Energetic asymmetry among hydrogen bonds in MHC class II·peptide complexes

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Comparison of crystallized MHC class II·peptide complexes has revealed that, in addition to pocket interactions involving the peptide side chains, peptide binding to MHC class II molecules is characterized by a series of hydrogen bonds between genetically conserved amino acid residues in the class II molecule and the main chain of the peptide. Many class II·peptide structures have two sets of symmetrical hydrogen bonds at the opposite ends of the class II antigen-binding groove (β-His-81, β-Asn-82 vs. α-His-68, α-Asn-69). In this study, we alter these peripheral hydrogen bonds and measure the apparent contribution of each to the kinetic stability of peptide·class II complexes. Single conservative amino substitutions were made in the I-A^d protein to eliminate participation as a hydrogen bonding residue, and the kinetic stability of a diverse set of peptides bound to the substituted I-A^d proteins was measured. Although each hydrogen bond does contribute to peptide binding, our results point to the striking conclusion that those hydrogen bonds localized to the amino terminus of the peptide contribute profoundly and disproportionately to the stability of peptide interactions with I-A^d. We suggest that the peripheral hydrogen bonds at the amino terminus of the bound peptide that are conserved in all class II·peptide crystal structures solved thus far form a cooperative network that critically regulates peptide dissociation from the class II molecule.

Major histocompatibility complex (MHC) class II molecules are composed of two noncovalently associated, polymorphic transmembrane proteins, termed α and β, of approximate molecular mass of 33 kDa and 28 kDa, respectively. They interact with T cell receptors to provoke an antigen-specific CD4$^+$ T cell response. Crystal structures of class II molecules have provided insight into the structural elements that control interactions with peptide. Both polymorphic and genetically conserved amino acid residues make contacts with the bound peptide (1–7). Most of the polymorphic residues face the interior of the peptide-binding groove, and thus contribute to allele-dependent peptide binding. Similar to what has been observed in class I molecules, MHC class II molecules appear to have "pockets" that are capable of binding to peptide side chains. In addition to these pocket interactions, MHC class II molecules bind peptide through a series of hydrogen bonds between genetically conserved amino acid residues in the class II molecule and the main chain of the peptide. These conserved residues bind at relatively regular intervals throughout the length of the peptide, and apparently stabilize the peptide in a fairly fixed polyproline type II conformation throughout the length of the peptide-binding pocket (3).

One striking aspect of class II·peptide structures noted in murine complexes (6, 7) are two sets of symmetrical hydrogen bonds at the opposite ends of the binding pocket (Fig. 1). The hydrogen bonds contributed by β-His-81 and β-Asn-82 are localized to the bound peptide's amino terminus and are symmetrical to those formed by α-His-68 and α-Asn-69 at the peptide's carboxyl-terminal end. This symmetry is a feature shared by most murine I-A and DQ class II molecules. Previous results from our laboratory indicated that substitution of amino acids β-His-81 and β-Asn-82 in I-A^d leads to defects in the intracellular sorting of class II molecules (8, 9) that are secondary to defects in peptide acquisition (10). In more recent experiments, we evaluated the contribution of a single hydrogen bond (β-His-81) to the kinetic stability of peptide·MHC class II complexes. Our studies revealed that all peptides studied displayed a profound loss in the stability of their interactions with the hydrogen bond-deficient variant relative to wild-type class II molecules (11). These were unexpected results, raising the question of whether all hydrogen bonds contribute equally to the stability of peptide·MHC class II complexes. In this study, we compare the magnitude of the contribution of hydrogen bonds from each of the symmetric amino acids at the periphery of the peptide binding pocket to the stability of peptide·class II complexes. Our results show that the hydrogen bonds at the peptide's amino terminus contribute asymmetrically and disproportionately to the stability of class II·peptide complexes, and point to a highly cooperative set of interactions between peptide and MHC class II molecules in that region of the class II antigen-binding site.

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Materials and Methods

Establishment of Cell Lines Expressing Hydrogen Bond-Deficient Class II Molecules. Genes encoding class II I-A^d α- or β-chains were expressed in the SV40 expression vector pcEXV (12). Constructs of class II I-A^d cDNA containing point mutations at positions of 81 (His → Asn, termed β-His-81-H$^-$) or 82 (Asn → Ser, termed β-Asn-82-H$^-$) in β have already been described (8–10). Homologous α-chain mutations at position 68 (His → Asn termed α-His-68-H$^-$) or 69 (Asn → Ser, termed α-Asn-69-H$^-$) were made in a similar fashion by primer overlap extension method using PCR (12).

Cells, Tissue Culture Conditions, and cDNA Transfections. The murine class II- and invariant chain-negative fibroblast cell line Ltk$^-$ was cotransfected with DNA encoding the I-A^d molecule [wild-type (wt) α-chain + wt β-chain or mutant β-chain or, alternatively, wt β-chain and mutant α-chain] and a plasmid containing a neoresistance gene (pSV2neo) by the calcium phosphate precipitation method as described (8). Class II-positive transfectants were obtained by sorting and were maintained in selective drug during all phases of culture with the exception of the expansion for large scale preparation of class II molecules, described below.

Purification of Class II Molecules. I-A^d protein (either wt or hydrogen bond-deficient variant) was isolated as described previously (11, 13). In brief, $\approx 1–2 \times 10^{10}$ cells expressing wt I-A^d or the hydrogen bond-deficient class II molecules were grown as nonadherent cells, then lysed in a solution of 0.1% Nonidet P-40 in

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Abbreviations: wt, wild type; DM, dodecyl maltoside; CLIP, class II-associated invariant chain peptides; HA, influenza hemagglutinin.

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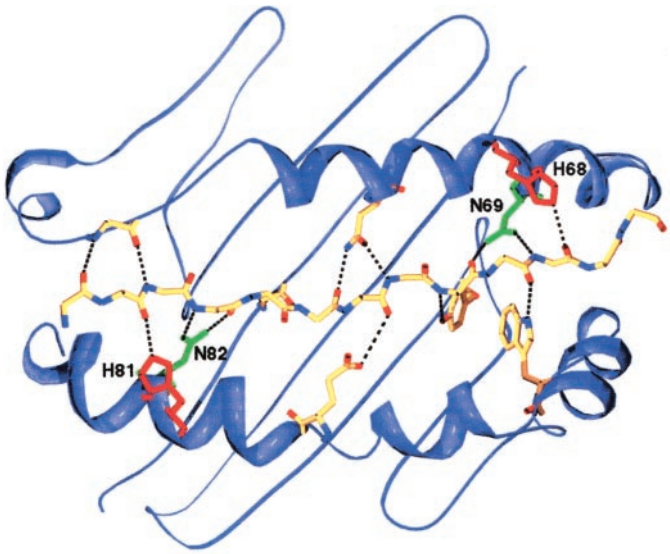

Fig. 1. Structure of the I-A^d MHC class II·peptide complex. This image is taken from the coordinates of the published crystal structure of I-A^d ovalbumin (323–339) (6). Peptide side chains are not shown, and peptide amino terminus is to the left. The Asn and His residues contributing the hydrogen bonds analyzed in the current study are highlighted in green and red, respectively.

PBS (pH 7.4). Class II molecules were isolated by affinity chromatography with a monoclonal anti-class II antibody M5114 (14) affinity column. After binding to the anti-class II antibody, the Nonidet P-40 detergent was exchanged for 0.2 mM dodecyl maltoside (DM) in PBS (pH 7.4) to preserve protein stability over long term storage of the isolated protein.

Synthesis and Labeling of Peptides. Peptides were synthesized and labeled with fluorescein at the amino terminus as described (11). C-terminal fluorescein labels were obtained by synthesizing an N-terminally acetylated peptide ending with an extra C-terminal lysine protected by an ivDDE group (4,4-dimethyl-2,6-dioxocyclohex-1-ylidiene-3-methylbutyl; Nova Biochem). A 5- to 10-min incubation of resin with 2% hydrazine resulted in selective deprotection of the ivDDE group. The peptide was then fluorescein-labeled, cleaved, and purified as described (11).

Determination of Dissociation Rates of Peptide·MHC Complexes. A solution of MHC protein (≈250 nM) and an excess of fluorescein-labeled peptide (3–5 μM) was incubated at pH 5.3 in PBS containing 0.2 mM DM (DM/PBS) buffered with citrate-phosphate buffer at 37°C. Samples were incubated until near maximal numbers of complexes accumulated (15 min to 24 h; differing incubation times had no effect on kinetic stability; data not shown). Excess peptide was removed by rapid size-exclusion chromatography. The sample containing MHC class II·peptide complexes was diluted and then incubated at 37°C. At different time intervals, 50-μl aliquots were injected onto a 30-cm by 7.5-mm TSK3000SWxl analytical size-exclusion column (Toso-Haas, Montgomeryville, PA) connected to a fluorescence detector. The fluorescence associated with the protein peak was taken as a measure of the amount of peptide bound. For short time intervals, some aliquots were briefly stored (1–2 h at most) at 4°C before injection. This procedure effectively stopped dissociation for all peptides with half-lives longer than 0.02 h (data not shown). Half-lives of dissociation were obtained from single exponential functions fit to the monophasic dissociation data. At least two experiments were run for each half-life measured.

Results

To evaluate the relative apparent contribution of individual peptide·MHC hydrogen bonds to the stability of peptide·MHC class II complexes, single conservative amino substitutions were made in the I-A^d protein by using PCR-mediated mutagenesis on the cDNA encoding the appropriate gene product. His (β-81 or α-68) was substituted with Asn, based on Dayhoff's finding that this is the most conservative substitution possible (15). Asn residues (β-82 or α-69) were changed to Ser, based on previous work by Glimcher's laboratory (16) and our own (9, 10) indicating that this substitution compromises the ability to acquire peptide but does not cause major changes in class II structure or conformation.

Genes encoding the wt or variant class II molecules were introduced into fibroblasts by calcium phosphate-mediated transfection, and class II molecules isolated from detergent lysates of the transfected cells were tested for peptide binding stability with a series of fluorescein-labeled peptides. The peptides used have a wide range of kinetic stability with wt I-A^d (see Table 2), with dissociation half-times ($t_{1/2}$) ranging from close to 200 h (Cys C) to under 12 h [class II-associated invariant chain peptides (CLIP)]. As shown in Table 1 and Fig. 2, several variant peptides with single amino acid changes that modulate the binding stability were also included in this analysis. Fig. 2 shows the dissociation kinetics of the test peptides for each of the four mutant molecules relative to the dissociation kinetics for the wt class II molecule. Each graph in Fig. 2 compares the dissociation kinetics displayed by the wt I-A^d molecule with class II molecules mutated at analogous amino acids (i.e., β-His-81 vs. α-His-68 or β-Asn-82 vs. α-Asn-69). With the exception of the highest-stability peptide (Cys C), all of the remaining peptides showed readily apparent enhanced dissociation rates when tested with each of the hydrogen bond-deficient variants. Cys-(40–54) stability was compromised with all hydrogen bond variants except the α-His-68-H$^-$ variant. As expected, in general, the loss of the potentially bidentate hydrogen bonds contributed by Asn (see Fig. 1) was more detrimental to stable peptide binding than the loss of the single hydrogen bond contributed by His (3, 17). We concluded from this first level of analysis that each individual hydrogen-bonding residue tested in these studies contributes measurably to the interaction between MHC and its bound peptide.

The magnitude of the apparent contribution of any given hydrogen bond to kinetic stability can be calculated as a rate

Table 1. Peptides analyzed for binding to I-A^d and hydrogen bond loss variants

Peptide	Sequence	Source
mIi-(85–99)	KPVSQMRMATPLLMR	Residues (85–99) of murine Ii
mIi-(85–99) P95 → A	KPVSQMRMATALLMR	Residue 95 Pro to Ala in murine Ii-(85–99)
CysC-(40–54)	DAYHSRAIQVVRARK	Residues (40–54) of cystatin C
E-α-(52–67)	ASFEAQGALANIAVDK	Residues (52–67) of I-E^d α-chain
HA-(126–138)	HNTNGVTAASSHE	Residues (126–138) of influenza hemagglutinin
HA-(126–138) T128 → M	HNMNGVTAASSHE	Residue 126 Thr to Met of HA (126–138)

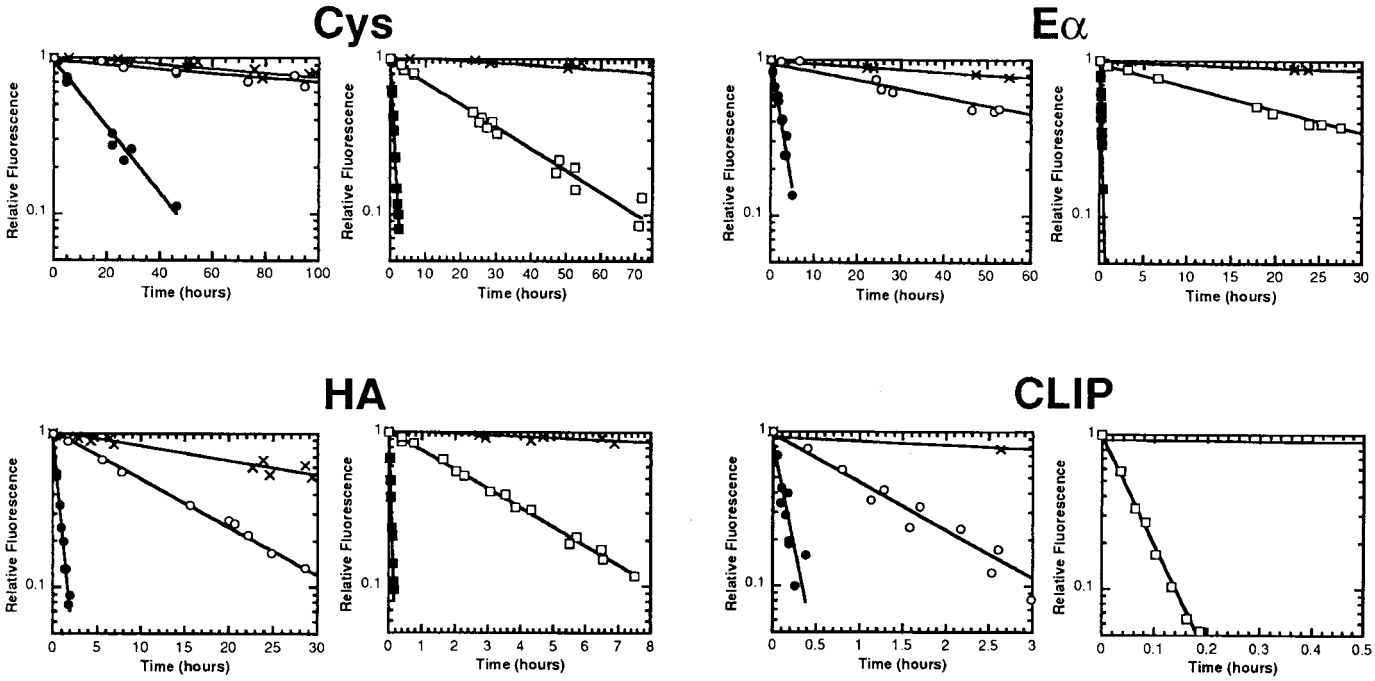

Fig. 2. Dissociation kinetics of peptides with hydrogen bond-deficient variants of class II molecules. The wt or hydrogen bond-deficient class II molecules were tested for their dissociation kinetics with the indicated peptide. Shown for comparison are the dissociation kinetics displayed by wt class II molecules (× symbols) with those displayed after mutation of hydrogen-bonding amino acids. In each pair, the left graph compares the loss of His hydrogen bonds [C-terminal α-His-68 (open circles) vs. N-terminal β-His-81-H$^-$ (filled circles)], whereas the right graph compares the loss of Asn hydrogen bonds [C-terminal α-Asn-69-H$^-$ (open squares) vs. N-terminal β-Asn-82-H$^-$ (filled squares)].

enhancement value, which is a measure of the increase in dissociation rate because of disruption of a hydrogen bond. This value is obtained by dividing the dissociation $t_{1/2}$ of any given peptide from wt class II molecules by the $t_{1/2}$ for the same peptide from the hydrogen bond-deficient molecule. This value represents the relative magnitude of destabilization induced by mutating a hydrogen-bonding residue. The rate enhancement values for each peptide on each variant are presented in Fig. 3. Both Figs. 2 and 3 point to the striking conclusion that, although each class II hydrogen bond does contribute to peptide binding, the location of the hydrogen bond is critically important to overall class II·peptide stability, and that the hydrogen bonds at the peptide's amino terminus (β-His-81 and β-Asn-82) contribute disproportionately. For example, the dissociation rate enhancement of the E-α-(52–68) peptide from α-His-68-H$^-$ is 3.3-fold (i.e., the peptide dissociates 3.3 times faster from the α-His-68-H$^-$ molecule than from wt I-A^d), whereas the loss of the hydrogen bond at β-His-81 causes a 90-fold rate enhancement. A similar conclusion is drawn when one compares the two hydrogen bonds contributed by Asn residues to their symmetric counterparts. For the E-α peptide, the rate enhancement value for loss of α-Asn-69-H$^-$ is 8.3, whereas the same peptide dissociates ≈1000-fold faster when the analogous hydrogen bonds from Asn at the amino terminal end of the peptide (β-Asn-82) are eliminated. This pattern holds true for every peptide tested: the apparent magnitude of the contribution from the hydrogen bonds at the amino terminus of the peptide (β-His-81 and β-Asn-82) far exceeds that contributed by the analogous amino acids (α-His-68 and α-Asn-69) at the carboxyl terminus. Binding of CLIP to the β-Asn-82-H$^-$ variant could not even be detected, presumably due in part to its very rapid dissociation rate. To estimate the contribution of the β-Asn-82 hydrogen bonds to CLIP stability, we performed the peptide dissociation at pH 7.4 rather than pH 5.3, which slowed dissociation sufficiently to allow detection of the β-Asn-82-H$^-$·CLIP complexes. Wild-type class II and the other hydrogen bond-deficient variants were analyzed in parallel (Table 2). Neutral pH stabilized wt·CLIP complexes, resulting in a dissociation half time of ≈75 h. With the loss of the hydrogen bonds contributed by β-Asn-82, the dissociation half time is 0.03 h (2 min), leading to a striking rate enhancement value of ≈2500-fold. The symmetric α mutation is also very destabilizing, giving a $t_{1/2}$ of 0.5 h, but its rate enhancement value (170) is less than 1/10th that displayed by β-Asn-82-H$^-$. We conclude that all of the hydrogen bonds between the class II α helix and the peptide main chain are important, but those localized at the peptides' amino termini most profoundly control the interaction with class II molecules.

Such large rate enhancements require confirmation that the peptides are binding to the hydrogen bond-deficient variants and wt class II molecules in the same register. We addressed this question by deriving and synthesizing variant peptides anticipated to have improved or diminished pocket interactions with class II. For example, the influenza hemagglutin (HA) peptide has been cocrystallized with I-A^d, and its binding register is known. We can thus predict, and then test, substitutions that are expected to have better or worse fits with the known pockets in I-A^d. If the pockets are preserved in the class II hydrogen bond loss variants and if the peptides bind in the same register, then a peptide variant with enhanced stability on wt will display similarly enhanced stability on the hydrogen bond variant. We have considerable knowledge regarding the structural elements that control binding of CLIP-(85–99) and HA-(126–138) to the I-A^d molecule (4, 6, 18–21), and we have identified a number of substitutions in these two peptides that either destabilize or enhance stability with I-A^d (unpublished results). Shown in Fig. 4 are the results of experiments using two variant peptides that display enhanced kinetic stability on wt I-A^d molecules, which were analyzed with each of the hydrogen bond-deficient I-A^d variants. Fig. 4 (*Top*) shows the enhanced binding stability of CLIP (Pro96 → Ala, enhanced P6) and HA (128T → M,

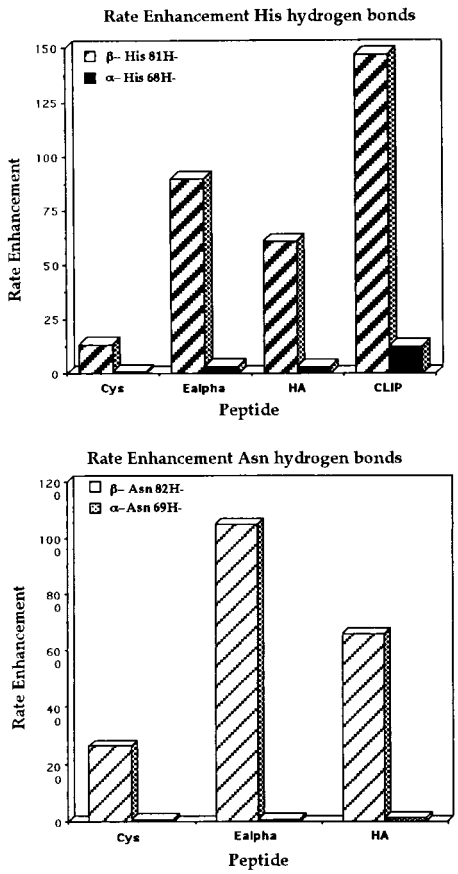

Fig. 3. Dissociation rate enhancements because of elimination of hydrogen-bonding residues. For each peptide, rate enhancement values were calculated by dividing the dissociation half time of a given peptide from wt class II molecules by the dissociation half time of the same peptide from a given I-A^d mutant (see Table 2). (*Upper*) The rate enhancement values for His hydrogen-bond loss at pH 5.3 [α-His-68-H$^-$ (filled bars) vs. β-His-81-H$^-$ (hatched bars)]. (*Lower*) The rate enhancement values for Asn hydrogen-bond loss at pH 5.3 [α-Asn-69-H$^-$ (stippled bars) vs. β-Asn-82-H$^-$ (hatched bars)]. Table 2 shows the rate enhancement values displayed by CLIP when tested with each of the variant and wt molecules at pH 7.4.

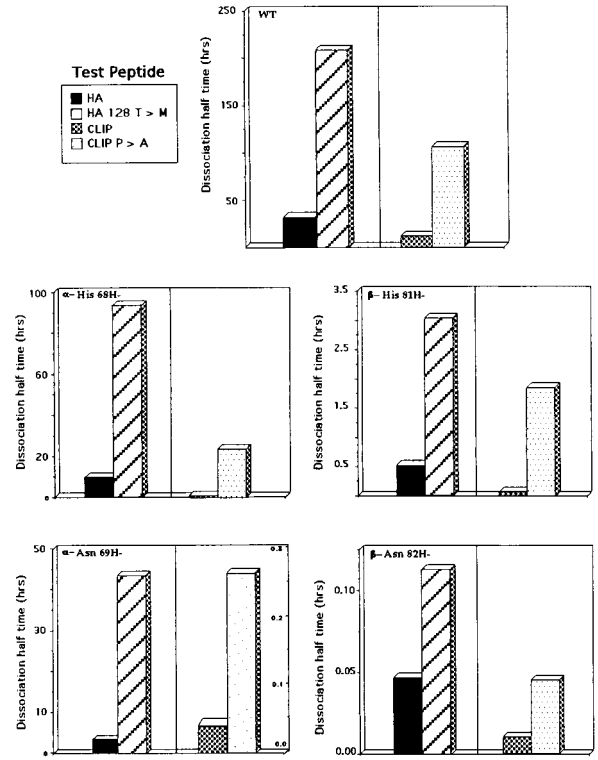

Fig. 4. Peptides bind to wt class II and hydrogen bond variants in the same register. Wild-type HA-(126–138) (filled bars) is compared with an HA mutant peptide stabilized at P1 (126–138, T^{128} → M) (hatched bars) on the *Left* side of each graph. The *Right* side of each graph compares CLIP-(85–99) (heavy stipples) with CLIP-(85–99) P$_{95}$ → A, stabilized at P6 (light stipples). The class II variant tested is indicated in the *Top Left* of each graph. The expected binding register for the peptides are CLIP-(85–99)·K-P-V-S-Q-M(P1)-R-M-A(P4)-T-P(P6)-L-L-M(P9)-R and HA-(126–138)·H-N-T(P1)-N-G-V(P4)-T-A(P6)-A-S-S(P9)-H-E

enhanced P1) for the wt I-A^d molecule. CLIP (Pro96 → Ala) displays a 3-fold enhanced stability relative to native CLIP on wt I-A^d and HA (128T → M) shows an 8-fold enhanced stability relative to native HA on wt I-A^d. The rest of Fig. 4 shows that each of the variant peptides displays an enhanced kinetic stability on each of the hydrogen bond-deficient class II molecules. We have also mutated HA and CLIP at other residues, and, in each case, the pattern displayed by wt class II molecules (i.e., enhanced or diminished kinetic stability) is displayed by the hydrogen bond-deficient variants (unpublished results). These experiments allow us to conclude that the initial complexes formed between wt I-A^d and the hydrogen bond variants have the same register. The results also indicate that the integrity of the MHC class II molecule is maintained in the variant I-A^d proteins.

Another question that may arise from our findings is whether fluorescein-labeling peptide at its amino terminus might poten-

Table 2. Peptides analyzed for binding to I-A^d and hydrogen bond-deficient variants

Peptide	wt $t_{1/2}$	81 $t_{1/2}$	81 RE	68 $t_{1/2}$	68 RE	82 $t_{1/2}$	82 RE	69 $t_{1/2}$	69 RE
CysC-(40–55)	190	14	13	220	1.1	0.71	270	22	9
E-α-(52–67)	170	1.9	90	51	3	0.16	1000	20	8
HA-(126–138)	30	0.5	62	9.7	3	0.05	670	2.4	13
mIi-(85–99), pH 5.3	12	0.08	150	0.95	12	ND	ND	0.04	290
mIi-(85–99) P95 → A (P6 ↑)	110	1.8	57	24	4	0.05	2100	0.27	390
mIi-(85–99), pH 7.4	76	0.4	190	8.4	9	0.03	2600	0.44	170
HA-(126–138) T128 → M (P1 ↑)	210	3	68	94	2	0.11	1800	44	5
E-α-(52–67) COOH	160	1.5	110	64	2.5	NT	NT	NT	NT
Ii-(85–99) COOH	6.1	0.05	120	0.9	6.8	NT	NT	NT	NT

The amino acid position in I-A^d where hydrogen bond has been eliminated. wt, wild type I-A^d; $t_{1/2}$, hours. RE refers to the enhancement factor of peptide dissociation displayed by the hydrogen bond variant relative to wt class II and is calculated by dividing the $t_{1/2}$ dissociation of peptide from wt class II by the $t_{1/2}$ displayed by the hydrogen bond-deficient variant. ND, binding not detected. NT, not tested. Peptides were labelled at their NH_2 terminus except where noted COOH. Anticipated changes in pocket interactions are indicated in parentheses for variant peptides.

tiate the destabilization of the peptide·class II complex in this region and provoke an asymmetric effect. To address this possibility, we compared dissociation kinetics of two representative peptides [CLIP-(85–99) and E-α-(52–68)] labeled either at their carboxyl or amino termini, as described in *Materials and Methods*. If the amino-terminal fluorescein modification were responsible for the greater apparent contribution of β-His-81 and β-Asn-82 compared with α-His-68 and α-Asn-69, peptides labeled at the carboxyl terminus would show less of an effect from the β-His-81-H$^-$ and β-Asn-82-H$^-$ variants. Table 2 shows that this is not the case—the position of the label has little effect on peptide dissociation from both wt molecules and hydrogen bond-deficient variants. These results confirm our conclusion that the hydrogen bonds between the MHC and the peptide main chain at the amino terminus of the bound peptide contribute uniquely and disproportionately to peptide binding stability.

Discussion

MHC class II·peptide complexes are characterized by a latticework of hydrogen bonds between the peptide main chain and the MHC class II α helices. The hydrogen bonds are spaced at regular intervals spanning the length of the peptide-binding groove. The experiments presented here were initiated to determine whether different hydrogen bonds make detectable contributions to the stability of class II·peptide complexes, and whether symmetric hydrogen bonds contribute equally to the stability of class II·peptide interactions. Our studies lead to the unexpected but compelling conclusion that, although each of the four residues studied do, in fact, contribute to peptide stability, those hydrogen bonds localized to the amino terminus of the peptide contribute profoundly and disproportionately to the stability of peptide·class II interactions. This conclusion is consistent with observations made with DR molecules, using chemical modifications of the peptide to assess the contribution of hydrogen bonds in peptide·class II stability (22) and with conformational analyses showing that formation of the peptide amino-terminal backbone hydrogen bonds is critical to the nucleation of a mature MHC protein conformation (23).

There are several potential mechanisms that might account for such an apparent disparity between the intermolecular hydrogen bonds at the two ends of the peptide binding groove. The first is that proximity to a dominant anchor-pocket interaction determines the relative contribution of neighboring hydrogen bonds. If, for example, I-A^d molecules primarily use the P1 pocket for anchoring the peptide, destabilization close to this site, such as elimination of the hydrogen bonds contributed by β-81 or β-82, might disproportionately destabilize peptide binding to I-A^d. Several pieces of data argue against this possibility. First, the crystal structure of HA-I-A^d suggests that this peptide does not engage the P1 pocket any more than it does the P4 or P9 pocket, yet this peptide demonstrates the profound impact of loss of hydrogen bonds close to the P1 pocket. Second, our studies point to the general importance of hydrogen bonds at the peptides' amino termini, and yet the recognized peptide binding motif of I-A^d appears to rely mainly on interactions at the P4, P6, and P9 pockets (6, 24). Our own experiments using variant peptides also argue against the idea that dominant pocket interactions dictate sensitivity to particular hydrogen bonds. The variant CLIP peptide shown here had an enhanced P6 interaction and the variant HA peptide had an enhanced P1 interaction, but both peptide variants maintained disproportionate sensitivity toward amino-terminal hydrogen bonds.

Two non-mutually exclusive possibilities can explain the profound apparent contribution of the hydrogen bonds at the peptides' amino termini. It is possible that the carboxyl-terminal region of the peptide has more mobility or rotational freedom than the amino-terminal region. Accordingly, the hydrogen bonds at the carboxyl terminus would be less fully engaged and contribute less to the overall stability of class II·peptide interactions. Loss of these hydrogen bonds would have a relatively minor impact. The finding that there is no obvious difference in B-values for atoms at the N and C termini of the bound peptides in class II (6, 7) may argue against this possibility, but better understanding of this issue requires a specific assessment of the dynamics of peptide·class II complexes in solution. Alternatively or additionally to this first possibility, one can speculate that the high impact of hydrogen bond loss at the peptides' amino termini reflects a particularly pronounced degree of cooperativity among the interactions between peptide and class II molecules in this region. By this model, individual bonds between class II molecules and peptide would be critically dependent on the integrity of neighboring interactions. Destabilization of one bond such as that contributed by β-His-81 would cause a rapid destabilization of adjacent (although chemically distinct) bonds. Although we have not addressed what sites in the class II molecule might cooperate with β-His-81 and β-Asn-82, one particularly appealing candidate is the α-chain region immediately across the P1 peptide binding pocket (see Fig. 1). This region, between α-51 and α-54 is in an extended conformation, unlike the majority of the MHC class II α helices. This segment is also markedly different from the analogous region (residues 58–80) in the MHC class I molecules (1, 17), which maintains α helical structure. A noteworthy element of this segment of class II α-chains, observed in crystal structures, is a set of genetically conserved hydrogen bonds between α-53 and the peptide main chain. Unlike the hydrogen bonds studied in our experiments, this set of hydrogen bonds connects the main chain of the class II α helix to the bound peptide, and its participation in class II·peptide interactions is insensitive to MHC sequence. We speculate that the three sets of hydrogen bonds at the amino terminus of the bound peptide (i.e., those contributed by α-53, β-81, and β-82) that are conserved in all class II·peptide crystal structures identified thus far form a cooperative network that critically regulates peptide dissociation from the class II molecule.

Although our study clearly demonstrates that the hydrogen bonds between β-His-81 and β-Asn-82 and the bound peptide are critical for peptide·class II complex stability, it is not yet possible to conclude whether these hydrogen bonds are uniquely important in this regard. We have not yet explored the contribution of other highly conserved hydrogen-bonding residues, such as those localized closer to the interior of the binding pocket (e.g., β-71 or Asn-α-62) or other amino acids at the periphery, such as Trp-β-61. It should also be pointed out that we do not know whether all allelic and isotypic forms of the class II molecules will be equally sensitive to the loss in hydrogen bonding residues. I-A^d has been suggested to depend less on strong pocket interactions (6), and a question that might be raised is whether this allelic form of murine class II molecules relies to an atypically high degree on hydrogen-bonding interactions. Our recent results suggest that this will not be the case. First, improving pocket interactions between I-A^d and the peptide does not render a peptide more resistant to the hydrogen bond losses (Fig. 4 and unpublished results), nor does weakening of pocket interactions make a peptide more dependent on hydrogen bonds (unpublished results). Secondly, we have initiated studies with the I-A^k molecule, which is thought to have strong pocket interactions with its bound peptides (7, 25). Our analyses thus far involving intracellular trafficking studies suggest that, like the I-A^d molecule, I-A^k molecules with hydrogen bond losses at β-82 or β-81 have a severely compromised ability to stably acquire peptide (unpublished results). Our findings are consistent with work analyzing the binding of the HEL-(42–61) peptide to I-A^k. In this study (26), when hydrogen bonds were disrupted by carbonyl reduction of the peptide, active peptide binding to the class II molecule was lost. It is conceivable that

there may be some peptide·class II complexes that will pass a sufficiently high threshold in cumulative binding energy such that they become resistant to hydrogen bond losses, but our experiments performed to date suggest that these peptides will be very rare.

The surprisingly large contribution of hydrogen bonds at the peptides' amino termini is interesting to consider in light of the speculated mechanism of peptide exchange catalyzed by HLA-DM. The relative enhancement of peptide dissociation rates observed for β-His-81 and β-Asn-82 is comparable to DM-catalyzed rate enhancements for other class II molecules (27–29). This observation suggests that DM could function by stabilizing a peptide·MHC intermediate in which one or more hydrogen bonds between the MHC molecule and the peptide are disrupted. Our findings of energetic asymmetry support the speculation by Wiley and colleagues (30) that the primary activity of DM may be the destabilization of one or two hydrogen bonds at the amino terminus of the peptide. This possibility is supported by several independent experimental observations. The CerCLIP antibody, which reacts with the amino terminus of the Ii-derived CLIP segment, blocks DM function (31). Also, a single amino acid mutation in the DR P1 pocket that alters the conformational state of the class II molecule also modulates its interactions with DM (32). Finally, and most convincingly, random mutagenesis of DR molecules has identified a lateral face of DR that is a critical site of productive interaction of DR molecules with DM (33, 34). This region of DR is in close proximity to the amino terminus of the bound peptide. Thus, the DM mechanism may involve a cooperative, progressive disruption of binding interactions initiated at the amino terminus of the bound peptide. Regardless of whether destabilization of hydrogen bonding is indeed the mechanism by which DM promotes peptide exchange on the class II molecule, our results suggest that physiologically relevant enhanced peptide dissociation rates can be achieved without any initial global changes in MHC conformation by disrupting a single, solvent-exposed, amino-terminal hydrogen bond.

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