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ENTHALPY-ENTROPY COMPENSATION AND COOPERATIVITY AS THERMODYNAMIC EPIPHENOMENA OF STRUCTURAL FLEXIBILITY IN LIGAND-RECEPTOR INTERACTIONS

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Abstract

Ligand binding is a thermodynamically cooperative process in many biochemical systems characterized by the conformational flexibility of the reactants. However the contribution of conformational entropy to cooperativity of ligation needs to be elucidated. Here we perform kinetic and thermodynamic analyses on a panel of cycle-mutated peptides, derived from influenza H3 HA₃₀₆₋₃₁₉, interacting with wild type and a mutant HLA-DR. We observe that within a certain range of peptide affinity, this system shows isothermal entropy-enthalpy compensation (iEEC). The incremental increases in conformational entropy measured as disruptive mutations are added in the ligand or receptor are more than sufficient in magnitude to account for the experimentally observed lack of free energy decrease cooperativity. Beyond this affinity range, compensation is not observed, and therefore the ability of the residual interactions to form a stable complex decreases in an exponential fashion. Taken together, our results indicate that cooperativity and iEEC constitute the thermodynamic epiphenomena of the structural fluctuation that accompanies ligand/receptor complex formation in flexible systems. Therefore, ligand binding affinity prediction needs to consider how each source of binding energy contributes synergistically to the folding and kinetic stability of the complex in a process based on the trade-off between structural tightening and restraint of conformational mobility.

Keywords

Antigen Presentation; MHC class II; Ligand-Receptor Interaction; Cooperativity; Thermodynamics

Introduction

Predicting the affinity of a ligand for the binding site of a protein with known structure is an important goal, yet one which remains elusive. Several reasons may explain this failure. First of all, prediction of thermodynamic parameters for non-covalent interactions, as in the

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case of ligand binding to a specific site, is inherently difficult¹. This difficulty arises in part because a majority of these interactions occur in water, whose physical-chemical features constitute a hurdle to the prediction of thermodynamic parameters based only on structural data².

Second, the historical model for explaining specificity of ligand-receptor interaction has been the docking mechanism, which relies on structural rigidity and complementarity of shape (and possibly charge)³. The evidence that proteins show conformational plasticity and may undergo structural rearrangement upon engagement with a ligand or a substrate favors an “induced fit” model in lieu of a “lock and key” model⁴. However, while the former mechanism takes into account protein plasticity, the idea still remains that initial complementarity in shape between protein and ligand is important for binding.

The stress put on structural and charge matching in both these models underlines the enthalpic component of the binding process. However, this may be misleading, since a critical role is played by the entropy of the system. Recent evidence has led to the idea that in many biologically relevant situations the interaction between ligand and receptor would be favored by a “sloppy” conformation. Such a fit would be structurally sound enough to provide an enthalpic contribution but loose enough to avoid freezing out conformational mobility and increasing conformation search space. Indeed it has been experimentally shown that the entropy and the enthalpy of intermolecular complexation compensate each other, and the trade-off between intermolecular motion and enthalpic interactions accounts for this general observation⁵. This evidence, which established the principle of entropy-enthalpy compensation (EEC), was initially considered an experimental artifact due to the limitation of indirect techniques to assess free energy values⁶. The introduction of direct calorimetric measurements has provided a reliable confirmation of the occurrence of EEC.

In systems that feature multiple molecular interactions, EEC has been strictly related to cooperativity of binding. This is the phenomenon for which multipoint binding between reactants usually display considerably different binding affinities than one would expect from simply summing the association energies of all the respective parts^{7, 8}. Drawing on the behavior of enzymes and the enhanced substrate binding of vancomycin upon dimerization, Williams has proposed that the enthalpy of binding events can be significantly weakened by the background thermal movements between a ligand and its substrate⁹. Multipoint binding dampens such motions, and consequently all of the individual interactions are strengthened, providing a positive enthalpic enhancement to cooperative binding. More recently Hunter and Tomas¹⁰ have shown that *artificial* molecular recognition systems feature negligible positive binding cooperativity compared to *biological* systems, possibly because the rigidity of artificial ligands cannot accommodate numerous partially bound states that are the initial phases of binding. In each of these partially bound states, a balancing between loss of enthalpy and gain of conformational freedom takes place. The authors related the cooperativity observed in biological systems, but not in artificial systems, to the linear amplification across multiple interactions of such a balancing.

A paradigm of the difficulty in predicting ligand-receptor affinity by a reductionist, enthalpy-based approach, is the interaction of pathogen-derived peptides with class II Major Histocompatibility Complex molecules (MHCII). Since the recognition of peptide/MHCII (pMHCII) complexes by CD4+ T cells is a key event in the initiation of an immune response, the ability to identify which peptides can bind to particular MHCII is critical for predicting immune responses and thus for vaccine development¹¹. MHCII binds peptides in a groove defined by a β -sheet floor and two helical sides. The groove is characterized by hydrophobic pockets and by a network of H-bonds that can form between MHCII side chains and the peptide backbone¹². MHCII shows extensive polymorphism predominantly

restricted to the peptide-binding groove. The contacts observed in available MHCII structures have helped cement the classical interpretation of MHCII-peptide binding specificity based on hydrophobic pocket structure and charge matching. However, biochemical and biophysical analyses have indicated an enhanced conformational heterogeneity or flexibility of the protein in the absence of a peptide^{13; 14}. Several other studies indicated that MHCII molecules undergo a conformational transition during peptide binding or exchange from a heterogenic state to a more rigid and stable state^{15; 16; 17; 18; 19}. These latter reports suggest that the mechanistic details of the peptide loading process are more complicated than the ones provided by a simple enthalpy-based docking model.

We have been reexamining the question of peptide binding and have shown a power law relation between cooperativity and the total energy available to the complex^{20; 21}. Thus, formation of a pMHCII complex can be seen as a process in which the peptide and to some extent the groove fold together, whose probability of success is exponentially related to the number of interactions established between the reactants and the ability of the system to maximize these interactions until the lowest free energy level is reached.

Here we report the extension of these studies to the thermodynamics associated with peptide-binding-induced MHCII folding. By measuring the energetics involved in the binding of peptides derived from the sequence of HA₃₀₆₋₃₁₉ via cycle-mutation to the human MHCII HLA-DR1 (DR1), we show that, within a peptide K_D range, the enthalpic component of cooperativity does not translate into free energy decrease cooperativity because it is compensated by the entropy of the system. However, this compensatory mechanism is not complete, therefore as the peptide worsens in terms of enthalpic contributions, the residual flexibility is not sufficient to maximize the few interactions available. This phenomenon is evidenced by the observation that a lower dissociation constant threshold value exists, below which additional sources of interaction result in decreased affinity. Similarly, an upper constant can be determined, above which disrupting one sole H-bond determines loss of complex formation.

Taken together, our results indicate that cooperativity and EEC constitute the thermodynamic epiphenomena of the structural fluctuation that accompanies pMHCII complex formation, indicated as entropy of binding. Therefore, peptide affinity prediction needs to consider how each source of binding energy contributes synergistically with the others to the folding and kinetic stability of the complex in a process based on the trade-off between structural tightening and restraining of conformational mobility.

Results

Structural Basis for the Construction of the Mutant Box Applied to DR1/HA₃₀₆₋₃₁₉

Cooperativity in a multipoint ligand-receptor binding event is evidenced by a disproportion between the observed affinity value and the expected value based on summation of the affinity contributions of single interactions. A general strategy to probe the occurrence of such a phenomenon is the mutant cycle approach engineered by Horovitz and Fersht (Figure S1A)²². This consists in introducing multiple substitutions in the sequence of the reactants and assessing their binding parameters. If the effect on the binding free energy of the double (or triple) mutation is not equal to the sum of effects of the single mutations, then the two (or three) residues are coupled (cooperative)²². We applied this strategy to determine the extent of cooperativity the DR1/HA₃₀₆₋₃₁₉ system undergoes during complex formation or depletion. Stable peptide binding is postulated to involve the sequestration of hydrophobic side chains in polymorphic pockets of the groove (Figure S1B) and formation of a hydrogen-bond (H-bond) network between conserved residues of the MHCII and the peptide backbone (Figure S1C)¹². Substitutions have been introduced at peptide positions with

either low (here indicated as P1, P6, and P9) or intermediate (P2, P3, P7, and P10) solvent exposure. The peptides substituted at low solvent-accessible sites constitute the subset that disrupts hydrophobic interactions²¹. The intermediate subset is thought to affect the hydrogen bond network²⁰. In addition to the substitutions in the HA peptide we included a His→Asn substitution at position β 81 of the DR1 molecule (β 81mut). This substitution can disrupt a H-bond to the main chain carbonyl oxygen of the P(-1) peptide residue (Figure S1C). The rationale for the mutations has been explained previously^{20; 21} and is summarized in Table SI. The peptides used in this work are listed in Table I.

Cooperativity in Association and Dissociation as a function of the Source of Binding Energy—We have previously measured the extent of cooperativity to determine the impact of system flexibility on peptide/MCHII complex formation. We have shown that cooperativity as a function of complex kinetic stability or of peptide affinity can be described by a power law^{20; 21}. We wish to investigate the relationship between cooperativity and the source of binding energy. To this aim, we measured cooperative effects on affinity for every multiple-substituted peptide. Our analysis encompassed both peptide subsets as well as the β 81mut (The K_D values are reported in Table I). By this approach we can describe the folding process in terms of the traditional view, which considers the burial of non-polar surface area as the most important contribution to the folding energetics, and the alternative hypothesis that the H-bond network established during a ligand-receptor interaction drives the conformational search. The plot of cooperativity $\mathcal{C}$ vs. K_D (Figure 1) shows that the two subsets have different slopes. The different slope of cooperativity versus affinity measured for either peptide subset indicates that the extent to which the flexibility of the pMHCII system contributes to the free energy decrease is a function of the different sources of interaction during the folding process.

The data in Figure 1 were derived by binding values measured at equilibrium. Therefore, we could not establish whether the different contribution to the free energy of folding from reactant desolvation and H-bonding can be explained with a preferential involvement in either association or dissociation phase. To determine the relationship between each source of interaction on the cooperativity of different phases of the binding process, we quantified cooperative effects on association and dissociation rates for those complexes for which we were able to measure peptide release at pH 5 and 37 °C. Binding and peptide release data for P1P6P9 substituted complexes at five different temperatures are shown in Figure S2a-r, but only the values measured at 37 °C are reported in Figure 2A; binding and peptide release data for β 81P2P3P10 substituted complexes are shown in Figure S3a-o, and the values measured at 37 °C are reported in Figure 2B. Cooperativity in complex kinetic stability was calculated by comparing the expected half-life of multiple-substituted complexes with the observed values. Cooperative effects on association constants were determined from $k_{on} = k_{off}/K_D$. In Figure 2C and 2D the $\ln\text{-}\ln$ plots of cooperativity vs. either k_{on} or $t_{1/2}$ are shown for both set of peptides. Clearly, reduction of peptide desolvation area affects folding and unfolding of the complex with different magnitude when compared with loss of H-bonding abilities. Indeed, in the cooperativity vs. k_{on} plot, the data relative to the P1P6P9 peptides (desolvation) are fit by a line that is four-fold steeper than the β 81P2P3P10 regression line (1.74 vs. 0.42). On the other hand, comparison of cooperativity vs. $t_{1/2}$ reveals that destabilization of the H-bond network affects complex kinetic stability to a greater extent than the reduction of the hydrophobic surface (slope 0.84 vs. 0.43). These results indicate that desolvation of the peptide and the binding groove is the main factor promoting the initial association whereas the H-bonding network is responsible for the strengthening of the newly forming hydrophobic milieu and for the overall kinetic stability of the complex.

EEC and cooperativity

Of interest is the cluster of peptides with low cooperativity observed in Figure 1. We decided to examine this region by plotting the $\mathcal{C}$ vs. K_D (Figure 3). For both peptide subsets a K_D range can be identified, for which little or no cooperativity can be measured, followed by a steep increase of cooperativity with peptide affinity decreasing. This leads to the novel question of the significance of peptides of different K_D that show no cooperativity. To address this question, we correlated the structural and thermodynamic perspectives by expressing the cooperativity as a function of ΔH and ΔS .

Cooperativity $\mathcal{C}$ is the ratio of the observed over calculated differential of free energy decrease for a multiple- (for instance triple-) substituted peptide. Free energy is expressed in terms of enthalpic and entropic contributions:

$$\mathcal{C} = \text{obs } \Delta\Delta G / \text{exp } \Delta\Delta G = \Delta\Delta G_{1,6,9} / (\Delta\Delta G_1 + \Delta\Delta G_6 + \Delta\Delta G_9) =$$

$$(\Delta\Delta H_{1,6,9} - T\Delta\Delta S_{1,6,9}) / ((\Delta\Delta H_1 - T\Delta\Delta S_1) + (\Delta\Delta H_6 - T\Delta\Delta S_6) + (\Delta\Delta H_9 - T\Delta\Delta S_9)) =$$

$$(\Delta\Delta H_{1,6,9} - T\Delta\Delta S_{1,6,9}) / ((\Delta\Delta H_1 + \Delta\Delta H_6 + \Delta\Delta H_9) - (T\Delta\Delta S_1 + T\Delta\Delta S_6 + T\Delta\Delta S_9)) = 1$$

Therefore:

$$(\Delta\Delta H_{1,6,9} - T\Delta\Delta S_{1,6,9}) = ((\Delta\Delta H_1 + \Delta\Delta H_6 + \Delta\Delta H_9) - (T\Delta\Delta S_1 + T\Delta\Delta S_6 + T\Delta\Delta S_9)),$$

and, rearranging both terms,

$$(T\Delta\Delta S_1 + T\Delta\Delta S_6 + T\Delta\Delta S_9) - T\Delta\Delta S_{1,6,9} = (\Delta\Delta H_1 + \Delta\Delta H_6 + \Delta\Delta H_9) - \Delta\Delta H_{1,6,9}.$$

The latter indicates that when the difference between expected and observed enthalpic contributions is balanced by an exaggerated gain in conformational mobility, the effect of cooperativity on K_D is reduced, as this balance is calculated for $\mathcal{C}=1$. Based on this equivalence, we hypothesize that the flat portion of the $\mathcal{C}$ vs. K_D plot coincides with the range of peptide K_D for which the enthalpic component of cooperativity is counterbalanced by an increased entropic component. In what follows we will generate an experimental confirmation of this hypothesis.

Entropy-Enthalpy Compensation Effects Participate in the Interaction of Peptides to MHCII

We investigated the thermodynamics of DR1/HA interaction by measuring the affinity and half-life of HA-derived peptides substituted at positions with low or intermediate solvent accessibility at five different temperatures (4, 16, 27, 37, 41 °C). The affinity constant $K_A = 1/K_D$ values, measured by competitive binding, appears to be temperature-dependent for all the pMHCII interactions investigated. This dependence is represented in the form of $\ln K_A$ versus T^{-1} (*van't Hoff*) plots for each peptide tested (Figure S2a-r, S3a-o). The non-linear regression observed in all cases indicates that reactions are heterothermic, which is common when non-covalent interactions are the main source of binding energy. Therefore the corresponding change in heat capacity ΔC_p is 0 and, under these circumstances, changes in enthalpy and entropy are expected, reflecting the molecular flexibility of the reactants.

In Figure 4A we show the thermodynamic measures for P1P6P9 substituted peptides binding to wtDR1 calculated from the thermal binding data (Figure S2). Figure 4B shows the same measures for P2P3P10 mutated peptides in binding to either DR1 or $\beta 81\text{mut}$ (Figure S3). The energetic profile of interaction is described by change in binding energy and its enthalpic and entropic components. Notably, the trend in the standard

thermodynamic parameters points to the possibility that variations in binding enthalpy are compensated by variation in entropy.

Previous discussions of entropy-enthalpy compensation have focused on the tendency of ΔH° and $T\Delta S^\circ$ to oppose and compensate each other as the temperature varies^{23; 24; 25}. Figure 4C shows the plots of ΔH° versus $T\Delta S^\circ$ for several complexes; in each plot the data points are obtained at various temperatures. The linearity of the relationship between ΔH° and $T\Delta S^\circ$ is evident between 16 and 41 °C. This “temperature-dependent” enthalpy-entropy compensation is required by basic thermodynamics for any process in which the change in heat capacity ΔC° is nonzero, albeit only in the restricted range of temperatures where $|\Delta S^\circ| \ll |\Delta C^\circ|$.

Isothermal Enthalpy-Entropy Compensation

It is well known in small-molecule “host-guest” chemistry that if the binding of a homologous series of ligands is compared under otherwise constant conditions including constant temperature, ΔH° and $T\Delta S^\circ$ often show compensating changes. We investigated the occurrence of this phenomenon, which is sometimes referred to as “extra-thermodynamic” enthalpy-entropy compensation. Figure 4D shows the enthalpic and entropic contributions to binding free energy calculated at 300 °K for all the complexes tested. It is evident that the thermodynamic strategies for achieving a favorable ΔG are also diverse. For some proteins a favorable enthalpy change compensates for an unfavorable entropy change whereas others take advantage of a favorable $T\Delta S$ to bind. For both sets of peptides, enthalpy has an impressively large range, about 48 kJ/mol, and over 30 kJ/mol for entropy.

It may appear surprising that the line in Fig. 4d fits most of the data ($r = 0.92$), but there is a relatively simple explanation.²⁶ For this set of pMHCII complexes, the mean $\Delta G^\circ_{\text{bind}}$ is -32.76 ± 8.18 kJ/mol, with a range from -43 kJ/mol to -17 kJ/mol. Regardless of the numerical values (which depend on experimental conditions), $\Delta G^\circ_{\text{bind}}$ varies over a smaller range than either ΔH° or $T\Delta S^\circ$. Thus, the familiar Gibbs relationship $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ may be re-expressed as $\Delta H^\circ = T\Delta S^\circ + \Delta G^\circ$ so that in a plot (Figure 4D) of ΔH° versus $-T\Delta S^\circ$ for systems where $\Delta G^\circ_{\text{bind}}$ varies in a relatively narrow range ($\Delta G^\circ \pm \Delta G^\circ$), we expect to see a linear relationship with slope -1 and an intercept of ΔG° . For the data in Figure 2E, the slope is -1.23 and the intercept is -27.85 kJ/mol. The negative deviation of the slope from -1 indicates that $T\Delta S^\circ$ “undercompensates” for $\Delta\Delta H^\circ$; that is, as sequence context worsens, the benefit in entropy ($T\Delta S^\circ < 0$) is about 20-30% smaller than the penalty in enthalpy ($\Delta\Delta H^\circ < 0$), so that $\Delta G^\circ_{\text{bind}}$ becomes more negative. The strong correlation (0.92) reflects the clustering of the $\Delta G^\circ_{\text{bind}}$ values and is internally consistent. Thus, pMHCII interactions are also profoundly influenced by a “isothermal” enthalpy-entropy compensation (iEEC)²⁶.

The linear relationship in Fig 4D may represent true isothermal enthalpy-entropy compensation or may instead be simply an artifact of correlated errors in measuring ΔH° and ΔS° by the van't Hoff method, where both parameters are extracted from the dependence of $\ln K$ on temperature⁶. A number of statistical tests have been devised to distinguish these alternatives^{27; 28}, one of which is that for true compensation the slope in a plot of ΔH° versus ΔS° (the so called “compensation temperature”) should be significantly different from the experimental temperature; this is true here for all complexes. A second test²⁸ is that true isothermal enthalpy-entropy compensation is indicated by a linear relationship in a plot of ΔH° versus $\Delta G^\circ_{\text{bind}}$, whereas correlated errors alone yield a scattergram. Such an analysis (Figure S4) shows a linear relationship indicating that the sequence-context data represent true compensation.

These results support our explanation for the power law trend of $\mathcal{C}$ vs. peptide affinity or complex kinetic stability (Figure 3). On the left side of the curve we observe the range of K_D for which the enthalpic component of cooperativity does not translate to ΔG cooperativity because the residual motility is sufficient to optimize the available interactions. The compensatory mechanism identified in this window would be responsible for the permissiveness of peptide binding. For values of peptide affinity above this range, the loss of enthalpy is not balanced by the increased conformational mobility (entropy). As a result, negative cooperativity increases dramatically. This indicates a greatly reduced probability for stable interaction of these sequences. Therefore, the slope of the ln-ln plot, which is the exponent of the power law, is related to the extent of compensation the system can provide, given a certain allele and a peptide sequence.

Analysis of the Modification in Peptide Affinity for MHCII due to the $\beta 81$ H-bond Disruption

We further test the importance of EEC by analyzing the contribution of one specific source of binding energy. Disruption of one particular bond should result in decreasing the favorable enthalpic contribution to ΔG decrease. In this context, peptides with multiple binding interactions elsewhere would form a stable complex regardless of the presence of this bond, but adding one source of binding energy would decrease the entropic contribution. In contrast, peptides with fewer favorable interactions are more likely to depend on the contribution of energy from this bond to maintain the conformational change; therefore, as sequence context worsens, the benefit in entropy would be smaller than the penalty in enthalpy due to under compensation, so that $\Delta G^\circ_{\text{bind}}$ becomes less negative. To address this issue, we quantified the variation of peptide affinity due to the loss of the $\beta 81$ H-bond. To increase the statistical significance of our analysis, we considered binding to and release from wtDR1 and $\beta 81$ mut of all the peptides, regardless of whether they were mutated at positions with low or intermediate solvent exposure. These experiments also included peptides containing a P7 L→P substitution that was not considered in the above analysis since this position features an intermediate degree of solvent exposure but usually prefers hydrophobic residues (peptide binding and release studies for P7 substituted peptides are reported in Figure S5). Table I and II show that the K_D of peptides with high affinity for wtDR1 further decreases as they bind to $\beta 81$ mut. On the contrary, the K_D of peptides with reduced ability to fold wtDR1 increases when measured for the binding to $\beta 81$ mut. By plotting the ratio of peptide affinity for $\beta 81$ mut over affinity for wtDR1 as a function of the log of affinity for wtDR1, we identified a sigmoid relation with a significant correlation ($r^2 = 0.83$). The value of peptide affinity for wtDR1 at which the $\beta 81$ mut/wtDR1 affinity ratio is 1 was identified in the 200-250 nM range. This indicates the K_D threshold value below which peptides are able to mediate the conformational changes of DR1 to a stable complex even though missing a source of binding energy from the MHCII (Figure 5A).

Modification of peptide affinity in binding to either wtDR1 or $\beta 81$ mut may be due to modification of complex kinetic stability (k_{off}) or modification of the association rate (k_{on}), which, in a cooperative model of peptide binding, may be related to changes in the rate of unfolding or folding of the complex. To assess the impact of the $\beta 81$ H-bond disruption on either aspect of pMHCII interaction, we plotted the ratio of peptide K_D for $\beta 81$ mut over peptide K_D for wtDR1 as a function of both $k_{\text{on}\beta 81\text{mut}}/k_{\text{onwtDR1}}$ and $t_{1/2\text{wtDR1}}/t_{1/2\beta 81\text{mut}}$ (since $t_{1/2}$ is inversely proportional to k_{off}). For this analysis, we considered affinity and kinetic stability values measured at 37 °C. As shown in Figure 5B, peptides with a preferential binding to $\beta 81$ mut (brown dots) are clustered in a space where the association rate to $\beta 81$ mut is faster than to wtDR1 but the resulting complexes show similar stabilities. For peptides whose K_D is greater in binding to $\beta 81$ mut than to wtDR1 (blue dots), the association rate to wtDR1 is comparable with the association rate to $\beta 81$ mut, but the kinetic stability of the $\beta 81$ mut/peptide complex is 2- to 3-fold lower than that of wtDR1. Thus, in

the absence of an H-bond, high-affinity peptides can fold the binding groove at a faster rate than in the presence of the β 81 H-bond, without significant changes in complex kinetic stability (group 1). Peptides with poorer folding properties associate with the β 81mut at a similar rate as to wtDR1, but the resultant complex is less stable (group 2). Finally, in Figure 5C we show modifications of entropic (top panel; $-\Delta\Delta S_{\text{DR1} \rightarrow \beta 81 \text{mut}}$) and enthalpic (mid panel; $\Delta\Delta H_{\text{DR1} \rightarrow \beta 81 \text{mut}}$) contributions to free energy decrease in complex formation due to disruption of the β 81 H-bond. For peptides of group 1, enthalpy of complex formation does not change significantly, whereas the entropy of the system increases, with the consequence that binding of those peptides to β 81mut is favored as compared with wtDR1, mainly because of an enhanced residual flexibility (Figure 5C – bottom panel, negative red bars). On the contrary, reduction in enthalpy for peptides of group 2 is greater than any gain in favorable entropy (Figure 5C – bottom panel, positive blue bars). In these complexes, loss of an H-bond is “undercompensated” by the residual flexibility, resulting in reduced ΔG and, consequently, in decreased peptide affinity.

These results confirm the occurrence of incomplete (because undercompensating) iEEC in pMHCII complex formation and the relationship between the phases of peptide binding and the different sources of binding energy.

Discussion

When biochemical processes occur in conjunction with structural changes, it is common to explain the occurrence of thermodynamic cooperativity solely in terms of the structural change. However, this practice focuses exclusively on changes in system enthalpy, and it implicitly assumes that the cooperative mechanism is enthalpy-driven²⁹. We have probed the peptide/MHCII system to understand the relationships among molecular flexibility/rigidity, binding cooperativity and the role of entropy therein. Our analysis indicates that in a multipoint ligand/receptor system undergoing substantial conformational rearrangements, the ability of conformational entropy to maximize all the interactions available to complex formation opposes cooperativity. This evidence suggests that the link between nonadditivity in conformational entropy and flexibility/rigidity within molecular structure is both the thermodynamic driving force of cooperative binding and the substrate of the compensatory mechanism between entropic and enthalpic components of binding free energy decrease. Therefore, when cooperativity is accompanied by structural changes, it is probably unwise to assume that the structural changes contribute in a positive manner to cooperativity without specific and explicit support for this conclusion.

A localized modification in a ligand/binding complex during a multipoint binding event could be described in terms of a thermodynamic system, which is defined by all the atoms within a core whose radius is associated with the structural features of the same protein³⁰. The effect of this first modification on the next cluster of interactions (i.e. cooperativity), or their independence from it, is related to the flexibility of the system and the distance between the two. Since the conformation of the protein fluctuates, the boundary of the system can have a more or less wide range and frequency of fluctuations, depending on the number of interactions that the protein and the ligand can provide for the given core. The enthalpic term is the stored energy in the protein due to the conformational change associated with the binding, whereas the fluctuations of the entire protein manifest themselves in the entropy of ligation. The free energy associated with the localized change is determined by the contribution from all the neighboring atoms in the vicinity that defines the system, as well as a mean force acting on the system from the atoms further away³⁰.

Fluctuating interactions are each associated with a component energy and entropy. Although total energy is the sum of constituent energy components, the conformational entropy is

non-additive due to correlated motions. Additive models neglect correlations in atomic motion that extend throughout the protein and therefore underestimate conformational entropy³⁰. We relate the nonadditivity of conformational entropy to rigid and flexible regions within molecular structure depending on the numbers and types of interactions that form. For instance, cross-linking disulfide bonds and H-bonds critically affect the formation of flexible and rigid regions. Of particular importance are interactions that break and form due to thermodynamic fluctuations. A reduction in conformational entropy does not occur when atomic interactions form within rigid regions because atomic motions are not further restricted.

The same driving force behind cooperativity manifests itself in the compensatory mechanism between conformational entropy and enthalpic contribution to free energy decrease in complex formation. In our system, the ligand/receptor interaction starts with the initial sequestration of peptide hydrophobic side chains into regions of the binding groove with a greater than average degree of exposure of hydrophobic groups is driven by the entropically dominated solvation free energy. This solvent reorganization is partially compensated by the increased enthalpy of H-bonding network between water molecules. The ability of the peptide to promote desolvation of the binding groove depends on steric and electrostatic complementarity as well as total hydrophobic surface.

However, when a ligand simply replaces water molecules upon binding and does not yield additional interactions, its binding affinity will be small. Once enough water molecules are displaced to allow an initial association, the tightening of the complex results from formation of the intermolecular H-bond network. Thus, the complex would be held together by multiple weak interactions between peptide and MHCII. For every interaction site of the complex there is a free-bound equilibrium, and the global bound state is a collection of various differently complexed states in equilibrium. Each interaction would undergo a trade-off between the favorable enthalpy available by maximizing the intermolecular interactions and the entropic cost of restricting the conformational freedom of the system¹⁰. Consequently, loss of enthalpy due to the disruption of each single H-bond in mutated peptides is compensated by a gain in conformational entropy. In weakly bound complexes, as is it the case for multiple substituted peptides, a large part of the available enthalpy is dissipated in the population of entropically favorable partially bound states; however, since the compensation is not complete, the half-lives of these complexes are reduced. When the balance between enthalpy and entropy results in an acceptable binding ΔG , the result is structural tightening and loss of ΔG cooperativity.

Our analysis of the $\beta 81$ mutation exemplifies some aspects of the proposed model. We have determined an enthalpic threshold above which adding the $\beta 81$ H-bond results in higher entropic costs without any significant improvement in affinity. The distributed energy provided by peptides of group 1 ($k_{DwtDR1} < 200nM$) is sufficient to stably fold the $\beta 81mut$ binding groove; thus, adding one more interaction (such as in the wtDR1) results only in higher entropic costs (as evidenced by $k_{onwtDR1} < k_{on\beta 81mut}$). On the other hand, peptides of group 2 rely on the presence of the $\beta 81$ H-bond to close the complex ($t_{1/2wtDR1} > t_{1/2\beta 81mut}$), as the small enthalpic contribution to free energy decrease from the peptide is compensated only partially by the residual structural flexibility; thus, adding one source of interaction has a positive effect on complex formation. We have also observed the threshold K_D value ($\approx 4 \times 10^3 nM$) above which disrupting the $\beta 81$ H-bond compromises formation of a stable complex. Indeed, two peptides (P2,3 SD and P3,10 DG) could bind in a stable fashion to wtDR1 allowing off-rate measurements, whereas this was not possible with the $\beta 81mut$ (Table I). This analysis suggests an approach to define the contribution of the receptor and its polymorphism to cooperativity, as one can identify for each allele the energy threshold

value below which increasing/decreasing desolvation area in a specific pocket or ability/inability to form a particular H-bond can be compensated.

One intriguing question we intend to address is whether the thermodynamic model of binding proposed here has a structural counterpart. There is evidence in the literature indicating that this may be the case. We hypothesize that the extent of “tightness” generated by the relative enthalpic and entropic contributions determines the probability with which a complex assumes a stable conformer otherwise known as “compact”³¹. In a situation where the pMHCII interaction significantly relies on the entropic component, then the number of possible states assumed by the resulting complex is greater than that assumed by an isoenergetic complex in which the enthalpic component determines the net kinetic stability. In turn, the fraction of complexes with an incomplete folded conformation (“floppy”) will increase³¹. Preliminary experiments performed by our group seem to indicate that a correlation between peptide K_D , cooperativity in peptide affinity for MHCII, thermodynamic signature of the complex and probability of the complex to assume either conformer does exist. Validating such an hypothesis would have great biological relevance, as further analysis suggests that the “peptide editing” molecule HLA-DM, which is considered the final arbiter of the epitope selection process, facilitates peptide exchange by targeting specifically the “floppy” conformer³². If these results are confirmed, they will provide the thermodynamic foundation for a unifying theory for peptide binding and DM-mediated peptide exchange.

Material and Methods

Peptide Synthesis

Peptides derived from the sequence GPKYVKQNTLKLAT, representing residues 306-319 of the hemagglutinin protein from influenza A virus (H3 subtype), are described in Table 1. The N-terminal Gly facilitated labeling. Side chains in the HA peptide are numbered relative to the P1 Tyr residue¹². N-terminal labeling with FAM (Molecular Probes) or LC-LC biotin (Pierce) was performed on the resin before deprotection, and then peptides were cleaved and purified by HPLC and confirmed by MALDI-TOF mass spectrometry (Protein Nucleic Acid Shared Facility- MCW).

Expression and Purification of Recombinant Soluble DR1 Protein

Recombinant soluble empty (peptide free) DR1 was produced and immunoaffinity purified from a stably transfected *Drosophila* S2 insect cell line essentially as described³³. It is known that DR1 produced in S2 cells might have insect-derived peptide loosely bound once purified. Nevertheless the increased peptide binding capacity, increased binding rate, and decreased pH dependence of peptide binding as compared with mammalian-derived DR1 indicate that, as isolated, the antigen binding site is largely empty (>85%)³³. DR1 proteins were purified and buffer exchanged into PBS (7 mM Na⁺/K⁺ phosphate, 135 mM NaCl, pH 7.4) using centrifugal ultra-filtration (Amicon). Purity (>95%) was confirmed by SDS-PAGE stained with GelCode Blue Stain Reagent (Pierce). DR1 proteins were quantified by measuring the UV absorbance @ 280 nm using an E_{280} of 56340 M⁻¹ cm⁻¹ before use.

Fluorescence Polarization Dissociation Measurements

DR1/peptide complexes were formed by incubating 1 μ M DR1 protein with a 10-fold molar excess of FITC-labeled peptide in 50 mM NaH₂PO₄ and 50 mM of sodium citrate (pH 5.3) and protease inhibitors for 16 h @ 37° C. DR1/peptide complexes were then purified from unbound peptide by buffer exchange into PBS with a Centricon-30 spin filter that had been pre-incubated with 25 mM MES (pH 6.5). Purified DR1/peptide complexes were then quantified by reading the UV absorbance @ 280 nm, factoring in an E_{280} of 1280 M⁻¹ cm⁻¹

for the Tyr residue and $10846 \text{ M}^{-1} \text{ cm}^{-1}$ for the fluorescein present in the bound peptide. 100 nM of purified DR1/peptide complexes were incubated with 100-fold excess of unlabeled HA₃₀₆₋₃₁₉peptide. Reactions were performed at 37 °C in 50 mM sodium citrate/sodium phosphate buffer at pH 5.0-5.3 and were covered with mineral oil to prevent evaporation. To avoid non-specific adherence of the protein, black polystyrene 96-well plates were used (Corning). Measurements were performed using a Wallac VICTOR counter (PerkinElmer Wallac) with the excitation wavelength = 485 nm and emission wavelength = 535 nm. Specific control groups included (a) protein only, (b) peptide only, and (c) buffer only, and were used for background correction. FP and anisotropy are mathematically related ways of expressing parallel/perpendicular emission ratios and are easily interconverted. Although FP is approximately linear with respect to the ratio of free:bound peptide, FP was converted to anisotropy (which is exactly linear) by the following equation $A = 2 \cdot \text{FP} / (3 - \text{FP})$ where A is anisotropy and FP indicates fluorescence polarization in mP units. Anisotropy values were fitted either according to a single- or a bi-exponential decay model. Each experiment was performed in triplicate, and the reported dissociation rate reflects the mean $\pm$ SD of three independent experiments.

Competitive Peptide Binding Assay

DR1 (20 nm) was incubated with 20 nm biotinylated HA peptide in PBS (0.1% BSA, 0.01% Tween 20, 0.1 mg/ml 4-(2-aminoethyl)-benzene sulfonyl fluoride, 0.1 mM iodoacetamide, 5 mM EDTA, 0.02% NaN₃, pH 7.2) in the presence of varying amounts of inhibitor peptides at 37 °C. Bound biotinylated peptide was detected using a solid-phase immunoassay and Eu²⁺ labeled streptavidin. Plates were read using a Wallac VICTOR counter (PerkinElmer Wallac). Data were fit to a logistic equation $y = a / [1 + (x/x_0)^b]$. IC₅₀ values were obtained from the curve fit of the binding data and converted to K_D values by using the Cheng-Prusoff equation $K_D = (\text{IC}_{50}) / (1 + [\text{bHA}] / K_{D,\text{bHA}})$ in which $K_{D,\text{bHA}}$ was set equal to 14 nM on the basis of the results of the direct binding of bio-HA peptide to DR1. Each point represents the mean and SD of three independent experiments performed in quadruplicate. Because pMHCII binding represents a multistep reaction, the IC₅₀ for a competitive binding assay may not be directly proportional to the K_D . While this can be offset by long incubations relative to half-life, we study low-affinity peptides where half-lives are impossible to determine. Therefore, the values of affinity reported herein should be considered as apparent K_D values.

Calculation of Cooperative Effects

We utilized a multiple substitution strategy previously used to identify interacting partners during protein folding^{7; 22}. To normalize the $t_{1/2}$ values of a given pMHCII complex, we define the effect of each substitution as the ratio of the substituted measurement over that of the DR1/wtHA value ($\Delta t_{1/2}$). For calculating cooperativity, the effect of multiple substitutions is measured directly (observed value). The expected value for a combination of substitutions is calculated as the product of the individual substitutions [e.g. $\Delta t_{1/2,\text{exp}} x,y = (\Delta t_{1/2}, x) \times (\Delta t_{1/2}, y)$]. For peptides with three substitutions, the expected value would be the product of all the different substitutions [e.g. $\Delta t_{1/2,\text{exp}} x,y,z = (\Delta t_{1/2}, x) \times (\Delta t_{1/2}, y) \times (\Delta t_{1/2}, z)$]. The cooperativity is the ratio of the expected to observed ($\mathcal{C} = \text{obs/exp}$) values for $\Delta t_{1/2}$. A value of 1 for the ratio of observed/expected ratio indicates no cooperativity, for it would suggest an independent energetic contribution from each substitution. Cooperativity is evidenced when the ratio is not equal to 1. Since each measurement (both expected and observed) is affected by an error and cooperativity is calculated as their ratio, its value is affected by the propagation of the relative errors. Thus, error on cooperativity is calculated through standard error propagation: $\Delta \mathcal{C} = \mathcal{C} \times \sqrt{(\Delta \text{exp/exp})^2 + (\Delta \text{obs/obs})^2}$ and, in a ln plot, the error is calculated as: $\Delta \mathcal{C}_{\ln} = \Delta \mathcal{C} / \mathcal{C}$.

Calculation of Thermodynamic Parameters

Equilibrium thermodynamics at 27 °C were analyzed by measuring K_D values over a range of temperatures, calculating $\Delta G = RT \ln(K_D)$ and fitting the non-linear form of the van't Hoff equation to these data:

$$\Delta G^\circ = \Delta H_{T_O} - T \Delta S_{T_O} + \Delta C_p (T - T_O) - T \Delta C_p \ln (T/T_O)$$

where R is the gas constant, T is the temperature in Kelvin, T_O is an arbitrary reference temperature (300.15 °K), ΔG° is the standard free energy of binding at T, ΔH_{T_O} is the enthalpy change upon binding at T_O , ΔS_{T_O} is the standard entropy change upon binding at T_O , and ΔC_p is the change in heat capacity, assumed to be temperature independent.

Thermodynamic parameters for interaction at 37 °C were calculated as follows:

$$\begin{aligned}\Delta G_{37^\circ\text{C}} &= -RT \ln (K_A) (37^\circ\text{C}) \\ \Delta H_{37^\circ\text{C}} &= \Delta H_{27^\circ\text{C}} + 10 * \Delta C_p \\ T \Delta S_{37^\circ\text{C}} &= T \Delta S_{27^\circ\text{C}} + 310^\circ\text{K} * \Delta C_p (\ln (310^\circ\text{K}/300.15^\circ\text{K}))\end{aligned}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Correlation analysis of cooperativity and peptide affinity in binding to DR1
 Natural log (ln) plot of cooperativity $\mathcal{C}$ vs. K_D for each pMHCII complex tested at 37 °C. Because we defined $\mathcal{C}$ as the ratio of the observed to expected values for K_D , and K_D is inversely proportional to affinity, positive cooperativity in affinity is indicated on the y-axis by values <0 and negative cooperativity by values >0 . Horizontal error bars represent the SD of the K_D measurement. Vertical error bars represent the error of cooperativity as calculated through SE propagation. The line indicates the fit of the data to a linear regression. White dots represent P1P6P9 substituted peptides, whereas black dots show $\beta_{81}P_2P_3P_{10}$ substituted complexes.

Figure 2. Reactant desolvation promotes peptide association with MHCII whereas the H-bonding network is responsible for the overall kinetic stability of the complex

(A) Binding affinity and kinetics of DR1 interaction with a range of peptides generated by cycle mutation from the sequence of HA₃₀₆₋₃₁₉ at P1, P6 and P9 position was measured at 37 °C by equilibrium based-competitive binding assay and FP. Effect of peptide substitution on binding affinity and kinetics is presented as a fold difference compared with the wtHA. Substitutions that negatively affect binding have relative affinities and kinetics lower than 1. Values shown are the mean $\pm$ SD of three independent experiments performed at least in triplicate. (B) Binding affinity and kinetics of wtDR1 and β 81mut interaction with a range of peptides generated by cycle mutation from the sequence of HA₃₀₆₋₃₁₉ at P2, P3, and P10 positions was measured at 37 °C by equilibrium based-competitive binding assay and FP. Bar graph as in (A). (C) - (D) Natural log (ln) plot of cooperativity vs. association (C) and dissociation rates (D) for each multiple substituted pMHCII complex whose peptide release was measurable. White dots represent P1P6P9 substituted peptides, whereas β 81/P2P3P10 data are indicated as black dots. The lines indicate the fit of the data to a linear regression. Error bars as in Figure 1.

Figure 3. Identification of peptides with little or no cooperativity in binding to DR1

Plot of cooperativity $\mathcal{C}$ vs. K_D for each pMHCII complex tested at 37 °C. Because we defined $\mathcal{C}$ as the ratio of the observed to expected values for K_D , and K_D is inversely proportional to affinity, the cooperative effect is positive if $\mathcal{C} \gg 1$, while if $0 < \mathcal{C} \ll 1$ the cooperative effect is negative. The line indicates the fit of the data to a single exponent power law. White dots represent P1P6P9 substituted peptides, whereas black dots show $\beta_{81}P_2P_3P_{10}$ substituted complexes.

Figure 4. Isothermal entropy-enthalpy compensation effects participate in binding of peptides to DR1 binding groove

(A) Comparison of binding thermodynamics of wtDR1 interaction with P1P6P9 substituted peptides. Energetic profile of interaction is described by change in binding energy, and its enthalpic and entropic component. (B) Comparison of binding thermodynamics of wtDR1 and β81mut interaction with P2P3P10 substituted peptides. Energetic profile of interaction is described as in (A). (C) Values of ΔH° are plotted against TΔS° where each data point represents values calculated from the van't Hoff data for the actual temperature at which equilibrium binding measurements were made. The predicted ΔH° and TΔS° as a function of temperature were calculated from $\Delta H = \Delta C_p (T - T_H)$ and $T\Delta S = T\Delta C_p \ln(T/T_S)$. (D) The enthalpic (ΔH°) and entropic (-TΔS°) contributions to binding free energy are shown at 300.15 K for all the complexes reported in Table I. The strong apparent correlation is related to the clustering of the ΔG°_{bind}.

Figure 5. Analysis of kinetic and thermodynamic modifications consequent to disruption of the $\beta 81$ H-bond

(a) Plot of the K_d variation upon loss of the $\beta 81$ H-bond (expressed as the ratio of peptide affinity for $\beta 81$ mut over wtDR1) versus the logarithmic K_d to wtDR1 for all peptides tested. The broken line (ratio = 1) identifies the K_d value (205 nM) above which peptide binding to wtDR1 is energetically favored if compared with $\beta 81$ mut. Blue dots indicate peptides with greater affinity for $\beta 81$ mut than for wtDR1. Brown dots represent peptides with greater affinity for wtDR1. (b) Peptide K_d variation upon loss of the $\beta 81$ H-bond (expressed as the ratio of peptide affinity for $\beta 81$ mut over wtDR1) is analyzed as variation of on and off rates (here indicated as pMHCII half-lives). Color code as in panel (a). (c) Analysis of the modifications of the entropic (top panel) and the enthalpic (middle panel) contributions to free-energy decrease for group 1 (brown bars) and group 2 (blue bars) peptides in binding to either wtDR1 (dark bars) or $\beta 81$ mut (light bars). Bottom panel shows the extent of compensation between enthalpy and entropy consequent to the loss of the $\beta 81$ H-bond. Negative bars indicate a productive compensation, resulting in increased or unchanged ΔG . Positive bars indicate undercompensation, as the loss of enthalpy is not balanced by increased entropy. The consequent reduced ΔG indicates less favorable binding to DR1 as compared with $\beta 81$ mut.

Table I

Dissociation e association constants, dissociation and association rates, complex half-lives, free energy of binding, thermodynamic parameters and cooperativity values for all peptide/MHCII reactions reported in Figure I and II measured at 37 °C

Name	pMHC peptide sequence	K_D (nM)	K_A (mM ⁻¹)	k_{off} $\times 10^3$ (min ⁻¹)	$t_{1/2}$ (min)	k_{on} (M ⁻¹ min ⁻¹)	ΔG (kJ mol ⁻¹)	ΔH (kJ mol ⁻¹)	$-\Delta S$ (kJ mol ⁻¹ K ⁻¹)	ΔC (kJ mol ⁻¹ K ⁻¹)	c
DR1/HA	PKYVQNTLQIAT	73.4 ± 3.8	1.36 E+04	0.16	4638 ± 202.3	2.11 E+06	-42.34 ± 4.9	-54.12 ± 3.64	11.78 ± 1.26	-3.94 ± 0.37	*
DR1/P1 L	PKLVQNTLQIAT	96.5 ± 4.4	1.02 E+04	0.19	3338 ± 156.9	1.96 E+06	-41.58 ± 5.14	-52.35 ± 4.16	10.77 ± 0.98	-3.63 ± 0.27	*
DR1/P1 V	PKVQNTLQIAT	279.6 ± 7.7	7.72 E+03	0.22	2876 ± 129.2	1.67 E+06	-40.87 ± 4.15	-49.77 ± 3.39	8.90 ± 0.76	-3.21 ± 0.41	*
DR1/P1 P S	PKYVQNTLQIAT	188.8 ± 10.3	5.30 E+03	0.48	1160 ± 54.5	2.51 E+06	-39.90 ± 4.69	-47.41 ± 4.07	7.51 ± 0.62	-2.72 ± 0.34	*
DR1/P1 P S	PKYVQNTLQIAT	182 ± 8.5	5.49 E+03	0.28	2614 ± 108.7	1.51 E+06	-39.99 ± 3.96	-48.63 ± 3.22	8.64 ± 0.74	-3.48 ± 0.25	*
DR1/P1 S	PKYVQNTLQIAT	6.71 ± 0.02 E+03	1.49 E+02	6.65	195 ± 8.1	9.83 E+07	-39.70 ± 4.04	-36.79 ± 3.55	6.09 ± 0.49	-3.94 ± 0.37	*
DR1/P1,9 LA	PKLVQNTLQIAT	250.3 ± 11.3	4.00 E+03	0.19	2039 ± 92.5	7.72 E+07	-39.18 ± 5.18	-43.82 ± 4.52	4.64 ± 0.66	-2.68 ± 0.18	1.078
DR1/P1,9 VA	PKYVQNTLQIAT	694.5 ± 18.3	1.44 E+03	0.48	862 ± 37.4	6.83 E+07	-36.55 ± 3.0	-42.51 ± 2.68	5.96 ± 0.32	-3.70 ± 0.22	0.793
DR1/P1,9 LS	PKLVQNTLQIAT	1.19 ± 0.03 E+03	8.40 E+02	1.16	356 ± 13.7	9.74 E+07	-35.16 ± 3.24	-38.80 ± 2.95	3.64 ± 0.29	-4.17 ± 0.34	0.680
DR1/P1,9 VS	PKYVQNTLQIAT	5.72 ± 0.13 E+03	1.93 E+02	3.58	99 ± 4.6	6.91 E+07	-31.37 ± 4.83	-37.02 ± 4.38	5.65 ± 0.45	-4.05 ± 0.24	0.407
DR1/P1,9 KA	PKYVQNTLQIAT	2.44 ± 0.06 E+04	4.10 E+01	49.5	14 ± 3.2	2.03 E+06	-27.37 ± 3.67	-30.01 ± 3.49	2.64 ± 0.18	-4.88 ± 0.49	0.388
DR1/P1,9 KS	PKYVQNTLQIAT	4.64 ± 0.12 E+04	2.16 E+01	*	*	*	-25.72 ± 1.72	-26.10 ± 1.68	0.38 ± 0.04	-4.24 ± 0.51	0.127
DR1/P1,6 LK	PKLVQNTLQIAT	1.22 ± 0.03 E+05	8.20 E+00	*	*	*	-23.23 ± 3.45	-24.24 ± 3.36	1.01 ± 0.09	-4.94 ± 0.65	0.085
DR1/P1,6 VK	PKYVQNTLQIAT	2.61 ± 0.07 E+05	3.83 E+00	*	*	*	-21.27 ± 1.97	-20.31 ± 1.85	0.96 ± 0.12	-5.79 ± 0.88	0.106
DR1/P1,6 LKA	PKLVQNTLQIAT	1.79 ± 0.11 E+05	1.15 E+00	*	*	*	-22.24 ± 2.59	-20.89 ± 2.21	-1.35 ± 0.38	-6.71 ± 0.47	0.129
DR1/P1,6 VKA	PKYVQNTLQIAT	3.16 ± 0.08 E+05	5.59 E+00	*	*	*	-20.76 ± 2.27	-18.29 ± 1.96	-2.47 ± 0.31	-5.54 ± 0.41	0.029
DR1/P1,6 LKS	PKLVQNTLQIAT	8.67 ± 0.02 E+05	9.35 E+01	*	*	*	-18.17 ± 1.99	-14.74 ± 1.84	-3.43 ± 0.15	-6.08 ± 0.53	0.042
DR1/P1,6 VKS	PKYVQNTLQIAT	1.07 ± 0.05 E+06	3.14 E+00	*	*	*	-17.63 ± 1.92	-12.50 ± 1.52	-5.12 ± 0.40	-5.23 ± 0.68	0.018
DR1/HA	PKYVQNTLQIAT	90.6 ± 2	1.98 E+04	0.17	3971 ± 244	3.45 E+06	-43.80 ± 2.91	-54.16 ± 1.25	11.12 ± 1.46	-4.34 ± 0.17	*
DR1/P2 S	PKYVQNTLQIAT	75.2 ± 4	1.33 E+04	0.26	2712 ± 108	3.40 E+06	-42.28 ± 3.41	-48.35 ± 2.50	5.98 ± 0.81	-3.52 ± 0.35	0.795
DR1/P2 S	PKYVQNTLQIAT	97.2 ± 4	1.03 E+04	0.21	3378 ± 101	2.11 E+06	-41.62 ± 5.07	-50.12 ± 3.74	8.50 ± 1.33	-4.80 ± 0.21	*
DR1/P2 D	PKYVQNTLQIAT	118 ± 5	8.45 E+03	0.34	2048 ± 56	2.86 E+06	-41.12 ± 2.54	-47.23 ± 1.66	6.21 ± 0.88	-5.24 ± 0.25	0.852
DR1/P2 D	PKYVQNTLQIAT	157 ± 6	6.33 E+03	0.18	3821 ± 265	1.15 E+06	-40.36 ± 1.75	-46.19 ± 0.83	5.45 ± 0.92	-4.50 ± 0.04	*
DR1/P3 D	PKYVQNTLQIAT	318 ± 12	3.14 E+03	0.24	2834 ± 215	7.67 E+07	-38.55 ± 3.08	-43.81 ± 2.50	5.20 ± 0.58	-4.71 ± 0.37	*
DR1/P2,10 SG	PKYVQNTLQIAT	273 ± 15	2.68 E+03	0.71	970 ± 151	1.91 E+06	-38.15 ± 2.38	-44.62 ± 1.66	6.58 ± 0.72	-4.49 ± 0.18	0.409
DR1/P2,10 SG	PKYVQNTLQIAT	588 ± 47	1.79 E+03	1.18	581 ± 59	2.10 E+06	-37.11 ± 3.35	-40.51 ± 2.50	4.95 ± 0.85	-3.97 ± 0.12	0.339
DR1/P3 D	PKYVQNTLQIAT	643 ± 36	1.55 E+03	0.53	1307 ± 222	8.23 E+07	-36.74 ± 4.51	-43.39 ± 3.74	6.56 ± 0.77	-3.29 ± 0.67	0.549
DR1/P2,3 SD	PKYVQNTLQIAT	3.57 ± 0.07 E+03	2.80 E+02	1.04	667 ± 50	2.91 E+07	-32.33 ± 1.43	-34.46 ± 1.25	1.85 ± 0.18	-3.67 ± 0.84	0.102
DR1/P3,10 DG	PKYVQNTLQIAT	4.62 ± 0.19 E+03	2.08 E+02	1.36	551 ± 66	2.61 E+07	-31.55 ± 1.8	-35.29 ± 3.33	2.78 ± 0.47	-4.67 ± 0.87	0.068
DR1/P2,3 SD	PKYVQNTLQIAT	9.17 ± 0.95 E+03	1.09 E+02	*	*	*	-29.90 ± 3.0	-28.74 ± 2.41	-1.94 ± 0.59	-4.72 ± 0.21	0.058
DR1/P3,10 DG	PKYVQNTLQIAT	1.40 ± 0.17 E+04	7.14 E+01	*	*	*	-28.81 ± 3.86	-26.58 ± 3.74	-2.58 ± 0.12	-4.34 ± 0.78	0.031
DR1/P2,3,10 SDG	PKYVQNTLQIAT	8.99 ± 0.32 E+04	1.12 E+01	*	*	*	-24.04 ± 1.61	-19.39 ± 0.83	-5.12 ± 0.78	-4.57 ± 0.13	0.007
DR1/P2,3,10 SDG	PKYVQNTLQIAT	2.61 ± 0.1 E+05	3.83 E+00	*	*	*	-21.27 ± 0.85	-15.84 ± 0.42	-6.04 ± 0.43	-5.12 ± 0.24	0.002

* non measurable; substitutions are marked in red; amino acid position within peptide sequence is indicated according to the P numbering system, assuming the Y308 as P1

Table IIStability and dissociation constant of P1P9 substituted peptides in binding to β 81mut

Peptide	$t_{1/2}$ (min)	k_D (nM)
HA ₃₀₆₋₃₁₈	4638 ± 202.3	50.6 ± 1.9
P1 L	3338 ± 156.9	67.9 ± 2.0
P1 V	2876 ± 129.2	89.4 ± 3.6
P9 S	1160 ± 54.5	130.2 ± 5.7
P9 A	2614 ± 108.7	125.7 ± 5.3
P1,9 LA	2039 ± 92.5	206.4 ± 7.9
P1,9 VA	862 ± 37.4	739.3 ± 22.0
P1,9 LS	356 ± 13.7	$1.88 \pm 0.06 \times 10^3$
P1,9 VS	99 ± 4.6	$8.17 \pm 0.23 \times 10^3$