Peptide-Binding Motifs for the I-A^d MHC Class II Molecule: Alternate pH-Dependent Binding Behavior†

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ABSTRACT: The ability of peptides to form stable complexes with MHC class II molecules expressed in the host determines their ability to recruit CD4 T cells during an immune response. In this study, we sought to define the features of the antigenic peptides that control their kinetic stability with I-A^d because of the diversity of peptides that this molecule is known to present. Peptide dissociation assays indicated that each pocket of I-A^d displays exquisite sensitivity to side chain structure, size, and charge. Most surprising were results related to the P1 pocket, which has been difficult to define by conventional competition assays. Our studies revealed a considerable degree of specificity in the P1 pocket but also an unexpected degree of structural flexibility. Amino acids with neutral side chains such as Met and the alternatively negatively charged Glu are both highly favored at P1. Interestingly, these two options at the P1 pocket in I-A^d display dramatically different pH-dependent interactions with the class II molecule. These findings are discussed in the context of a structural model to explain these data and in light of the immunological implications of pH-dependent behavior of class II−peptide complexes in acidic endosomal compartments, where DM-catalyzed loading of class II molecules takes place, and at the neutral pH of the APC cell surface, where class II−peptide complexes promote activation of CD4 T cells.

Following the discovery that MHC[1] class II molecules have the capacity to bind antigenic peptides, significant effort in identifying and quantifying the specific interactions between peptide and MHC molecules has been put forth. Many class II molecules have structural features that correspond well with known binding motifs of the peptides presented by these MHC molecules. For example, the P1 pockets of human DR1 (*1*, *2*) and of murine I-A^b class II molecules (*3*, *4*) are large and lined with hydrophobic residues, selecting strongly for amino acids with bulky hydrophobic side chains at this position, such as Phe and Tyr, while the I-A^k molecule has a strong preference for negatively charged amino acids at P1 (*5*), binding behavior that agrees with the cocrystal structure of I-A^k with bound peptide (*6*). In contrast, there are examples of MHC molecules for which the peptide binding motifs have been more difficult to define. These MHC molecules have been described as promiscuous (*7*−*10*). The extensively studied I-A^d molecule (*8*, *11*−*16*) is perhaps the best example of this. Early studies (*12*, *13*) defined a six-amino acid motif responsible for binding, now known to represent the P4, P6, and P9 pockets (*17*). The specificity of these pockets was apparently low, and results of cocrystallization of I-A^d−peptide complexes (*17*) were consistent with these early studies, which concluded that high-affinity interactions between peptide and this MHC molecule can be achieved without large side chain−pocket interactions.

Understanding the structural interactions that control the binding of antigenic peptides to MHC class II molecules is important in a number of regards. Better algorithms for predicting MHC binding would be extremely useful for vaccine design and epitope identification (*18*, *19*). Also, clarification of the nature of peptide−MHC class II interactions is important in understanding DM editing within antigen presenting cells (APC) (*20*−*25*). Our laboratory is particularly interested in understanding peptide−MHC class II interactions because of our recent evidence that immunodominance in CD4 T cell responses restricted by I-A^d can be both predicted and controlled by variation in the kinetic stability of peptide−class II complexes (*21*, *26*). This discovery has led us to perform a more comprehensive evaluation of binding of peptide to the I-A^d protein. In earlier work using several model peptides, we found that I-A^d has clear pocket preferences (*14*, *27*). In this paper, we describe the results of extensive studies using peptides known to be immunodominant in I-A^d-expressing mice. We show first through the use of peptide dissociation assays that the I-A^d molecule has exquisite preferences in each of its primary pockets. Second and most surprising was our finding that

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[1] Abbreviations: CHO, Chinese hamster ovary; CLIP, class II invariant chain-derived peptide; DDM, *n*-dodecyl maltoside; HA, hemagglutinin; HPLC, high-performance liquid chromatography; LACK, *Leishmania*-activated C kinase; MHC, major histocompatibility complex; MYO, myoglobin; OVA, ovalbumin; PBS, phosphate-buffered saline; PI, phosphatidylinositol; SEC, size exclusion chromatography.

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I-A^d has two strikingly different mechanisms for achieving stable peptide binding through its P1 pocket, leading to presentation of peptides that have opposite pH-dependent binding behaviors at the two pH values that are relevant to class II molecule function.

EXPERIMENTAL PROCEDURES

Purification of Soluble I-A^d Proteins. The soluble I-A^d molecules were isolated as described previously (*28*). Briefly, a total of 1×10^{10} I-A^d PI CHO cells were solubilized with 1.5 L of 50 mM Tris, 150 mM NaCl, 1 mM *n*-dodecyl maltoside (DDM), and 0.025% NaN_3 containing protease inhibitors. Class II molecules were isolated using antibody affinity chromatography. After PI cleavage, the soluble I-A^d was eluted from the column at pH 11.0 and fractions containing class II were pooled and dialyzed against PBS (pH 7.4) containing 0.2 mM DDM and 0.025% NaN_3 (DDM/PBS). The class II protein was concentrated with a Centriprep YM-10 device (Millipore Co., Bedford, MA) and stored at 4 °C in DDM/PBS.

Peptides. Peptides N-terminally labeled with fluorescein were purchased either from Invitrogen Life Technologies (Grand Island, NY) or from Biopeptide Co. LLC (San Diego, CA) or generously donated by C. Beeson (Medical University of South Carolina, Charleston, SC). Unlabeled Eα[52−68], synthesized in the core peptide facility at the University of Chicago (Chicago, IL), was included to prevent rebinding of the dissociating peptide. The peptides were subjected to mass spectrometry and reverse phase HPLC to verify a purity of >90%.

Binding Experiments. One microliter of 250 μM labeled peptide was added to 25 μL of 100 nM I-A^d molecules, with 5 μL of 10× pH 5.3 McIlvaines buffer, to a final volume of 50 μL adjusted with DDM/PBS, and then incubated at 37 °C for 2−20 h (*16*). The I-A^d−peptide complex was isolated from the free peptide by being passed over a Micro Bio-Spin 30 column (Bio-Rad Laboratories Inc.). For dissociation at different pH values, the Micro Bio-Spin 30 column was equilibrated with DDM/PBS and McIlvaines buffer at the dissociation pH. Dissociation was performed by incubating the complex for increasing periods of time at 37 °C in the presence of unlabeled 5 μM Eα. Aliquots were analyzed by HPLC with a BioSep SEC S 3000, 300 mm × 7.8 mm column (Phenomenex Inc., Torrance, CA), run at a flow rate of 0.8 mL/min with DDM/PBS. The emission intensity of the complex was measured at 525 nm with a fluorescence detector.

Molecular Dynamics Simulations. To explore the possibility of an interaction between Glu128 and a residue within the P1 pocket of a MHC class II molecule, molecular dynamics was performed in a manner similar to that described previously (*29*) using the cocrystal structure of an HA peptide bound to I-A^d [PDB entry 2IAD (*17*)]. The complex was subjected to 1.625 ps of molecular dynamics (0.0005 ps time steps) at 298 K and a dielectric of 40 with either charged or uncharged histidines using CNS (*30*). Harmonic restraints (10 kcal mol^{-1} Å^{-2}) were applied to the positions of Cα atoms, and waters were excluded from the simulation. The average *xyz* Cα displacement between the native peptide−MHC complex subjected to molecular dynamics and the native peptide−MHC complex crystal structure was calculated with CCP4 (*31*) and found to be less than 0.8 Å. Next, peptide position 128 was mutated to glutamic acid, and an appropriate side chain rotamer was chosen using the interactive graphics program O (*32*). Molecular dynamics was performed on the mutant T128E peptide−MHC complex using the aforementioned parameters except the mutated residue was not harmonically restrained. The resulting models were inspected for potential interactions involving the mutant Glu128 residue. Interactions were classified as hydrogen bonds if they demonstrated both reasonable geometry and a distance between the non-hydrogen atoms of polar character ranging from 2.65 to 3.60 Å.

RESULTS

I-A^d Exhibits Exquisite Pocket Preferences for Stable Peptide Binding. We initiated these studies to dissect the relationship between the kinetic stability of peptide−MHC class II complexes and the biological properties of peptide−class II complexes such as immunodominance in CD4 T cell response in vivo and DM editing that occurs in APC. To explore this issue, we used the kinetic stability of peptide−class II complexes as a highly sensitive measure of peptide−MHC "affinity" (*26*). To determine the impact of particular residues at defined pockets (P1, P4, P6, and P9), we used variants of the influenza HA[126−138] peptide that has a well-defined register with I-A^d (*17*) (Table 1). These studies show that although I-A^d was originally defined to have a highly "promiscuous" six-amino acid binding motif (*12*), encompassing P4−P9, all the pockets have a dramatic influence on binding of the peptide to I-A^d. For example, the amino acid residue at P4 in the peptide can influence the off-rate significantly, with the substitution of Ala for Val reducing stability by 40-fold. We found that P6 favored peptides with small side chains, with Ala preferred 4-fold over Val. Similarly, the amino acid residue at P9 in the peptide can dramatically alter kinetic stability with I-A^d, with Ser apparently most preferred, while Met and Asn are highly destabilizing, reducing kinetic stability by 20- and 100-fold, respectively. Most striking perhaps was the finding that the P1 pocket of I-A^d, which has been ignored (*9*, *11*−*13*) or described as promiscuous (*8*), has profound influences on binding to I-A^d. Met appears to be the most favorable for the I-A^d P1 pocket, while Gly is the most unfavorable. These P1 amino acids substituted for the Thr residue in the wild-type (WT) HA sequence can either enhance binding by 6-fold (Met) or weaken binding by 30-fold (Gly). Thus, a single amino acid change at P1 can alter binding to I-A^d by more than 150-fold. These studies allowed us to assemble a binding register hierarchy for I-A^d, shown in Figure 1, that shows the relative influence of amino acid side chains at P1, P4, P6, and P9. It should be noted that the primary substitutions that have been explored in our studies are those that are anticipated to maintain T cell recognition. Therefore, the data in Figure 1 most likely under-represent the magnitude and number of negative substitutions that can be made in I-A^d-binding peptides.

Peptides that Display High-Kinetic Stability Interactions with I-A^d and that Are Immunodominant in Immune Responses Bind to I-A^d with Glu at the P1 Position. The hierarchy map of pocket preferences was then used to tentatively align other immunodominant peptides that are

Table 1: Half-Lives of I-A^d−Peptide Complexes[a]

Peptide bound to I-A^d	SEQUENCE (Putative register) 1 4 6 9	$t_{1/2}$ (h) pH 5.3	$t_{1/2}$ (h) pH 7.4	Pocket
Myo[102-118]WT	K Y L E F I S E A I I H V L H S R	260	139	na
Eα[52-68]WT	A S F E A Q G A L A N I A V D K K	96	217	na
CLIP[85-99]WT	K P V S Q M R M A T P L L M R	7	89	na
T128M	H N M N G V T A A S S H E	165	365	P1
T128E	H N E N G V T A A S S H E	161	65	P1
T128V	H N V N G V T A A S S H E	85	160	P1
T128Q	H N Q N G V T A A S S H E	63	132	P1
HA[126-138]WT	H N T N G V T A A S S H E	26	59	na
V131L	H N T N G L T A A S S H E	19	32	P4
T128V, S136T	H N V N G V T A A S T H E	9	57	P1, P9
S136A	H N T N G V T A A S A H E	8	32	P9
A133V	H N T N G V T V A S S H E	6	21	P6
S136G	H N T N G V T A A S G H E	4	28	P9
T128D	H N D N G V T A A S S H E	2.7	0.6	P1
S136M	H N T N G V T A A S M H E	2	9	P9
T128G	H N G N G V T A A S S H E	1	13	P1
V131A	H N T N G A T A A S S H E	0.9	6	P4
S136N	H N T N G V T A A S N H E	0.3	2	P9
Lack[161-173]WT	S L E H P I V V S G S W D	200	70	na
E163T	S L T H P I V V S G S W D	14	72	P1
I166A	S L E H P A V V S G S W D	2	1	P4
OVA[273-288]WT	M E E R K I K V Y L P R M K M E	160	26	na
E275Q	M E Q R K I K V Y L P R M K M E	30	100	P1
E275T	M E T R K I K V Y L P R M K M E	14	60	P1

[a] The dissociation kinetics were determined at 37 °C and pH 5.3 or 7.4. Data represent the results of two to five different experiments.

presented by I-A^d, including MYO[102−118], OVA[273−288], and LACK[161−173] (Table 1). Surprisingly, the alignments that seemed most favorable placed the negatively charged Glu residue at the P1 position. This amino acid seemed unlikely to bind well to I-A^d, on the basis of previous studies that suggested that, although promiscuous, the side chains at P1 that tended to be favorable for binding were uncharged. However, each of these three peptides binds very stably to I-A^d, with dissociation half-times of 160 h (OVA), 200 h (LACK), and 260 h (MYO). Also arguing in favor of the possibility that Glu might occupy the P1 position in binding to I-A^d are recent studies by Unanue and colleagues who found that many peptides that are eluted from I-A^d have plausible registers with Glu at the P1 pocket (*33*). To test whether the Glu was indeed the P1 pocket residue for LACK-[161−173] and OVA[273−288], this residue was altered to Thr. Our studies with HA indicated that Thr was a relatively poor P1 pocket residue for I-A^d (ref *27* and Table 1), and we thus predicted that if the Glu in LACK or OVA was the P1 anchor residue for these peptides and they were changed to Thr, the modified peptides would display more rapid dissociation from I-A^d. Indeed, this was observed to be the case (Figure 2A,B). Both LACK[161−173] and OVA[273−288] exhibited dramatically reduced (10−12-fold) stability with I-A^d when Glu was changed to Thr. This result both supports the register of these peptides as indicated and suggests not only that Glu was tolerated but also that Glu was in fact a favored residue at P1. To further test the quantitative contribution of Glu at P1, the HA[126−138] peptide was altered at this residue. Figure 2C shows that

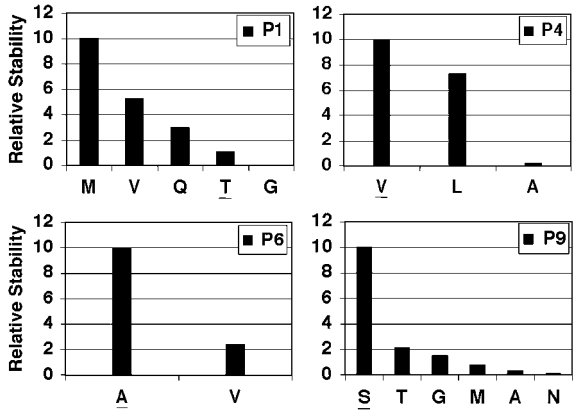

FIGURE 1: Class II molecule I-A^d displays a high degree of specificity in each of its four pockets. The sequence of HA[126−138] was changed at P1, P4, P6, and P9 to the amino acid shown on the abscissa. The indicated peptides were tested for their kinetic stability with I-A^d. The half-life for each peptide variant, expressed as a $t_{1/2}$ value in Table 1, is represented on an arbitrary scale, with the most stable residues at a given pocket indicated to the left and as a value of 10. Underlined residues at each pocket belong to the WT sequence of HA[126−138]. Data are representative of two to five different experiments.

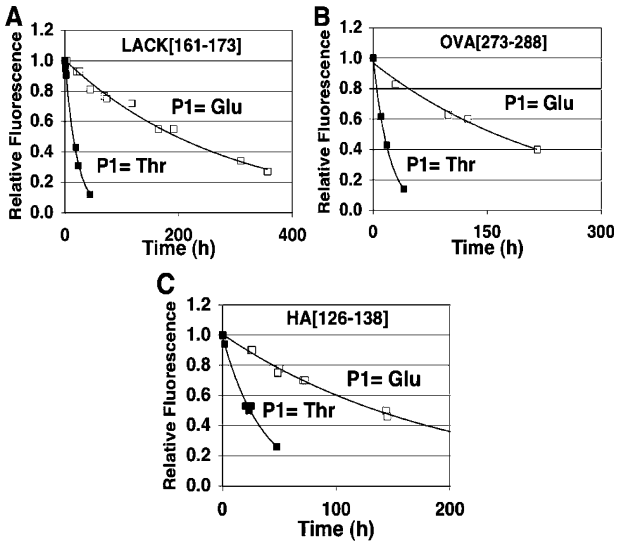

FIGURE 2: Glutamic acid at the P1 position of the peptide is highly favored for binding to I-A^d. Fluorescein-labeled peptides containing either Glu (□) or Thr (■) at P1 were incubated with soluble I-A^d at pH 5.3 and 37 °C for 16 h. In (A) LACK[161−178] and (B) OVA[273−288], Glu residues at positions 163 and 275, respectively, were changed to Thr residues. (C) In HA[126−138], the Thr at position 128 was changed to Glu. Shown are dissociation curves at pH 5.3 and 37 °C.

changing Thr to Glu at P1 enhances the stability of HA from a half-time of 25 to 160 h. This places Glu at P1 at the very top of the side chain hierarchy for binding to I-A^d, comparable to the uncharged Met.

Anomalous pH Binding Behavior with Peptides Containing Glu at P1. The finding that I-A^d forms very favorable interactions with the negatively charged Glu at P1 was surprising on the basis of the crystal structure of I-A^d with antigenic peptides. Neither of these structures revealed that a positively charged residue within the P1 pocket of I-A^d would be available to interact with Glu. A clue about the nature of the interaction between negatively charged residues

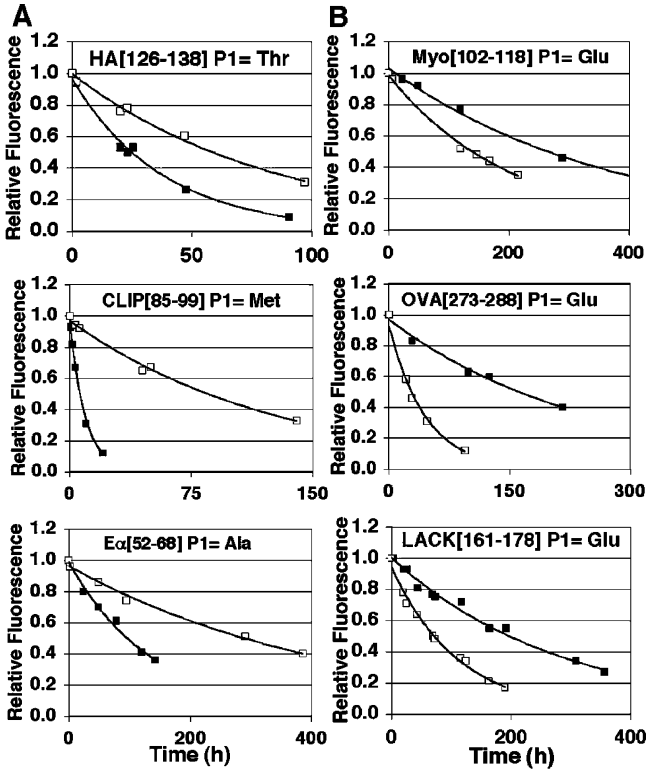

FIGURE 3: I-A^d−peptide complexes with Glu at P1 display atypical pH-dependent dissociation behavior. After incubation of the indicated fluorescein-labeled peptides with soluble I-A^d molecules at pH 5.3 and 37 °C for 16 h, complexes were isolated using a column pre-equilibrated with citrate/phosphate buffer at pH 7.4 (□) or 5.3 (■). Dissociation was performed by diluting the isolated complex in the same pre-equilibration buffer to allow the dissociation at 37 °C. Indistinguishable data were obtained when conditions used to form and isolate the complex were fixed at pH 7.4 or 5.3 (not shown).

at P1 with I-A^d came from our studies of the pH behavior of the peptide−class II complexes. Binding of peptide to MHC class II molecules shows interesting pH-dependent behavior. Peptide acquisition by class II molecules is typically facilitated by acidic pH (*34*−*38*), a behavior that is thought to reflect pH-dependent unfolding of class II molecules (*36*, *39*, *40*), allowing class II molecules to display a peptide-receptive conformation at acidic pH. This model is supported by the dissociation behavior of peptide−class II complexes. Typically, peptides display accelerated off-rates from class II at acidic pH and extended half-lives at neutral pH (*41*−*44*). Shown in Figure 3A are the pH-dependent dissociation curves of I-A^d with several well-known peptides (*11*, *14*). Each peptide displays slower dissociation from class II molecules at neutral pH than at acidic pH. When the peptides containing Glu at P1 were similarly examined, they all displayed the opposite behavior (Figure 3B). While MYO, OVA, and LACK all displayed very stable binding to I-A^d at acidic pH, they displayed accelerated off-rates at neutral pH, being from 3- to 10-fold faster.

To confirm that this unusual pH behavior was due to the amino acid residue at P1, rather than some other feature of the peptide−class II complexes, variant peptides were tested. The P1 residue of OVA[273−288] was changed from negatively charged Glu to the neutral Gln, to make a conservative change, while altering charge. Similarly, the

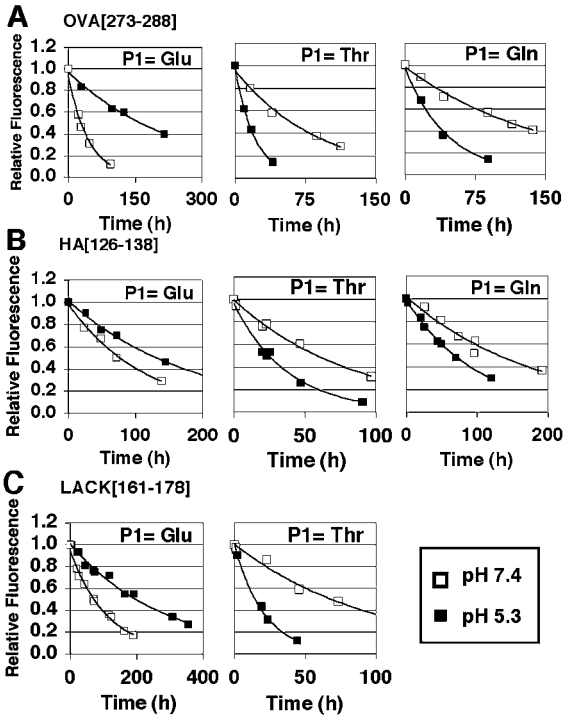

FIGURE 4: Glu at P1 is necessary and sufficient for atypical pH dissociation behavior of peptide−I-A^d complexes. MHC−peptide dissociation assays with soluble I-A^d at pH 7.4 (□) and 5.3 (■) were performed as previously described in Experimental Procedures using (A) OVA[273−288], where Glu 273 from the wild-type sequence was replaced with Thr or Gln; (B) HA[126−138], where Thr128 from the WT sequence was changed to Glu acid or Gln; and (C) LACK [161−173], where Glu163 was changed to Thr.

P1 Thr residue of HA was changed to either Glu or Gln. Finally, the P1 residue of LACK was changed from Glu to Thr. Each of these altered peptides was compared to the WT peptide to assess whether the substitution was favorable or unfavorable, and both types of peptides were analyzed for dissociation behavior at pH 5.3 or 7.4. Figure 4 shows the results of these experiments, from which two major conclusions were made. First, in all cases, Glu at P1 is associated with the atypical pH behavior of accelerated dissociation at pH 7.4, while all peptides with uncharged residues at P1 display stabilized binding to class II molecules at pH 7.4. For example, although HA maintains the typical extended half-life at pH 7.4 compared to that at pH 5.3 if its P1 pocket residue is changed from Thr to Gln, when Thr is changed to Glu, a reversal in patterns is observed. Now the HA peptide (Thr to Glu) displays accelerated off-rates at pH 7.4 compared to those at pH 5.3. Similarly, altering LACK's or OVA's P1 residue from Glu to Gln causes a reversal in pH behavior. Therefore, Glu at P1 is both necessary and sufficient for the atypical pH behavior of binding to I-A^d. The second major conclusion made is that Glu at P1 in a peptide sequence is very favorable for stable binding to I-A^d. By changing its normal P1 pocket residue from Thr to Glu, HA increases the stability of its interactions with I-A^d 6-fold. Conversely, LACK is destabilized more than 10-fold by the alteration from its normal Glu at P1 to Thr.

A Salt Bridge at the Base of the I-A^d P1 Pocket. The high-affinity binding of Glu-containing peptides to I-A^d at pH 5.3, but reversal of the advantage at pH 7.4, has important structural and biological implications. We presume that the class II molecule bound by these Glu-containing peptides displays the typical pH-dependent conformational states, with the pH 5.3 conformation relatively destabilized ("open") and the pH 7.4 conformation more stable ("closed"). Therefore, the accelerated off-rate of these peptides at neutral pH suggests that a favorable interaction within the P1 binding pocket at pH 5.3 becomes unfavorable at pH 7.4. The most likely scenario for explaining this behavior is that negatively charged Glu forms an intermolecular salt bridge with a positively charged residue in the class II binding pocket at acidic pH but that this salt bridge is lost at neutral pH. Because the pK_a of Glu (approximately 4.1) is outside the pH range changed in this experiment (from 5.3 to 7.4), it is likely that this residue in the peptide maintains its negative charge at both pHs. The only positively charged amino acid whose pK_a changes between pH 5 and 7 is His, which would be positively charged below approximately pH 6.5, while at pH >7, it would become deprotonated, thus returning the I-A^d P1 pocket to a neutral state, where Glu in the peptide would now become unfavored. Inspection of the I-A^d crystal structure does not reveal any His residues exposed in the P1 pocket when OVA[323−339] or HA[126−138] is bound, with their respective Ser or Thr side chains at P1. Possible interactions with Glu128 and MHC class II residues in the P1 pocket that could be responsible for the enhanced stability were considered. Notably, Hisα24 is in the proximity of the P1 pocket, as identified in the I-A^d crystal structure (*17*) as noted previously (*33*), and has the potential at low pH to interact with Glu by means of a positively charged imidazole ring (pK_a ∼ 6.8). To lend support to the possibility of an interaction between the charged form of Hisα24 and peptide mutant T128E, molecular dynamics simulations were performed. In simulations where Hisα24 was positively charged, Oε2 of Glu128 was predicted to be less than 3.2 Å from Nε2 of Hisα24. Furthermore, the orientations were consistent with a robust salt bridge interaction between the two formally charged side chains, enhanced by the hydrophobic local environment within the P1 pocket. In contrast, the simulations

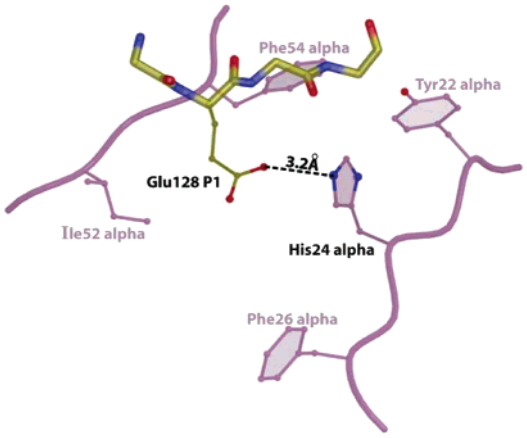

FIGURE 5: Orientation of Glu128 (P1) and Hisα24 side chains resulting from molecular dynamics performed under conditions of charged histidines. The peptide backbone from position P−1 to P3 is depicted as a yellow tube. Oxygens are colored red and nitrogens blue. Nearby hydrophobic P1 pocket residue side chains are depicted in light purple extending from a trace of the MHC backbone represented by the purple wire. A favorable salt bridge interaction between the Glu128 side chain and the charged Hisα24 imidazole ring is indicated by a dashed black line.

predicted only a weak hydrogen bond between Oϵ2 of Glu128 and Nϵ2 of Hisα24 when Hisα24 was uncharged. Therefore, we conclude that the positive interaction of Glu in the P1 pocket of I-A^d is likely due to an optional intermolecular salt bridge between the antigenic peptide and the MHC class II molecule that can occur when Glu is in the large P1 pocket. At neutral pH, it is likely that Hisα24 becomes uncharged and the negatively charged Glu has no complementary interactions in the now neutral P1 pocket. This shift in charge causes accelerated dissociation of these peptides at pH 7.4 despite the presumed return of the MHC class II pocket to the more relaxed and native state (*39*, *40*).

DISCUSSION

In this paper, we have explored the structural requirements for high-affinity peptide binding by the murine I-A^d molecule. We have found that each pocket displays exquisite sensitivity to side chain size, structure, and charge. Moreover, each of the four major pockets can contribute dramatically to the overall kinetic stability of peptide−class II complexes. In some cases, substitutions at anchor residues in the peptide that caused major alterations in kinetic stability make intuitive sense on the basis of the class II pocket size and shape observed in the crystal structure of I-A^d−peptide complexes (*17*). For example, the fit of the large P1 pocket was improved by substituting the small polar Thr residue with the larger polar amino acid Met. In other cases, the specificity of the pockets was more difficult to predict. For example, the P9 pocket appeared to be only partially filled by Ser in the I-A^d crystal structure (*17*), and we expected that the slightly larger Thr residue would more fully occupy this pocket and thus enhance binding. However, this substitution in the HA peptide weakened binding to I-A^d by more than 8-fold. These results suggest that in many cases, binding preferences between peptide and MHC class II molecules need to be quantified empirically. However, once established, the effect of alternate amino acids at a given position can be extrapolated to a new peptide. In the analyses of six independent peptides (refs *14*, *26*, and *27* and data not shown), a given substitution at a particular anchor residue leads to the predicted enhancement or attenuation of binding to I-A^d. From ref *26* and related studies (*14*, *27*), we can now rationally and reproducibly modulate the kinetic stability of peptide−class II complexes and cause a corresponding change in the immunodominance of that peptide in an immune response (*21*, *26*).

Most surprising of the results with pocket substitutions were those related to the P1 pocket, which has been difficult to define by conventional competition assays. Our dissociation assays revealed a considerable degree of specificity in the P1 pocket but also an unexpected degree of flexibility that allows stable binding of two chemically distinct classes of peptides, which can be distinguished by their pH-dependent interactions with the class II molecule. There are two different consequences to the choice of peptides selected by I-A^d, depending on the amino acid side chain in the P1 pocket. First, the area surrounding this pocket in I-A^d may have a different structure if Glu is present in this position rather than an uncharged amino acid, because formation of the stabilizing salt bridge may require local rearrangements in this region. These changes could modify the overall topology of the class II molecule in this region. Second, the kinetic stability at neutral and acidic pH will not parallel each other for these two different classes of peptides. Peptides with uncharged amino acids at P1 will display the most commonly observed pH-dependent behavior of class II−peptide complexes, with extended kinetic stability at neutral pH compared to that at acidic pH. In contrast, those peptides with Glu at P1 will more rapidly dissociate at neutral pH. Therefore, on an APC presenting peptides from a complex antigen expressing both types of peptides, the epitope density hierarchy will substantially change over time.

A similar type of anomalous pH behavior of peptide−MHC class II complexes displayed by Glu-containing peptides and I-A^d has been noted previously (*42*, *45*). An extensively studied example of this was reported by McConnell and colleagues for the DR4 MHC class II molecule (*42*) and showed that a negatively charged Glu residue at either a major (e.g., P9) or minor (e.g., P3) pocket led to accelerated dissociation at pH 7 compared to that at pH 5. Their model for explaining these data is that the pockets in the class II molecule available to these side chains are neutral. This interaction will tend to shift the pK_a of Glu or Asp to higher values, allowing these amino acids to largely be in the protonated and neutral state at pH 5. Because at acidic pH there will be an equilibrium between the protonated and nonprotonated state, it was hypothesized that the minor deprotonated acidic and thus unfavored form is primarily visualized in the dissociation reaction. At pH 7, the equilibrium shifts and heavily favors the deprotonated and charged state, thus accounting for even more rapid dissociation of the peptide at this pH. There are major differences between those peptide class II complexes and the examples described here. Most importantly, Glu is a favored residue in its respective binding pocket in I-A^d and represents one of the two most favored residues at the P1 position. Importantly, negatively charged Asp, with a shorter side chain, does not provide this favored interaction, suggesting that the high-stability binding at P1 has both size and charge requirements. Our interpretation is that the negatively charged residue, Glu, finds a favorable electrostatic interaction in the interior of the P1 pocket of I-A^d and that a local rearrangement in that region allows a stabilizing salt bridge to form between peptide and MHC. Our molecular dynamics study supports this model. When the pH is increased to 7.4, the His becomes neutral. Subsequently, the negatively charged Glu has no complementary charge, and dissociation of the peptide is therefore greatly accelerated. This behavior contrasts with that of many other MHC molecules with hydrophobic pockets where there is apparently no good option for accommodating a negatively charged amino acid, and therefore, these residues will always be unfavored.

The pH-dependent behavior of the different peptides presented by I-A^d has interesting immunological implications. The two different pH values that we have studied here represent the environments that are important in the biology of the class II molecule. In the acidic environment of endosomal compartments (pH 4.5−5.5), class II molecules replace CLIP with antigenic or self-peptides, an exchange reaction catalyzed by DM (reviewed in refs *46*−*48*). Here, DM edits the peptide repertoire presented by class II molecules by promoting dissociation of some of the newly acquired peptides (*20*, *25*, *49*−*51*). Binding of peptide to class II and interactions with DM are promoted by acidic

pH (*52*), and peptides that bind with high stability to class II molecules are likely to survive negative DM editing in endosomal compartments (*20*, *50*, *53*). Therefore, a greater fraction of these high-stability peptide−class II complexes will be exported to the cell surface, leading to their higher cell surface density relative to peptides that are more readily released from class II molecules by the activity of DM. Once peptide−class II complexes arrive at the cell surface, their spontaneous kinetic stability at neutral pH will determine their persistence and thus their availability to recruit CD4 T cells during the immune response. In most cases, peptides with high kinetic stability at acidic pH will have a competitive advantage at the DM editing phase and also at the cell surface, as the MHC molecule returns to its normal structure at neutral pH. In contrast, the second type of peptide that we have described in this report (with Glu at P1) will have only one of these two advantages. Peptides such as OVA[273−288] will have high kinetic stability at acidic pH and likely will resist negative DM editing in endosomal compartments and will be exported to the cell surface at high density. However, here they will become destabilized and will dissociate more rapidly than other high-stability peptides. The peptides examined in this study, MYO[102−118], LACK[161−173], and OVA[273−288], demonstrate extremely favorable interactions with class II ($t_{1/2}$ > 150 h at pH 5) that confer reasonable stability even at pH 7.4 (>24 h). It is possible that for peptides that have more modest stability with class II molecules, the accelerated dissociation at the cell surface may attenuate their overall immunodominance in a developing immune response.

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