, as defined through the enthalpic and entropic components of binding free energy decrease, even in the case of peptides with comparable affinity for the same MHCII. Whereas these energy variations maintain an enthalpy-based mechanism of complex formation, CD and MD simulation indicate that they are sufficient to affect the conformation and the lability of the resulting complex.

**FIGURE 5.** SPR assay of DM interaction with pMHCII complexes. Affinity measurements were performed by injecting the various pMHCII complexes or empty MHCII molecules in 2-fold dilutions and at seven concentrations from 8 \( \mu M \) and flowing them over DM-coated CM5 sensor chip at flow rate of 5 \( \mu l/min \) for 5 min and dissociated for 5 min. Binding to DM was analyzed for (A) unbound DR1, (B) unbound β81mut, (C) HA/DR1, (D) HA/β81mut, (E) HASG/DR1, and (F) HASG/β81mut. These experiments were repeated at least three times. Binding data were fit to a heterologous binding model using BIAevaluation software to derive the indicated \( K_D \) values.
SPR and FP-based analyses reveal that these conformational differences are correlated with the affinity of the complex for DM and its susceptibility to DM-mediated peptide exchange.

Peptide binding in the absence of DM is a flexible process and does not rely on independent contributions from pocket/anchor interactions and H-bonds, but it is a function of the synergism involving multiple single-point interactions (32, 33, 40). MHCII molecules feature conformational lability, and the α-subunit 3_{10} helical region with the adjacent extended strand, the β2 Ig-like domain, and the pronounced kink in the β-subunit helical region β62–71 are the most subject to conformational heterogeneity, either in the empty or in the bound state (41). We have previously shown that the effect of MHCII flexibility on the transition from the empty to the bound state (and vice versa) is evidenced by phenomena such as cooperativity and isothermal entropy–enthalpy compensation, with the latter also confirmed in the present work (32). These observations suggest a thermodynamic structural model by which peptides and MHCII bind by optimizing the available interactions through search of conformational space and, as they bind, system flexibility is restrained; the relative enthalpic and entropic contributions to binding free energy decrease determines the structure and conformational lability of the resulting complex.

How does the overall binding property of the peptide determine DM susceptibility of the complex? The correlation between thermodynamic mechanism of binding and complex structure strongly suggests that the interplay between entropy, enthalpy, and binding cooperativity are responsible for determining the probability by which a complex is generated and assumes a more or less DM-susceptible conformation. Indeed, the CD and MD analyses show that a complex, of which formation is correlated to a smaller entropic penalty, features greater conformational mobility and a secondary structure closer to the empty MHCII form as compared with an isoenergetic complex of which formation involves a larger enthalpic contribution. As a consequence, regions within the N-terminal side of the former complexes are expected to disengage from interactions with the peptide and be more amenable to interaction with DM at a higher frequency than the latter.

Studies performed with peptides unable to fill the P1 pocket have suggested a model by which DM susceptibility is a function of the interactions at the P1 region (9, 13, 15, 42, 43). This model has been further refined by a recent structure of DM/DR1 complex with DR1 covalently linked to a peptide lacking three N-terminal residues (14). According to this latter study, suboptimal P1 anchor residues would favor formation of a complex in which the peptide N-terminal dissociates from MHCII, and residues αW43, αF51, and βF89 would rotate out of the pocket, consequently becoming available for DM binding and not accessible to interactions with the peptide. The destabilized complex bound to DM would be able to exchange peptides and DM would dissociate from DR1 once the latter is bound to a peptide able to fill the P1 pocket and possibly capable of forming interactions at the other three major pockets. However, this model does not fully account for the observations that even peptides with optimal “anchor” residues are DM susceptible when bound to MHCII (44, 45). In a similar fashion, this same model is not able to explain the evidence that such peptides are unsuccessful in replacing completely a DM-sensitive ligand such as CLIP during a DM-mediated peptide exchange reaction (31). The alternative classical hypothesis of DM susceptibility based on disruption of one or multiple H-bonds, especially the one established between the His at β81 of the MHCII and the peptide backbone (46, 47), has been challenged by several studies in which complexes lacking one or more of these same H-bonds appeared to be more susceptible to DM than their wt counterparts (48–50). We have previously shown that the disruption of the β81 H-bond affects peptide binding and complex kinetic stability differently on the basis of overall energetics of the complex (32, 34). Our present analysis indicates that also the effect of the β81 H-bond on DM susceptibility varies across complexes, of which conformation and lability differ, as determined by the thermodynamic mechanism of binding.
The model we suggest can explain and reconcile all these conflicting observations: structural studies have mapped the DM/MHCII recognition site to the extended strand loop of the α-chain (14, 16, 51, 52), which is also one of the most dynamic regions of the complex (41). The occupancy state of the P1 pocket region ought to be one important determinant of the geometry of the DM/DR interface. However, due to cooperative effects, stable encapsulation of peptide side chains in the P1 pocket and concurrent formation of the H-bond network at the N-terminal side of the complex relies on interactions formed at other positions of the groove, including the C-terminal side. Indeed, a peptide featuring a suboptimal or poor P1 anchor, such as A for HA/DR1, not stabilized by other interactions across the groove, will be sufficient to promote those structural rearrangements leading to a DM-susceptible form of the complex. However, it would be theoretically possible to rescue suboptimal P1 anchors from causing DM susceptibility by modifying interactions at other positions of the peptide. For instance, we are currently examining the combined effect of the P1N substitution, which is significantly destabilizing in the context of HA peptides, with P4E and P7G, which appear to increase stability of the peptide in a DM-mediated exchange reaction. In the same vein, the role of the β81 H-bond and of any other H-bond in favoring the conformational rearrangement required for DM susceptibility are a function of the overall binding energy and conformational flexibility of the system. Indeed, DM susceptibility of HASG is increased by the β81 mutation, whereas HA is barely affected. We can also propose an explanation as to why the complex formed by a P1A-substituted HA peptide with the β81mut does not behave differently in terms of DM susceptibility as compared with a complex involving the wt MHCII: because the emptied P1 pocket, not sufficiently stabilized by other interactions across the binding groove, is already rearranged in the DM-susceptible form, the contribution to this rearrangement of the β81 H-bond loss is expected to be minimal (46). In addition to the effect of multiple interactions across the groove on the binding state of the peptide at the P1 pocket region, there is also a direct effect of each position on the conformation of the complex. Finally, the thermodynamic mechanism adopted by the system will determine the residual flexibility after complexation, hence the probability for the DM/DR interaction site to assume a conformation amenable to DM binding. Thus, we can define a spectrum of DM susceptibility in which one extreme is represented by complexes with reduced conformational mobility, fully occupied P1 pocket region (such is the case of HA_{306–319} bound to DR1), or suboptimally occupied but stabilized that do not bind DM and are not amenable to DM-mediated exchange. The opposite extreme would be represented by complexes of which structure and lability would permit DM interaction, but of which limited intrinsic stability cannot be further reduced by DM action, such as “anchor-less” HA_{306–319}. Between these two extremes are included all those pMHCII systems that can form a complex, of which overall structure and flexibility determined by respective binding thermodynamics define DM affinity and susceptibility.

Two aspects of the present study pose a limit to the generalization of our conclusions. First, this analysis is limited to four closely related DR/HA variant complexes, and it might be argued that the identified thermodynamic correlates of DM susceptibility are valid only in such instances where limited structural changes are applied to the system. This consideration should necessarily be taken into account if we attempted to derive rules of DM susceptibility on a peptide-sequence basis. Extending this study to a larger number of complexes formed by different MHC alleles and different peptides will help reach that goal. Nevertheless, this argument does not invalidate the correlation between thermodynamic mechanism of binding and DM susceptibility, irrespective of the structural features of the peptide, because the analyzed thermal parameters are state functions. Moreover, this argument would not nullify our observation that peptides able to fill the P1 pocket can be DM susceptible, which is a clear evidence against a P1-centric model of DM function.

The second limitation is that the DM dissociation experiments are correlative, and although supportive, the molecular dynamic simulation studies are not conclusive as to the structure of the conformers and the molecular aspects of the complex associated to DM activity. Electron paramagnetic resonance— and nuclear magnetic resonance–based experiments are underway to determine the structure of the conformers and mechanisms of DM interaction. Interestingly, our findings are consistent with a recent structural (and kinetic) study showing that DM susceptibility would be determined by a dynamic MHCII conformation, and indicating the modifications the complex undergoes as it switches from a DM-resistant to a DM-susceptible form (53). We expect to identify similar conformational rearrangements in our system by structural analysis.

The initial observations relative to DM activity indicating a role in facilitating CLIP release from MHCII have determined the adoption of kinetics-based approaches to the study of DM susceptibility (3, 21, 22, 54, 55). However, the conclusions reached from these experiments have been controversial, and they are not easily applicable to the problem of epitope selection in the context of the APCs. The panoply of peptides generated by endocytosed proteins includes a consistent number of ligands with low affinity for a given MHC allele, sequences with intermediate affinity and few high-affinity ones. At the end of epitope selection in DM-competent cells, MHCII molecules reach a thermodynamic equilibrium in which they are complexed with stable, high-affinity binders. It is difficult to explain how the MHC/peptide system may reach such equilibrium intrinsically in consideration of the evidence that the transit time of an MHCII through the MIC is comparable to the dissociation rate of many low- to intermediate-affinity peptides (56). Indeed, if this were the case, the expectation would be that most MHC molecules are bound to low- or intermediate-affinity peptides that in excess, as a consequence of a kinetic control of the selection process. We prefer the possibility that epitope selection is regulated by a mechanism able to enhance the thermodynamic control of the peptide binding process, with DM the likeliest factor determining the thermodynamic equilibrium of the endosomal machinery. This possibility is supported by the evidence that DM-deficient APCs present a significant amount of empty MHCII or MHCII bound to low-affinity peptides (55, 57). Our observations are a further confirmation of the correlation between thermodynamic signature of a pMHCII complex, its conformational flexibility, and susceptibility to DM-mediated peptide exchange. Thus, in conjunction with the published structural studies, our work provides a comprehensive theory to explain peptide binding and DM activity within the time frame allotted for epitope selection.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1 – SPR assay of DM interaction with pHLAII complexes at pH 7.0. Affinity measurements were performed by injecting the various pHLAII complexes in two fold dilutions and at seven concentrations from 8 μM and flowing them over DM-coated CM5 sensor chip at flow rate of 5μl/min for 5 min and dissociated for 5 min. Binding to DM was analyzed for (A) HA/DR1, (B) HA/β81mut, (C) HASG/DR1, (D) HASG/β81mut. These experiments were repeated three times.