Molecular Modeling and Design of Invariant Chain Peptides with Altered Dissociation Kinetics from Class II MHC†

Michael N. Liang,‡ Christopher Lee,‡ Yu Xia, and Harden M. McConnell*

Department of Chemistry, Stanford University, Stanford, California 94305-5080

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ABSTRACT: We have used molecular modeling to design substitutions in an invariant chain-derived peptide (CLIP), so as to alter the stability of its complex with class II major histocompatibility complex (MHC) proteins. We sought first to test whether CLIP binds in the same way to different class II MHC proteins. We designed destabilizing substitutions of two residues (Met 91 and Met 99) previously predicted to act as the major anchor residues for binding to all class II MHC and measured their effect on CLIP's dissociation rate from a series of three murine I-A MHC proteins. Even a conservative substitution preserving size and hydrophobicity but reducing flexibility (leucine, a branched residue) caused large accelerations in dissociation rates (up to 25-fold) at either position in all three MHC alleles, supporting the consistent role of these positions as the major anchors for MHC binding. These data also support the view that the special flexibility of the methionine side chains at these positions is essential for binding to diverse MHC molecules. We also used molecular modeling to design allele-specific enhancements of peptide binding. Designed substitutions of CLIP Pro 96 by Ala (for A^d), Glu (A^k), and Tyr (A^u) each yielded strong enhancement of binding (up to 128-fold) for their targeted allele and only moderate or destabilizing effects to the other alleles. These results demonstrate the accuracy of the molecular models and the predictive value of this modeling. Moreover, they provide strong evidence for the proposed general model of invariant chain association, indicating that it binds to all class II MHC in the same conformation.

Class II major histocompatibility complex (MHC) molecules are expressed on antigen-presenting cells of the immune system and serve to display foreign antigenic peptides to T-cells. They are composed of two polymorphic, noncovalently associated polypeptide chains ($\alpha\beta$). The first crystal structure of a class II MHC molecule showed that the binding site consists of eight antiparallel β-pleated sheets forming the floor and two α-helices making up the sides of the cleft (Brown et al., 1993). The subsequent crystal structure of a class II MHC−peptide complex showed that the main binding interactions are hydrogen bonds between the peptide backbone and protein side chains as well as various contacts between the peptide side chains and pockets within the protein (Stern et al., 1994). Despite differences in the sequence of peptides that bind to class II proteins, the conserved hydrogen bonds to the peptide main chain force peptide ligands into a similar conformation. The peptides bind in an extended conformation with a polyproline twist to the backbone (Jardetzky et al., 1996); in doing so, certain side chains of the peptide face into the hydrophobic pockets of the MHC molecule, while other side chains point up, presumably for recognition by the T-cell receptor.

An important processing step in class II presentation is the binding of invariant chain protein (Ii) to nascent class II molecules during their assembly in the endoplasmic reticulum (Brodsky & Guagliardi, 1991). Ii stabilizes class II MHC and acts as a chaperone during their transport to the cell surface (Romagnoli & Germain, 1994). The invariant chain also prevents binding of endogenously synthesized peptides to class II MHC molecules, so that their peptide binding sites remain available for presenting exogenous antigens (Bertolino et al., 1991; Elliott et al., 1994; Humbert et al., 1993; Peterson & Miller, 1990; Pinet et al., 1994; Stockinger et al., 1989). Upon arrival in a compartment specialized for the loading of class II molecules, Ii dissociates, permitting loading of foreign peptide antigens. Ii's MHC binding activity has been localized to a set of class II-associated invariant chain-derived peptides (CLIP)[1] spanning residues 83−107 of the extracytoplasmic domain of Ii (Chicz et al., 1992; Freisewinkel et al., 1993; Romagnoli & Germain, 1994; Rudensky et al., 1991).

Much work has addressed the question of how CLIP binds to MHC proteins. A number of studies have provided evidence that binding site polymorphism in both human and murine class II MHC affects CLIP binding as measured by different dissociation rates and IC_{50} values (Avva & Cresswell, 1994; Bangia & Watts, 1995; Geluk et al., 1995; Liang et al., 1995; Malcherek et al., 1995; Sette et al., 1995). We used molecular modeling to evaluate the possibility that CLIP binds within the conventional peptide binding groove for a series of human and mouse class II MHC proteins. We

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* To whom correspondence should be addressed.

‡ The first two authors contributed equally to the work.

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[1] Abbreviations: A^d, A^k, A^u, alleles of the class II MHC I-A molecule; CLIP, class II associated invariant chain peptides; DM, dodecyl maltoside; HPLC, high-performance liquid chromatography; Ii(86−100), peptide representing residues 86−100 (human numbering system) of the invariant chain (KPVSQMRMATPLLMR); fIi(86−100), CLIP peptide labeled with 5- (and 6-) carboxyfluorescein; fIi(86−100)-X*Y, labeled CLIP peptide with amino acid residue X substituted with residue Y at position *; PBS, phosphate-buffered saline.

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predicted a detailed structural model of how CLIP binds to the MHC (Lee & McConnell, 1995). Side chain packing calculations were used to model unknown MHC allele structures (A^d, A^k, A^u, and HLA-DR3) on the basis of the HLA-DR1 crystal structure (Brown et al., 1993) and to predict the structure of bound CLIP on the basis of the conformation of the hemagglutinin 306−318 peptide bound to HLA-DR1 (Stern et al., 1994). A crystal structure of CLIP bound to the human HLA-DR3 (Ghosh et al., 1995) has been solved and demonstrated that CLIP binds within the groove. The predicted computer model and crystal structure match closely, to within 1.2 Å rms for the HLA-DR3 model and 1.1 Å rms for the CLIP model.

Unlike other peptides, CLIP has the unique ability to bind all class II MHC molecules, despite their very divergent binding motifs. CLIP might bind to each MHC allele in a different conformation, so as to position residues that fit its motif within its anchor pockets. Such major shifts have been observed for the peptide thyroid peroxidase 535−551, which binds in different ways to HLA-DQ (Quaratino et al., 1995). Alternatively, CLIP might bind to all class II MHC in the same way, consistently employing the same residues as anchors. Molecular modeling studies of a series of human and mouse class II MHC proteins indicated that CLIP could bind in a consistent way to all (Lee & McConnell, 1995). In this general model, CLIP was predicted to place methionine, a less common amino acid with a highly flexible side chain, in the major anchoring pockets of the MHC (P1 and P9) and the P3 pocket, acting as "side chain adapters" to these polymorphic pockets. Methionine is the only large, hydrophobic side chain with independent torsional flexibility for every atom, essentially a long, snake-like chain useful for sequence-independent binding (Gellman, 1991). The CLIP-DR3 crystal structure (Ghosh et al., 1995) showed that this particular allele is consistent with the model, but the absence of structural information for other CLIP−MHC complexes has prevented verification of this general aspect of the model.

The present study tests the predictive value of our molecular modeling approach. Amino acid substitutions in CLIP were designed to yield specific alterations in the kinetics of peptide dissociation. Some substitutions were expected to stabilize binding and others to destabilize binding. To explore CLIP binding to multiple MHC alleles we designed CLIP substitutions to yield consistent kinetic changes across a series of MHC alleles and others to bind strongly only to specific alleles.

MATERIALS AND METHODS

Molecular Modeling. Models of the murine MHC proteins A^u and A^k from previous work (Tate et al., 1995) were used as the basis for modeling their complexes with the CLIP peptide. Peptide backbone coordinates of the hemagglutinin 306−318 peptide, from the crystal structure of its complex with the class II MHC protein HLA-DR1 (Stern et al., 1994), were superimposed on our A^u and A^k models, as a canonical framework for assessing different CLIP binding modes. To model each hypothetical alignment of CLIP's sequence onto the canonical peptide framework, the CLIP side chains and MHC side chains contacting the peptide were predicted *ab initio* by self-consistent ensemble optimization (Lee, 1994; Lee & Subbiah, 1991), as previously applied to MHC modeling (Lee & McConnell, 1995; Tate et al., 1995). All parameters and procedures were exactly as previously described (Lee, 1994). These initial models were evaluated as described in Lee and McConnell (1995).

The final CLIP−MHC models were modified to refine the region surrounding a two-residue deletion in the β-chain helix in the A^u and A^k alleles at β65−66. Tyr β65 was rebuilt in the context of the existing model by the program SEGMOD (Levitt, 1992), and the whole model was refined by constrained minimization in the program ENCAD (Levitt, 1983), using the LOOK software suite (Molecular Applications Group, Palo Alto, CA) with default settings and parameters. This consisted of 100 steps of constrained conjugate gradient minimization *in vacuo*, followed by an additional 400 steps without constraints. Initial constraints were applied to interatomic distances of Cα further than 20 Å apart, as described in Levitt (1983), using a force constant of 1 kcal mol $Å^{-2}$ and a range d_c of 1 Å. The cutoff distance for nonbonded interactions was 7 Å throughout these calculations, with a smooth truncation function ensuring no discontinuities in the energy gradient across the cutoff threshold (Levitt & Sharon, 1988).

CLIP amino acid substitutions were modeled by self-consistent ensemble optimization, using the LOOK software suite. Only the substituted position and surrounding MHC side chains were modeled *ab initio* in these calculations: for M91L, α28, α36, α47, α56, α58, β86; for P96A/P96E/P96Y, α14, α16, α69, α70, α73, β9, β11, β30; for M99L, α74, α77, β9, β30, β32, β38, β61; and for L97A, β30, β47, β61, β67, β70 (A^u numbering). The calculations were performed using heavy data collection and the linear cooling protocol (Lee, 1994). No conjugate gradient refinement was performed following the SCEO modeling.

Peptide Synthesis. Ii(86−100) (KPVSQMRMATPLLMR) and all CLIP mutants were synthesized on an Applied Biosystems 431A peptide synthesizer (Foster City, CA) using standard Fmoc chemistry. Protected peptides attached to the resin were labeled at their N-terminus using the *N*-hydroxysuccinimide ester of 5,6-carboxyfluorescein (Molecular Probes, Eugene, OR) in dimethyl sulfoxide. Labeled and unlabeled peptides were cleaved from the support resin with trifluoroacetic acid in the presence of radical scavengers ethanedithiol and thioanisole. All peptides were purified by reverse-phase HPLC to >95% using an acetonitrile/water gradient, and their identity was confirmed by mass spectrometry. The concentrations of labeled peptides used for the dissociation experiments were determined by mass and checked by UV spectrophotometry (ϵ_{495nm} = 68 000 M^{-1} cm^{-1}).

MHC Protein Purification. Procedures for class II MHC protein isolation have been described previously (Watts et al., 1984). A^d was obtained from the B-cell lymphoma A20−1.11, while A^k and A^u were obtained from BW5147.G.1.4 cells transfected with the appropriate α- and β-chain cDNA (Mason et al., 1995). Antibody affinity columns were used in the purification of protein; the monoclonal antibody MKD6 (Kappler et al., 1981) was used in the purification of A^d, while separate 10−2.16 columns (Oi et al., 1978) were used to purify A^k and A^u. Protein was eluted with solutions of either 0.2 or 1.0 mM dodecyl maltoside (DM)/0.5 M NaCl/0.1 M Na_2CO_3, pH 11.5. Protein concentrations were determined using a micro-BCA assay (Pierce, Rockford, IL).

Peptide Dissociation Kinetics. MHC protein was incubated with excess fluorescein-labeled peptide for various times (depending upon the time required to get the maximal

amount of binding). Excess peptide was removed from the sample with Sephadex G50-SF spin columns pretreated with a 10 mg/mL BSA solution to minimize the amount of nonspecific binding to the column. The complex was isolated in a pH 5.3 sodium citrate buffer, and the dissociation was incubated at 37 °C to begin the dissociation. Periodically, aliquots were taken from the heated sample and analyzed by high-performance size exclusion chromatography. Using a 7.5 mm × 60 cm Toso Haas TSK G3000SW column (Toso Haas, Montgomeryville, PA) with a guard column (7.5 mm × 75 mm), dissociation was monitored by a Shimadzu RF-551 spectrofluorometric detector and a standard UV detector connected in series (Witt & McConnell, 1991). The HPLC solvent was PBS/0.2 mM DM (pH 7.0) running at room temperature at 1.0 mL/min. The protein−peptide complex eluted at approximately 15 min. It should be noted that the fluorescein label at the N-terminus of each peptide had no effect on the kinetics. In assays where an equimolar amount of unlabeled peptide was added, the signal diminished by ∼50%.

In the dissociation experiments where time points less than 30 min apart were required, aliquots of the sample were taken and placed immediately at 4 °C. They were maintained on ice no longer than 20 min, after which time they would be injected onto the HPSEC apparatus. Controls were performed that demonstrate no significant dissociation of complex while at 4 °C (data not shown).

RESULTS

A key prediction of the computer model is that CLIP binds in the same way to all class II MHC, placing the unusually flexible side chains of residues Met 91 and Met 99 in the major anchor positions, P1 and P9 (Lee & McConnell, 1995). Thus, there should be a strong sensitivity to substitutions at these residues, affecting binding to any class II MHC. To test the proposal that the methionine side chain's flexibility is important for general binding, we selected the conservative amino acid replacement Met → Leu, which preserves the hydrophobicity and overall volume of the side chain but reduces its flexibility. Although these amino acids are considered very similar (by size and hydrophobicity or by amino acid similarity indices developed for sequence alignment scoring), suggesting little effect on binding, molecular modeling indicates the substitution could have a major effect at these positions in CLIP.

The substitutions Ii(86−100)M91L and Ii(86−100)M99L were modeled for CLIP bound to A^d, and in both cases the complex for leucine is strained relative to methionine. The strain energy in our calculation is on the order of magnitude of 1 kcal mol^{-1}. The structures of these complexes suggest probable causes for this strain. In the M91L mutant, Leu 91 must adopt a strained χ_1 torsion to point its branched side chain into the P1 pocket (Figure 1a), whereas the unbranched Met side chain has a normal rotamer state. In the P9 pocket, Met and Leu adopt similar conformations, but for Met the terminal methyl group is centrally placed deep within the pocket, whereas for Leu it is shifted to one side of the pocket, creating strain with the adjacent MHC residue α77 Leu (Figure 1b).

Figure 2 shows the experimental dissociation kinetics of CLIP M91L and M99L from three MHC alleles, A^d, A^k, and A^u. As summarized in Table 1, both substitutions cause large reductions in stability of the CLIP−MHC complexes, relative to that of wild-type CLIP, or a control substitution L97A. The M91L peptide displays reduced stability for all three MHC alleles. In A^d, the $t_{1/2}$ of dissociation drops from 5.5 to 0.23 h, a nearly 25-fold reduction (see Table 1).[2] Similar but weaker effects are seen in A^u (down from 4.2 to 0.68 h) and A^k (0.13 to 0.09 h). Qualitatively, M91L clearly dissociates more rapidly from A^k than does wild-type CLIP. However, since wild-type CLIP binds very weakly to A^k and a low signal is obtained, a destabilizing mutant leads to a further decrease in the amount of complex, and dissociation half-times are difficult to measure. These times are close to the minimum detectable in our assay. The M99L peptide shows similar stability decreases in A^d (5.5 → 0.77 h) and A^k (0.13 → <0.05 h), but none at all in A^u.

The negative control substitution L97A is shown by our model to be a surface position that interacts with the MHC much less than do the anchor positions. The dissociation data confirm this hypothesis, with very similar half-times of dissociation for the L97A mutant and for wild-type CLIP, for all three MHC alleles. The data for A^u show evidence of a biphasic, fast dissociating component, perhaps indicative of a kinetic intermediate, in several peptides including wild-type CLIP and L97A. The half-time of the dominant, slow component for L97A is 3.0 h vs 4.2 h for wild-type CLIP, only a 29% reduction (compared with a > 6-fold reduction for M91L).

Does variability in the stability reduction for M91L and M99L across the three MHC alleles indicate that CLIP binds each in a different way, or does this simply reflect polymorphic differences in the P1 and P9 pockets of these alleles? In particular, no reduction at all was observed for M99L/A^u. Our earlier modeling suggested the only $A^{d,k,u}$ polymorphisms affecting CLIP position 99 are at β38 and β61 and are A^u-specific (Lee & McConnell, 1995) (i.e., A^d and A^k are identical at these positions and A^u different). This is consistent with our experimental observation of a divergent response to M99L in A^u vs that in A^d and A^k, suggesting that it is related to the polymorphism. In particular, molecular modeling indicates that, in A^u, the β38 Leu side chain is longer than the short side chain present in A^d and A^k Val. β38 Leu reduces the size of the P9 pocket and contacts the distal methyl group of the Met 99 of CLIP (Figure 1c). Switching the CLIP Met 99 to Leu alleviates this unfavorable interaction. Thus, the similar off-rates of the native CLIP and the M99L peptides from A^u suggest that opposing effects contribute to complex stability—the mutation to Leu results in an adverse loss in flexibility but relieves the unfavorable steric strain at the base of the pocket. The larger effect of the M91L mutation in A^d compared to the two other alleles appears to be related to the A^d-specific polymorphism at position 86 on the MHC β-chain (Pro for A^d, Thr for A^k and A^u). The Pro at β86 was modeled previously as the residue that affects Met 91 of the peptide (Lee & McConnell, 1995). It is predicted to create increased space for the long Met side chain at the base of the pocket, increasing the preference for it over the shorter leucine.

In addition to creating destabilizing mutations, we have designed a stronger test of the general model by designing

[2] The dissociation half-time of CLIP (86−100) peptide from A^d reported in this work differs from the value reported previously (Liang et al., 1995). The value here is correct ($t_{1/2}$ = 5.5 h, at pH 5.3); the other dissociation experiment was performed at a higher pH (pH 5.8).

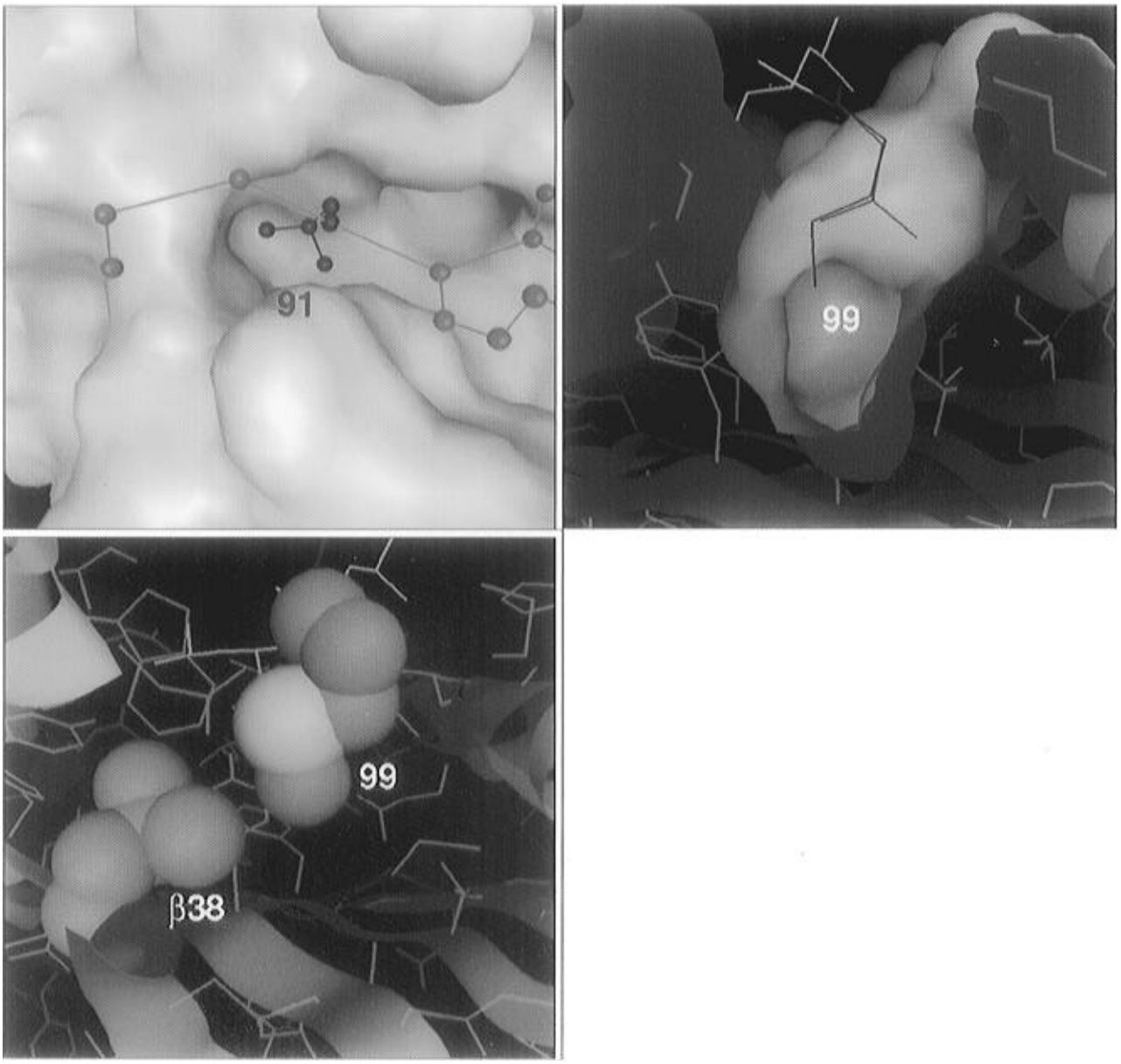

FIGURE 1: Molecular modeling of CLIP M91L and M99L substitutions. (a, top left) The Leu 91 side chain is shown in red within the context of the M91L peptide (green) and the A^d molecular surface (white). (b, top right) The Leu 99 side chain (dark blue) of the M99L peptide is shown in comparison with the Met 99 side chain (red) of wild-type CLIP. (c, bottom) The A^u polymorphism β38 Val → Leu contacts the distal methyl group of CLIP Met 99 (both shown in CPK spheres).

peptides that should show enhanced binding. Our original CLIP/I-A models suggested one strained interaction, between CLIP Pro 96 and the MHC P6 pocket (Lee & McConnell, 1995). The bulky proline side chain has two effects at this position. First, it blocks one of the canonical peptide−MHC hydrogen bonds, between the backbone N of this residue, and Asn α62. Second, the rigid, cyclic proline side chain appears to have some steric strain within the P6 pocket. We therefore designed a substitution, CLIP 96 Pro → Ala, to enhance CLIP binding by alleviating both of these effects. Alanine's small side chain fits the P6 pocket without steric strain and also can hydrogen bond normally to Asn α62.

The mutation P96A yields a complex with MHC with marked enhancement in stability (Figure 3 and Table 1). The largest effect was observed in A^d, where the half-time of dissociation increased to 85.0 h (from 5.5 h for wild-type CLIP), a 15-fold enhancement. A^k also showed a large (10-fold) enhancement. However, in A^u this peptide mutation had no detectable effect on dissociation rate. These results contrast strikingly with the equivalent replacement at the next residue (L97A), which results in no change in dissociation kinetics in all three alleles. Since alanine's small side chain has less stabilizing interaction with the P6 pocket than the larger proline side chain, the large enhancement seen for P96A suggests that this substitution alleviates strain due to 96 Pro.

In our initial estimation of the effect of the P96A mutation, we did not attempt to distinguish potential differences in response between the $A^{d,k,u}$ alleles. Is the markedly different response of A^u at P6 due simply to polymorphic differences between the P6 pockets of these alleles? We analyzed these differences within the CLIP/I-A models (Figure 4). There are two major differences, first in the depth of the P6 pocket (very shallow in A^d and deep in A^u) and second in its hydrophobicity (polar in A^d and A^k, hydrophobic in A^u). Two polymorphisms, at α70 and β9, give rise to these differences. In A^d, the polymorphism α70 Glu (vs glycine in A^k and A^u) makes the P6 pocket very shallow and its floor strongly polar (Figure 4a). Both characteristics disfavor the large, hydrophobic side chain of proline. In A^k, the polymorphism β9

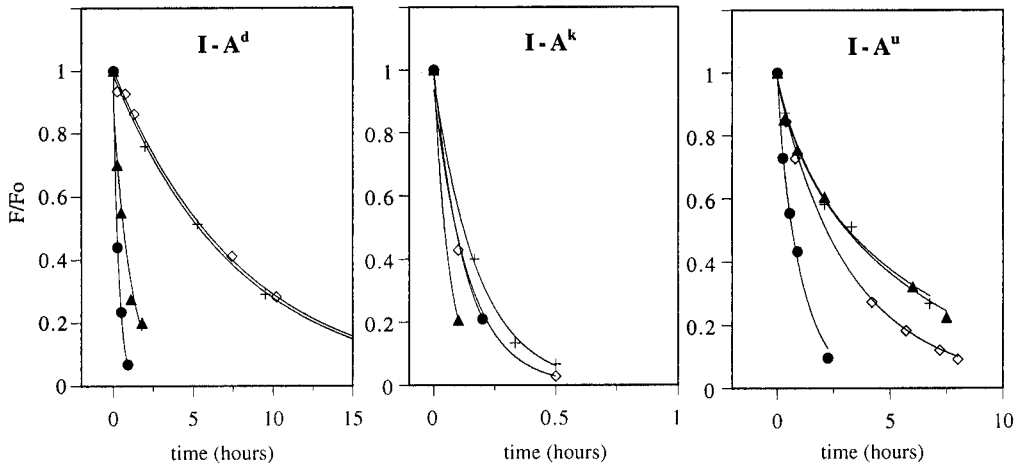

FIGURE 2: Dissociation kinetics of fIi(86−100) (+) and CLIP mutants fIiM91L (●), fIiM99L (▲), and fIiL97A (◇) from MHC class II molecules. Incubations were performed at 37 °C, pH 5.3, in a sodium citrate buffer. The solid lines are single exponential fits through the data, except for the A^u dissociation curves, which were fit to a double exponential function (see text). The curves were normalized to the first time point, F_0.

Table 1: Dissociation Data for CLIP and CLIP Mutants, at 37 °C, pH 5.3

designed effect	peptide	A^d $t_{1/2off}$ (h)	A^k $t_{1/2off}$ (h)	A^u $t_{1/2off}$ (h)
wild type	Ii(86−100)	5.5	0.13	0.3, 4.2[a]
decrease in complex stability across all alleles	M91L	0.23	0.09	0.68
	M99L	0.77	<0.05	*0.5, 4.2*[b]
	M91LM99L	0.1	<0.05	0.68
negative control	L97A	5.6	0.1	0.7, 2.9
increase in stability across all alleles	P96A	85.0	1.3	*0.3, 4.2*
increase in stability for specific alleles	P96Y	no binding	0.47	**22.3**[c]
	P96E	0.37	**16.0**	0.6, 3.7

[a] In some cases, the dissociation curves were biphasic, with a fast and a long-lived component; both half-times are listed here. [b] The dissociation half-times in italics do not follow the predicted kinetic effect (see text for explanation). [c] Data in bold indicate allele-specific enhancements in complex stability.

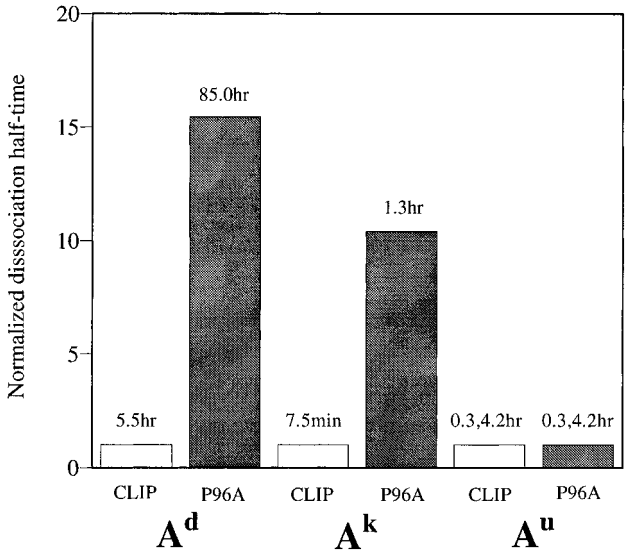

FIGURE 3: Summary of dissociation half-times for CLIP compared to the fIiP96A mutant for the A^d, A^k, and A^u alleles. For each MHC allele, the two half-times were normalized to that of the native CLIP peptide, in order to relate the interallelic effects of the mutation. The actual half-times of dissociation are listed above each bar.

His (vs valine in A^d and A^u) again introduces a polar character to the pocket and reduces its size (Figure 4b). In A^u, the P6 pocket is deep (Figure 4c) and mostly hydrophobic.

Evidence from the crystal structure of the CLIP−DR3 complex (Ghosh et al., 1995) suggests that Pro 96 shifts downward in the pocket about 1 Å relative to typical antigenic peptides in order to replace its lost hydrogen bond, by allowing Asn α62 to transfer it to the side chain of the previous residue Thr 94. In A^u, the P6 pocket can accommodate Pro 96 well, but not in A^d and A^k. These analyses suggest that the I-A alleles bind CLIP in the same general way but differ in their response to mutation of Pro 96 due to P6 pocket polymorphisms. The kinetic differences might have been predicted from the models, but we did not originally do so.

Allele-specific polymorphisms, positions in the MHC where one allele differs from the residue shared by the other two alleles, can complicate interpretation of multiallele experiments. However, these polymorphisms can be used to test a binding model, if an unusual polymorphism suggests a potential specific interaction with a modified peptide. We have exploited this possibility by designing strong allele-specific enhancements, i.e., a CLIP mutation that improves binding to a specific allele. We focused on the P6 pocket because in our models it is an important anchor pocket, not filled by a deeply buried CLIP side chain. Also, this pocket contains the polymorphism β9 Val (A^d and A^u) vs His (A^k), the only polymorphic positively charged residue inside a binding pocket in these alleles. Modeling indicated that the substitution P96E should stabilize CLIP's complex with A^k, via electrostatic interaction between the introduced carboxylic acid group and the buried histidine (Figure 5a). Furthermore, the deep, hydrophobic P6 pocket in our A^u model suggested

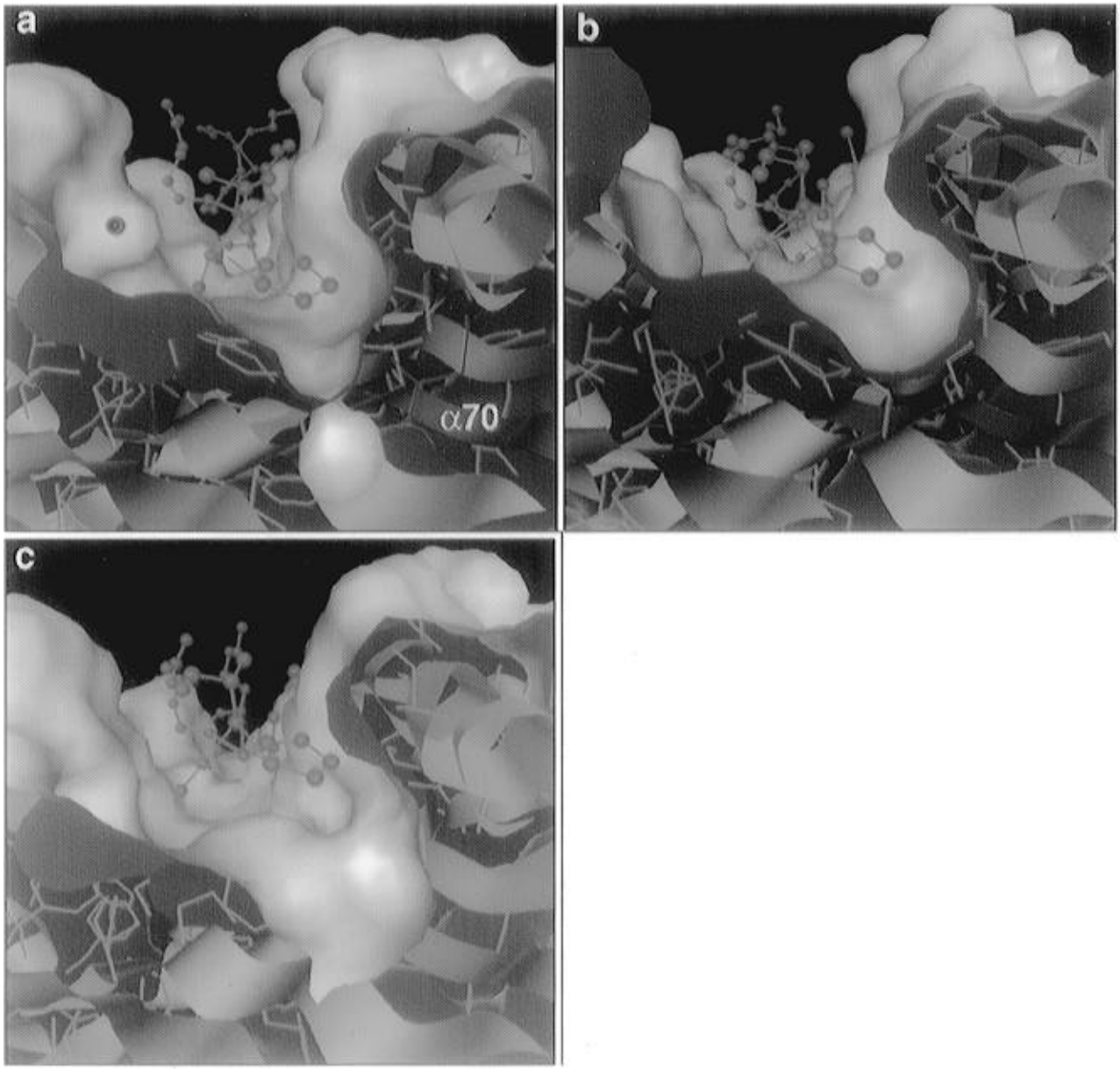

FIGURE 4: Molecular modeling of the P96A peptide, bound to (a) A^d (the polymorphism α70 Gly → Glu is shown in red), (b) A^k, and (c) A^u.

enhancement of CLIP binding by replacing 96 Pro with a hydrophobic anchor residue that could fill the pocket. We modeled several hydrophobic amino acids at this position and selected tyrosine on the basis of its fit to the pocket (Figure 5b). Phe, Leu, and smaller hydrophobics also fit in our model, but Trp had many bad contacts, due both to its large size and to its shape.

These substitutions gave large, allele-specific enhancements in CLIP binding (Figure 6 and Table 1). The peptide P96E showed an increased stability of 128-fold over the CLIP−A^k complex. This peptide bound more weakly to A^u and much more weakly to A^d. Intriguingly, A^d and A^u differ in the P6 pocket by the polymorphism α70 Glu (A^d) vs Gly (A^u), suggesting a clear explanation for the large difference in their response to this peptide, by repulsive electrostatic interaction with this residue. By contrast, the mutation P96Y caused a 5.3-fold increase in the dissociation half-time in A^u (to 22.3 h), and a substantial increase in A^k (to 0.47 h, a 3.7-fold rise). It completely abrogated binding of CLIP to A^d (Figure 6 and Table 1). The molecular model shows that a large tyrosine does not fit in the shallow P6 pocket of A^d, which accounts for the lack of any binding of the P96Y peptide to A^d.

DISCUSSION

These results show molecular modeling of MHC−peptide interactions to have predictive value. We did not synthesize any modified CLIP peptides other than those reported here. It is evident that our design goals could not have succeeded so well by chance, for both enhanced binding and destabilization, as well as negative controls. Modeling allowed us to design either uniform changes across all the alleles or to target specific alleles. The models showed positions where pocket composition was conserved, and the packing calculations predicted the effects of even conservative substitutions. For example, mutations of the anchor residues Met 91 and Met 99 to Leu maintained the size and hydrophobicity of the side chain but were predicted to cause destabilizing steric effects due to reduced flexibility of the leucine side chain (see Figure 1 and Table 1). The observed decreases in stability confirm this. The models also identified sites for allele-specific changes and allowed us to forecast which

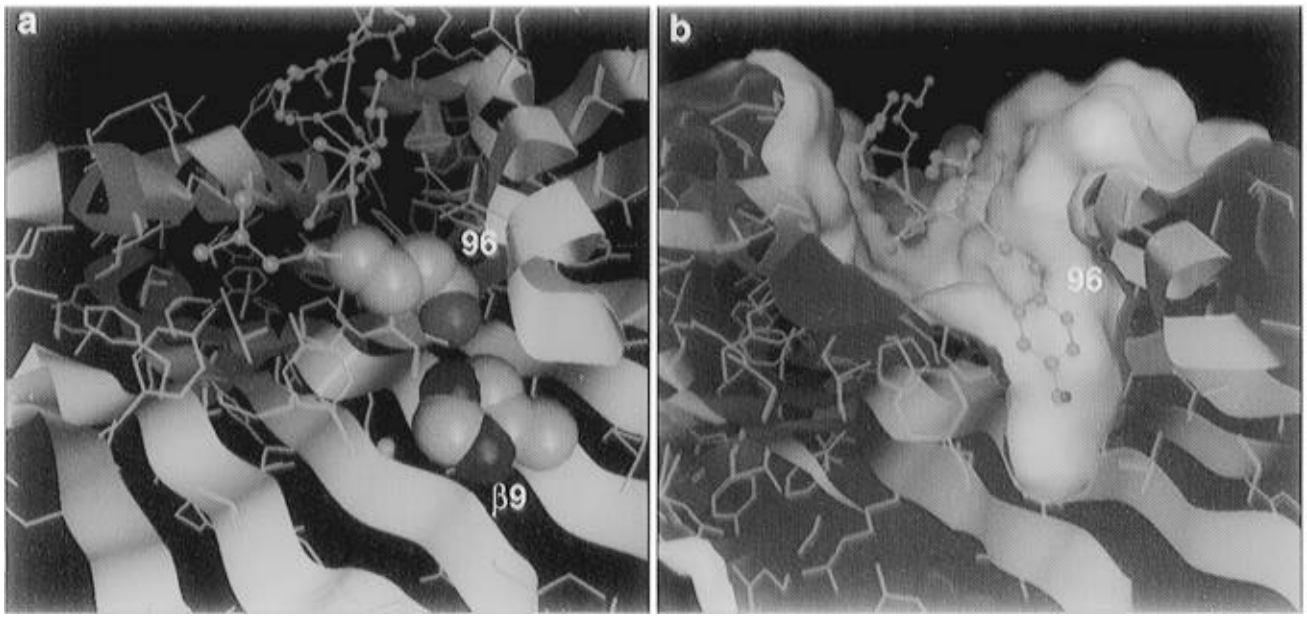

FIGURE 5: Molecular modeling of (a) the CLIP P96E peptide in A^k and (b) the CLIP P96Y peptide in A^u. The CLIP 96 Glu side chain and A^k-specific polymorphism β9 His are shown in CPK spheres.

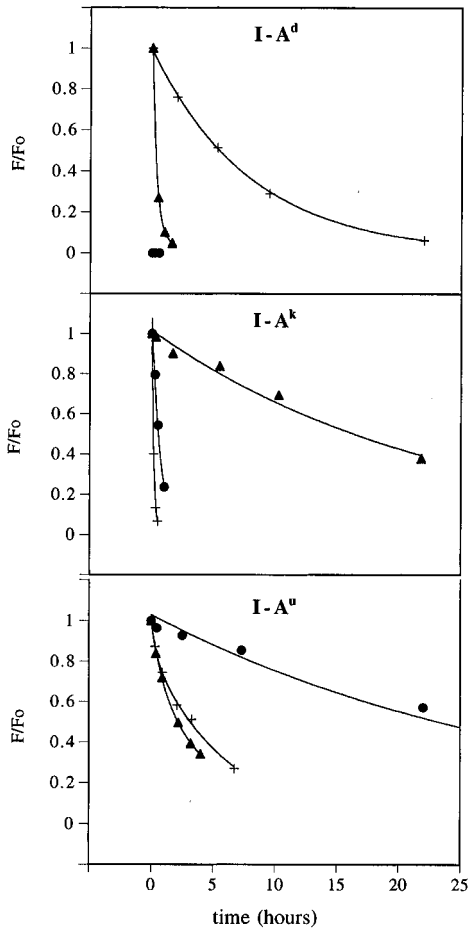

FIGURE 6: Dissociation of fIi (+), fIi P96Y (●), and fIiP96E (▲) from MHC molecules at 37 °C, pH 5.3. The plots have been scaled to the same time axis (0 → 25 h), and each curve has been normalized to the first time point, F_0. See Materials and Methods for more details on the dissociation procedure.

substitutions improve binding to an individual allele. The designed peptide mutations that anchor at P6 (see Figures 4 and 5, and Table 1) yield the expected allele specificity. These results are important, as strong enhancements achieved in only one allele but not in the others provide a stringent test of this molecular modeling.

Our results are consistent with other evidence of the value and limitations of the molecular modeling method employed (Koehl & Delarue, 1994; Lee, 1994, 1996; Lee & Levitt, 1991; Koehl & Delarue, 1995; Lee & Levitt, 1996). For mutant protein and MHC modeling, the coordinate predictions for side chains modeled *ab initio* (typically because of amino acid differences between the template structure vs the target protein to be modeled) match crystal structures to within 0.5−1.0 Å rms, only slightly higher than the rms difference observed for the conserved regions. Overall, the models' accuracy is similar to the level of agreement between independently solved crystal structures of the same MHC protein from different laboratories (Fremont et al., 1992; Zhang et al., 1992). We have used this method to predict the structures of MHC−peptide complexes for CLIP (Lee & McConnell, 1995) as well as MBP Ac1−11 (manuscript in preparation). This capability may be general to all peptides binding to class II MHC, because of class II's extensive hydrogen-bonding network holding the whole peptide backbone in place. The modeling scores the steric fit of peptide substitutions as a predictor of their effect on binding and includes simple considerations of solvation and electrostatic interactions. We have thereby been able to design kinetic changes with good qualitative success. However, since we model these substitution effects in the context of structures which are themselves models (our I-A MHC structures, which were modeled originally from the HLA-DR1 crystal structure), we have not attempted to predict these stabilizations quantitatively as we have in other systems (Lee, 1996; Lee & Levitt, 1991). Modeling of class I MHC−peptide complexes is also possible (Barber et al., 1996) but more difficult because of the increased conformational variability observed in the center of peptides bound to class I.

Our kinetic results provide evidence that CLIP binds to the murine MHC I-A alleles in the conformation predicted by modeling. We have successfully designed a series of kinetic effects on the basis of these models. Furthermore, the kinetic data suggest interactions between individual CLIP side chains and specific MHC polymorphisms: CLIP Met 91, β86; CLIP Met 99, β38; CLIP Pro 96, α70 and β9. These

were predicted by the models [see Table 1, (Lee & McConnell, 1995)]. Our results show that CLIP position 96 is a strong potential mediator of allele-specific targeting, consistent with the P6 pocket in the I-A models. Further evidence comes from studies on the interactions with the N-terminal peptide of myelin basic protein (MBP Ac1−11) and A^u P6 pocket residue β9. Site-directed mutagenesis of this position, replacing the A^u β9 Val with the A^k His, switched the specificity from one allele to the other, in a way that mirrors the specificity effects seen at position 96 in CLIP: Glu preferred in A^k and in A^u β9 Val → His, and Tyr in A^u and in A^k β9 His → Val (manuscript in preparation). β9 is at the base of the P6 pocket, supporting the model's placement of CLIP 96 in this pocket.

We believe this general model of CLIP association holds across all class II MHC. It was originally developed in modeling CLIP with a series of human (DR3) and murine $A^{d,k,u}$ MHC proteins. The recent crystal structure of a human HLA-DR3−CLIP complex (Ghosh et al., 1995) is consistent with this hypothesis. Alanine substitutions at positions Met 91 and Met 99 reduced the ability of CLIP to inhibit antigenic peptide binding to HLA-DR1 and HLA-DR17 (Malcherek et al., 1995), as well as with other DR alleles (Geluk et al., 1995). Related human HLA-DR and, by extension, mouse I-E alleles probably bind CLIP in the same way. Sensitivities to MHC polymorphism, consistent with this model, were seen in the mouse alleles E^d and E^k (Bangia & Watts, 1995; Liang et al., 1995). Furthermore, data showed that T-cell hybrids specific for human CLIP bound to the mouse molecule A^b in a manner indistinguishable from that of antigenic peptides (Morkowski et al., 1995). These data in conjunction with the work presented here strongly suggest that CLIP binds in a single consistent way across all mouse and human MHC. Furthermore, it seems likely that the CLIP sequence plays the same role within the full-length invariant chain protein (Ii). The consistent pattern of binding suggests a functional basis for this sequence to provide general class II MHC binding activity essential for the invariant chain. In support of this hypothesis, both CLIP (Bangia & Watts, 1995; Liang et al., 1995) and whole Ii (Germain & Rinker, 1993) show a weaker interaction with A^k compared to other alleles.

The results in this work support the hypothesis that CLIP binds to all MHC because this peptide lacks the allele-specific contacts that would optimize its binding to one particular allele. Malcherek and co-workers showed that the introduction of a DR17-specific contact residue in CLIP (Asp 93) interfered with its binding to another class II molecule (Malcherek et al., 1995). Similarly, our kinetic data show that CLIP residue 96, a proline, is not optimal for binding any of the individual $A^{d,k,u}$ alleles. With three different mutations (Pro → Ala, Glu, or Tyr), we have enhanced the peptide−protein complex for each allele (A^d, A^k, and A^u, respectively). Although the introduction of allele specificity enhanced binding for one allele, it also decreased the stability of the complex in the others, and it is evident that use of an allele-specific anchor at this position prevents binding to other alleles.

The dissociation rate of the peptide from MHC protein is our best measure of complex stability. Since the MHC protein is isolated from the surface of B-cell lymphomas acting as antigen presenting cells, a variety of endogenous peptides occupy the binding groove and must dissociate before labeled peptide can bind (Buus et al., 1988). This leads to difficulty in the determination of on-rates; only indirect methods provide measures of the on-rates (Mason & McConnell, 1994). As a further complication, inactivation of the peptide-free protein occurs relatively rapidly, so dialysis and Scatchard plots do not yield accurate equilibrium constants (Beeson & McConnell, 1995). Thus, the most reliable stability information comes from analysis of MHC−peptide complex dissociation. At the onset of this present study, we were uncertain whether structural models of the peptide−MHC complexes would correlate with dissociation kinetics. One could imagine a multitude of complicating factors, such as kinetic intermediates, subtle physical inaccuracies of the molecular structures, or various other complications that might interfere with correlation of the models with kinetic data. In particular, the activation energy for dissociation might bear little relation to the binding energy. On the basis of upon the ability of the models to guide the design of peptide mutants with particular kinetic effects, our results show that the first-order dissociation kinetics do reflect the complex binding energy.

In most cases, the dissociations follow a single exponential decay; however, in some instances with A^u, the dissociation curves can only be fit to a double exponential. The biphasic dissociation of CLIP peptides from A^u represents short- and long-lived MHC−peptide complexes. This has been previously attributed to kinetic intermediates formed in the binding reaction (Beeson & McConnell, 1994; Sadegh-Nasseri & McConnell, 1989; Sadegh-Nasseri et al., 1994). In other instances heterogeneity in dissociation kinetics may be due to isomeric complexes (Liang, unpublished results; Beeson et al., 1996). The presence of short-lived species in certain situations does not affect the present analysis of the kinetic results. In all dissociations where two species were present, the vast majority (>85%) of the complex was long-lived. We have focused on this long-lived dissociating species, which is *ipso facto* the most stable conformation of the MHC−CLIP complex. The molecular models were based on the coordinates of the DR1-hemagglutinin crystal structure and closely resemble the DR3-CLIP crystal structure (Ghosh et al., 1995; Stern et al., 1994). The MHC−peptide crystal structures are likely representative of the stable terminal complexes rather than kinetic intermediates.

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