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Crystal Structures of HLA-A*0201 Complexed With Antigenic Peptides With Either the Amino- or Carboxyl-Terminal Group Substituted by a Methyl Group

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ABSTRACT **The crystal structures of class I major histocompatibility complex (MHC) molecules complexed with antigenic peptides revealed a network of hydrogen bonds between the charged amino- and carboxyl-termini of the peptides and conserved MHC residues at both ends of the peptide binding site. These interactions were shown to contribute substantially to the stability of class I MHC/peptide complexes by thermal denaturation studies using synthetic peptides in which either the amino- or carboxyl-terminal group is substituted by a methyl group. Here we report crystal structures of HLA-A*0201 complexed with these terminally modified synthetic peptides showing that they adopt the same bound conformation as antigenic peptides. A number of variations in peptide conformation were observed for the terminally modified peptides, including in one case, a large conformational difference in four central peptide residues that is apparently caused by the lattice contact. This is reminiscent of the way binding a T-cell receptor changed the conformation of central residues of an MHC-bound peptide. The structures determined identify which conserved hydrogen bonds are eliminated in terminally substituted peptides and suggest an increased energetic importance of the interactions at the peptide termini for MHC-peptide stability. Proteins 33:97–106, 1998.** © 1998 Wiley-Liss, Inc.

Key words: antigenic peptides; class I MHC molecules; HLA-A2 complexes; hydrogen bonds; protein structure

INTRODUCTION

Short peptides (~9mers) of intracellularly proteolysed proteins are presented by class I MHC molecules on the surface of cells to specific receptors on T cells.[1,2] This peptide-dependent recognition is critical for the development of the T-cell repertoire and for activation of cytotoxic T lymphocytes (CTLs). Class I MHC molecules have a structure that enables the assembly of stable complexes with half-lives over 50 hours with a large number of different antigenic peptides. Crystallographic analyses of class I MHC/peptide complexes have shown that the charged peptide amino- and carboxyl-termini form networks of hydrogen bonds at the two ends of a binding groove with MHC residues that are conserved in all murine and human classical class I molecules (Fig. 1). These hydrogen bonds appear to provide a general mode of binding for short peptides that is MHC-allele and peptide-sequence independent. Interactions between a few peptide anchor residues and polymorphic MHC residues forming pockets along the binding site provide peptide-sequence specificity (reviewed in References 3 and 4). Most of the central residues of the peptides are exposed in the MHC complexes and are recognized by T-cell receptors (TCRs) (e.g. Reference 5).

Estimates of binding free energies between class I MHC molecules and peptides have been made for both the peptide-sequence dependent and peptide-sequence independent interactions. Structural comparisons of the binding pockets of HLA-A*0201 (HLA-A2), HLA-B*2705 (HLA-B27), and HLA-A*6801 (HLA-Aw68) specific for different side chains at the second position of the peptide (P2) allowed an estimate of the binding energies between the polymorphic MHC residues forming that pocket and the P2 peptide anchor residues.[6] Substitutions at the

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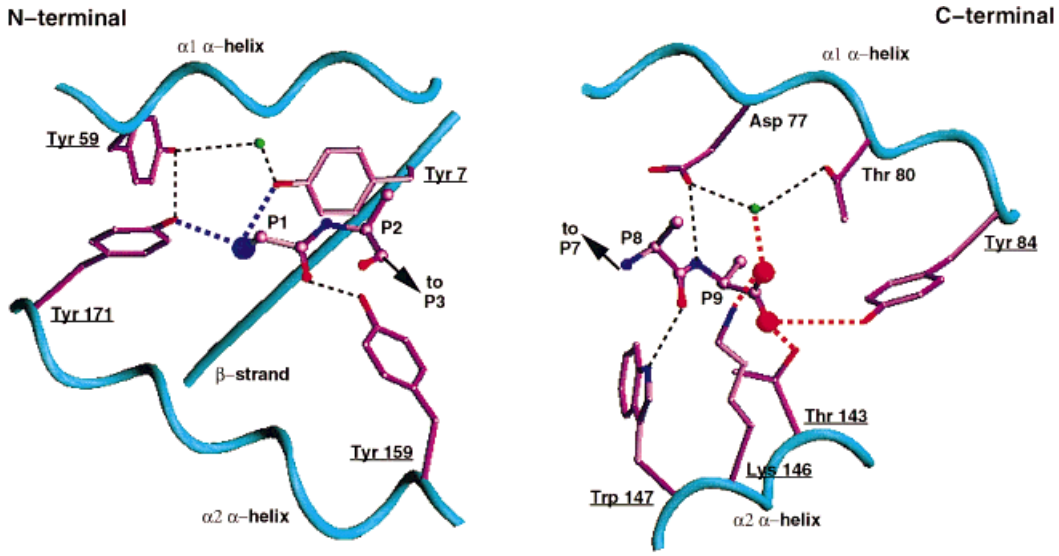

Figure 1.

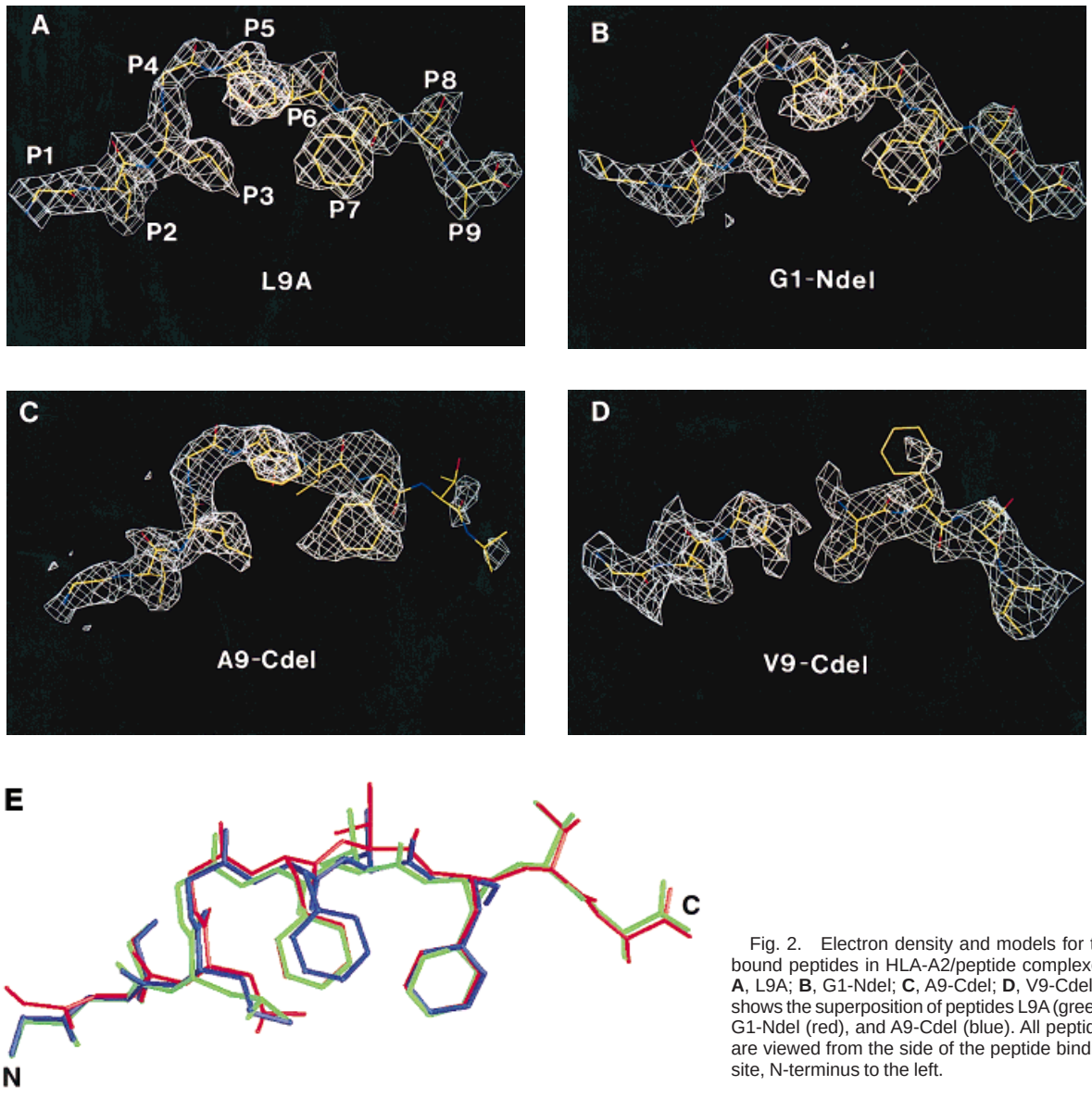

Fig. 2. Electron density and models for the bound peptides in HLA-A2/peptide complexes. **A**, L9A; **B**, G1-Ndel; **C**, A9-Cdel; **D**, V9-Cdel; **E** shows the superposition of peptides L9A (green), G1-Ndel (red), and A9-Cdel (blue). All peptides are viewed from the side of the peptide binding site, N-terminus to the left.

TABLE I. Peptides Used in the In Vitro Assembly and Crystallization of HLA-A2 Complexes[†]

	Position									
Peptide	P1	P2	P3	P4	P5	P6	P7	P8	P9	T_m[a]
Matrix	G	I	L	G	F	V	F	T	L	64.9°C
L9A	G	I	L	G	F	V	F	T	A	65.9°C
G1-Ndel	CH_3CH_2CO	I	L	G	F	V	F	T	A	44.9°C
G1A+L9A	A	I	L	G	F	V	F	T	A	67.5°C
A9-Cdel	G	I	L	G	F	V	F	T	$NHCH(CH_3)_2$	42.8°C
L9V	G	I	L	G	F	V	F	T	V	71.9°C
V9-Cdel	G	I	L	G	F	V	F	T	$NHCH(CH(CH_3)_2)CH_3$	44.0°C

[†]Sequences are based on the influenza virus matrix peptide (GILGFVFTL).
[a]Melting temperatures (T_m) measured previously (except for V9-Cdel) (See Reference 9).

TABLE II. Crystallographic Data

Peptide	Space group	Cell parameters						Number of molecules per asymmetric unit
L9A	P1	a = 50.4Å,	b = 63.1Å,	c = 74.9Å,	α = 81.9°,	β = 75.7°,	γ = 78.0°	2
G1-Ndel	P1	a = 50.3Å,	b = 63.5Å,	c = 74.8Å,	α = 81.8°,	β = 76.1°,	γ = 77.7°	2
A9-Cdel	$P2_1$	a = 63.7Å,	b = 88.5Å,	c = 79.7Å,	β = 90.2°			2
V9-Cdel	$P2_12_12_1$	a = 49.6Å,	b = 74.4Å,	c = 121.4Å,	α = β = γ = 90.0°			1

Peptide	Resolution	R_{merge}[a]	Completeness	I/σ
L9A	25–2.5Å (2.64–2.5Å)	0.102 (0.229)	0.97 (0.87)	9.7
G1-Ndel	25–2.6Å (2.74–2.6Å)	0.076 (0.183)	0.83 (0.75)	9.3
A9-Cdel	15–2.5Å (2.59–2.5Å)	0.056 (0.201)	0.69 (0.64)	11.3
V9-Cdel	15–2.9Å (3.00–2.9A)	0.068 (0.192)	0.89 (0.92)	17.9

[a]$R_{merge} = (\Sigma_h |I - \langle I \rangle|)/(\Sigma_h \langle I \rangle)$ for the intensity (I) of observations of reflections h.

peptide anchor and non-anchor positions have also been used to estimate the binding energies contributed by each sequence-dependent interaction.[7,8] A study in which either the amino- or carboxyl-terminal group of an antigenic peptide was substituted by a methyl group provided an estimate of the binding energies contributed by these sequence-independent functional groups.[9] However, a detailed analysis of the energetics contributed by these individual substitutions requires knowledge of the structural changes that each of these peptide modifications may have caused.

To address this issue, we have studied 7 synthetic peptides, compared the thermodynamic stability of their complexes with HLA-A2, and determined crystal structures of four of these related nonameric peptides, including three with either the charged amino- or carboxyl-terminal group substituted by a methyl group (Table I). The peptide sequences are based on the influenza virus matrix peptide (GILGFVFTL), a dominant antigenic peptide presented by HLA-A2 during human infections caused by influenza virus.[10] This study was designed to examine the consequence of deleting six hydrogen bonds between the charged peptide terminal groups and conserved MHC residues (bold dashed lines in Figure 1). The structural observations provide the means to interpret the thermodynamic data obtained for each of these class I MHC molecule/peptide complexes by thermal denaturation measurements made in this study and our previous study.[9] Some unanticipated conformational arrangements of the modified peptides and of two MHC residues, lead us to increase our estimates of the importance of the hydrogen bonds to the peptide termini.

Fig. 1. Conserved hydrogen bonds between the charged NH_2- and COOH-termini of influenza virus matrix peptide and MHC residues. Side chains at P2 (Ile), P8 (Thr), and P9 (Leu) are shown to the β carbon. Substituting the peptide $^+NH_3^-$ (large purple atom) or COO^- (large red atoms) terminal groups by a methyl group would eliminate two (dashed purple lines) or four (dashed red lines) hydrogen bonds, respectively. Both α-helices and a β-strand of HLA-A2 are shown in turquoise with conserved MHC residues underlined.

MATERIALS AND METHODS

Synthesis of Peptides

Peptides L9A, G1-Ndel, and A9-Cdel were synthesized, purified, and characterized as described previously.[9] The synthesis of V9-Cdel peptide was identical to that of A9-Cdel peptide except for the last cleavage-coupling step where (R)-1,2-dimethylpropylamine was used to provide the desired (R)-1,2-di-

methylpropylamino group (Cdel) (Table I). (R)-1,2-dimethylpropylamine of sufficient optical purity ($[\alpha]^{23}D$ -2.44° (neat amine)) was obtained by recrystallization (four times) from methanol of the d-tartrate salt of (R)(S)-1,2-dimethylpropylamine (Aldrich Chemicals Co.).[11] All purified peptides showed the expected sequence compositions and molecular weights, as determined by amino acid analysis and fast atom bombardment (FAB) mass spectrometry, respectively.

In Vitro Assembly of Class I MHC Molecule/Peptide Complexes

Class I MHC complexes were reconstituted by diluting *Escherichia coli*-expressed heavy chain (HLA-A*0201) (1 μM) and β_2-microglobulin (β_2m) (2 μM) in the presence of excess synthetic peptides (10–30 μM). The mixture was incubated at 10°C for 48 hours, concentrated, and purified by gel filtration chromatography.[12] Purified HLA-A2 complexes were analyzed by FAB mass spectrometry to confirm the molecular weight of the bound peptides.

Crystallization of HLA-A2/Peptide Complexes

Equal volumes of protein solutions (3–7 mg/mL) and 16% poly(ethylene glycol) (PEG) 6000 buffered with 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5 containing 0.1% sodium azide, were mixed to form hanging drops suspended over well solutions containing 13%–17% PEG 6000 buffered with 25 mM MES pH 6.5 containing 0.1% sodium azide. Microseeding using crystals of HLA-A2/matrix peptide complex was used to promote the growth of crystals for complexes with L9A, G1-Ndel, and A9-Cdel peptides.[13] Crystals of these three complexes were harvested into a well solution containing 20% PEG 6000. Shortly before data collection, crystals were placed in dialysis buttons and dialyzed stepwise against the harvest buffer supplemented with progressively higher concentrations of glycerol. Each transfer step corresponded to a 5%–10% increase in glycerol concentration (up to 20% glycerol) and varied from 3 hours to several days. No correlation was observed between changes in transfer procedures and quality of the diffraction data. Crystals from complexes with the V9-Cdel peptide were flashed-cooled in a well solution containing 20% glycerol.

Data Collection and Processing

Oscillation data were collected from crystals mounted in a thin film of harvest buffer containing cryoprotectant supported by a loop made of dental floss fiber and cooled in a stream of cold nitrogen. Diffraction data were integrated and corrected using XDS and XSCALE programs[14] for data collected on Siemens area detector system (L9A and G1-Ndel), and DENZO and SCALEPACK programs (Z. Otwinowski and W. Minor, personal communication) for data collected using either a MAR-research imaging plate system (18 cm diameter) (V9-Cdel) or an imaging plate system at CHESS (A9-Cdel). The A9-Cdel data are incomplete, so that structure is less well determined (Table II). The space group of each crystal was determined by analyzing the differences in intensities of pairs of reflections across putative mirror planes within the data,[13] confirmed by molecular replacement (MR), and by examining the electron-density maps. Space group and data statistics are shown in Table II. A lower resolution data set (2.9 Å) was also collected for a triclinic A9-Cdel crystal using an Elliott GX-13 X-ray source (data not shown).

Phase Determinations

Initial phases were determined by MR using either the computer programs AMoRe[15] for L9A, G1-Ndel, and A9-Cdel crystals or X-PLOR[16] for V9-Cdel crystal. The search model for the triclinic G1-Ndel crystal was the 1.9 Å structure of HLA-Aw68[17] (was the best-refined model available at the time) with Ala substitutions for the 11 side chains differing between HLA-Aw68 and HLA-A2 (except for Gly62 and Gly107). The search model for the monoclinic A9-Cdel crystal was the 2.5 Å structure of the isomorphous HLA-A2/influenza virus matrix peptide.[18] Two clear MR solutions, corresponding to the two molecules in each asymmetric unit, were found for both of these crystals. For each structure, 10% of observed reflections were excluded from the refinement for monitoring R_{free} throughout the model building and refinement.[19] Rigid body refinement with X-PLOR[16] was then carried out on the orientations of these two non-crystallographically related molecules. The search model for the orthorhombic V9-Cdel crystal was the 2.5 Å structure of HLA-A2/hepatitis B nucleocapsid peptide (peptide excluded).[18]

Model Building and Refinement

For the G1-Ndel crystal, the residues differing between the search model (HLA-Aw68) and HLA-A2 were rebuilt with difference Fourier maps. For the V9-Cdel crystal, MHC residues Arg97, Tyr116, Lys146, and Trp167 were removed from the model prior to refinement to avoid model bias, and were then rebuilt with difference (2Fo−Fc and Fo−Fc) Fourier maps. For all four structures, refinement protocols were used which included rigid body, overall temperature factor, positional, individual atomic temperature factor refinement, and simulated annealing using a slow-cooling protocol.[20] For the L9A, G1-Ndel, and A9-Cdel crystals, iterative real space two-fold phase averaging[21] was used to improve the electron-density maps in the alternate rounds of rebuilding and automated refinement using X-PLOR. Although solvent flattening was applied, no bulk solvent corrections were used. Initially, strict non-crystallographic symmetry (NCS) constraints were applied, and in later stages of refinement, tight NCS

TABLE III. Refinement Statistics†

	Peptide			
	L9A	G1-Ndel	A9-Cdel	V9-Cdel
Resolution (Å)	6–2.5 (2.6–2.5)	6–2.6 (2.71–2.6)	6–2.5 (2.6–2.5)	6–2.9 (3.03–2.9)
R_{free}	0.349 (0.459)	0.351 (0.471)	0.300 (0.408)	0.314 (0.424)
R_{cryst}	0.273 (0.383)	0.263 (0.365)	0.255 (0.372)	0.253 (0.360)
DBond (Å)	0.014	0.014	0.014	0.011
DAngle (°)	3.1	3.0	3.2	1.7
⟨B⟩ (Å²)	11.5	11.5	21.9	15.1
Main-chain atoms	11.1	11.1	21.6	12.8
Side-chain atoms	11.8	11.9	22.3	17.2
Peptide atoms	14.4	18.0	25.4	24.9
Number of missing side chains	4	2	2	6
Number of atoms	6,257	6,266	6,248	3,094
Procheck[a]	90.4	89.6	90.7	84.1
Number of reflections				
Working set	24,399	18,365	17,637	9,539
Free set	2,629	1,976	1,918	857

†R_{free} is R factor calculated using randomly chosen 10% of the reflection data. R_{cryst} is $(\Sigma h|Fo - Fc|/(\Sigma Fo)$, where Fo and Fc are the observed and calculated structure factors, respectively.
[a]See Reference 34.

restraints were applied separately to three segments (α1 and α2, α3, and β2m), excluding residues that were involved in crystal contacts. Prior to building any peptide model, the refined structure of HLA-A2 complexed with the G1-Ndel peptide was used as the starting model for structure refinement of the isomorphous L9A crystal.

Peptide Modeling

After a few cycles of model rebuilding and refinement, clear electron density could be seen for L9A and G1-Ndel peptides, however, no clear electron density could be seen at P4 and P5 positions of V9-Cdel peptide (Fig. 2d) and at the P8 and P9 positions of A9-Cdel peptide (Fig. 2c). When the R_{free} appeared to have reached a minimum (0.370, 0.368, 0.319, 0.319, for L9A, G1-Ndel, A9-Cdel, and V9-Cdel, respectively), a model of the appropriate peptide was then built where electron density permitted and subject to further refinement (use of methods to improve the electron density such as solvent flattening, histogram mapping, and weighted maps did not provide unambiguous electron density at the P4 and P5 positions of V9-Cdel peptide, which appear disordered as the result of a crystal contact).

Final Structures

The statistics for all refined structures show excellent peptide geometry (Table III). No water molecules have been included in any of the structures. The quality of the structures of some of the complexes is only moderate due to the limited availability and quantity of crystals of complexes made from the synthetic ligands. Therefore, we have restricted interpretation to those structural differences that are dramatic and completely obvious in the electron density maps. Refinement protocols were aimed at decreasing the R_{free} rather than the conventional R_{cryst} to avoid errors introduced by overfitting.

RESULTS

Synthetic Peptides

The sequences of the seven peptides studied here are listed in Table I. Thermodynamic data were previously reported for five of the peptides (Matrix, L9V, G1-Ndel, A9-Cdel, L9A) (Table I and Reference 9) and those results indicated that the terminal modifications reduced the thermal denaturation temperature of class I MHC/peptide complexes by about 22 °C . Since substitution of Ala for Leu at P9 (L9A) of the matrix peptide had a negligible effect on the thermal stability of the HLA-A2/peptide complex (Table I, Fig. 2a), the modifications to the N- and C-termini (G1-Ndel and A9-Cdel) that substituted methyl groups for the amino- and carboxyl-groups, respectively, were originally made to L9A to facilitate synthesis.[9] After the N-terminal methyl group of G1-Ndel was found to rotate out of normal position occupied by the N-terminal amino group in the structure of the HLA-A2/G1-Ndel complex (Fig. 2b), a second peptide G1A+L9A was synthesized substituting a methyl group for the hydrogen atom "side chain" of Gly at P1 and determined to form complexes with HLA-A2 with the same thermal stability as those with G1-Ndel peptides (Table I). After another unanticipated conformational change was

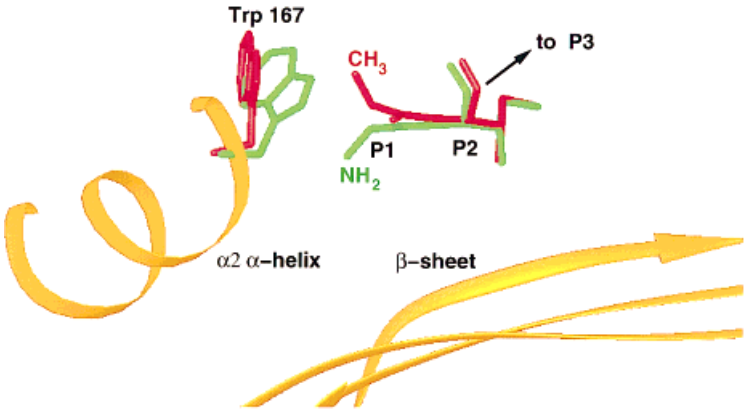

Fig. 3. Positions of MHC side chain Trp167 and N-terminal two peptide residues P1 and P2 in the HLA-A2/L9A (green) and HLA-A2/G1-Ndel (red) complexes.

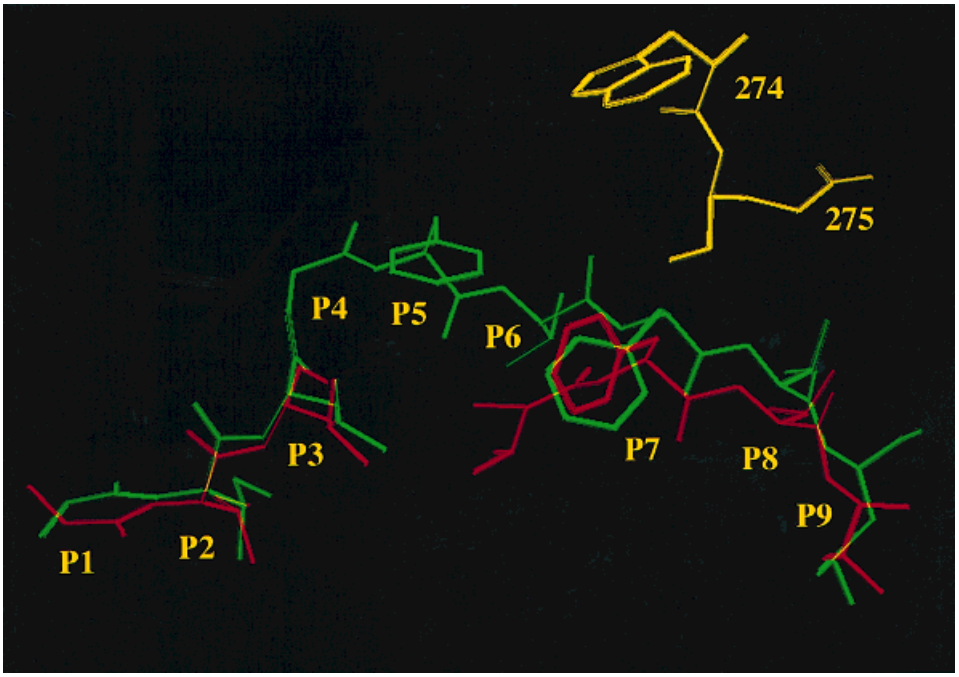

Fig. 4. Comparison of the matrix peptide (green) and V9-Cdel peptide (red) conformations as bound to HLA-A2. A lattice contact from residues 274 and 275 (yellow), which is the C-terminus of the HLA heavy chain, distorts the V9-Cdel peptide conformation at P6-P8 and results in disorder at P4-P5.

observed at the C-terminus of A9-Cdel in the HLA-A2/A9-Cdel complex, where the last two peptide residues were disordered (Fig. 2c), a modified peptide (V9-Cdel) was synthesized with the carboxylate group substituted by a methyl group, but with the optimal HLA-A2 anchor residue valine at P9 to stabilize the C-terminus in the peptide binding site (Table I, Fig. 2d). Overall, the bound conformations of these peptides, an extended conformation with a kink near P3 and P4 (Fig. 2), are very similar to all other peptides bound to class I MHC including a mixture of endogenous peptides bound to HLA-B27,[22] and single viral peptides bound to H-2K^b,[23–25] HLA-Aw68,[26] HLA-A2,[18,27] HLA-B35,[28] and HLA-B53.[29]

Structure of HLA-A2/L9A Complex

The conformation of the L9A peptide bound to HLA-A2 (Fig. 2a) is very similar to that of HLA-A2 complexed with the matrix peptide,[18] except that P9 binding is an alanine rather than a leucine. The rms difference between the main-chain atoms of the peptides is 0.23 Å, and that between the two HLA-A2 structures is 0.40 Å. These structural results are consistent with the thermal denaturation experi-

ments which showed that both HLA-A2 complexes have nearly identical thermal stability (Table I).[9] A small structural difference in the MHC binding site was observed; the orientation of MHC side chains Arg97 and Tyr116 have rotated from pointing towards the amino-terminal end of the binding site in the structure of the matrix peptide complex[18] to pointing towards the carboxyl-terminal end of the binding site in the structure of the HLA-A2/L9A complex (see Figures 8b and 8c of Reference 18). The same difference has been observed previously in three HLA-A2/peptide complexes (of five studied) and appears to be an accommodation to the orientation of the side chain at P7 or to a side chain smaller than leucine at P9.[18]

Structure of HLA-A2/G1-Ndel Complex

The structure of HLA-A2/G1-Ndel complex is similar to that of HLA-A2/L9A complex. The rms difference between the main chain atoms from P2 to P9 is 0.42 Å, and that between the two HLA-A2 structures is 0.40 Å (Fig. 2b). There is a slight change in the conformation of the peptide at the N-terminus. The P1 torsion angle ψ has rotated about 110°, rotating the methyl group that replaced the terminal amino group into the position normally occupied by the P1 side chain (Fig. 3). This rotation creates a cavity in the site at the position usually occupied by the amino-terminal group of the peptide which appears to be filled by one water molecule (a peak greater than 3.5σ and within 3.5 Å of the hydroxyl groups Tyr7 and Tyr171 was observed in the Fo−Fc difference Fourier maps). The bound water apparently makes the same pattern of hydrogen bonds as the substituted terminal amino group (Fig. 1).

The peptide G1A+L9A, with an alanine methyl group replacing the P1 glycine hydrogen "side chain," formed complexes with HLA-A2 that have approximately the same thermal denaturation temperature (T_m = 67.5 °C) as HLA-A2/L9A and HLA-A2/matrix (T_m = 65.9 °C and T_m = 64.9 °C), which both have Gly at P1 (Table I and Reference 9). This suggests that the rotation, itself, of the G1-Ndel methyl group out of the terminal amino-group binding site and into the P1 side chain pocket has no major thermodynamic effect, because it makes no difference whether the P1 side chain pocket is occupied by an alanine methyl group or a glycine hydrogen. In contrast, the loss of the hydrogen bonds to the charged N-terminus, ΔT_m = −21.0 °C (Table I and Reference 9), has a large effect on the thermodynamic stability of the HLA-A2/G1-Ndel complex.

A least-squares superposition of the main-chain atoms in the α-helices and β-strands of the peptide binding site (α_1 and α_2 domains) reveals that rotation of the substituted methyl group causes a movement of MHC side chain Trp167 (Fig. 3) to the position that it has been observed to occupy in the structures of HLA-A2 complexed with peptides having residues other than glycine at P1.[18] The P1 alpha-carbon atom of G1-Ndel is also shifted approximately up out of the binding site by 0.8 Å (Fig. 3). Substituting the amino-terminal group by a methyl group thus results in the loss of two hydrogen bonds between the charged amino-terminal group and conserved MHC side chains Tyr7 and Tyr171, and of a third hydrogen bond via a water molecule to Tyr59 (Fig. 1). The structure of HLA-A2/G1-Ndel complex also has a difference at the MHC side chains Arg97 and Tyr116, which point towards opposite ends of the binding site, a unique configuration in comparison to other structures of HLA-A2 complexed with single antigenic peptides (this study and Reference 18).

Structure of HLA-A2/A9-Cdel Complex

The conformation of A9-Cdel bound to HLA-A2 also had an unanticipated change relative to the matrix peptide and L9A. Although the first seven residues of A9-Cdel are essentially identical to those of L9A (rms deviation = 0.16 Å), no connected electron density was observed for P8 or P9, suggesting disorder at these positions (Fig. 2c). This disorder could have resulted partly from the lack of chirality at the P9 alpha-carbon of A9-Cdel (Table I), which allows rotation around the torsion angle Φ to position either of the two methyl groups in the site occupied by the P9 side chain. However, the lack of electron density in the P9 pocket, corresponding to these methyl groups, is inconsistent with this possibility. More likely, it is the absence of both the four hydrogen bonds to the terminal carboxylate, resulting from the methyl substitution in A9-Cdel (Fig. 1), and the absence of a large P9 anchoring side chain like Val, that prevents P8 and P9 from binding stably in the site, resulting in their conformational disorder.

MHC side chains Arg97 and Tyr116 are both pointing toward the amino-terminal end of the peptide binding site in the HLA-A2/A9-Cdel (e.g. Fig. 8b in Reference 18), an orientation opposite from that observed in the HLA-A2/L9A complex (above). It is unclear why these MHC residues adopt this orientation when P8 and P9 are disordered. We note that the X-ray data for this complex are less complete (Table II) than for the other crystals.

Structure of HLA-A2/V9-Cdel Complex

Because P8 and P9 of A9-Cdel were disordered in the MHC/peptide complex, the optimal P9 anchor for HLA-A2, valine, was substituted for the alanine at P9 of A9-Cdel (creating V9-Cdel [Table I]) in an effort to stabilize the bound conformation of a peptide lacking the terminal carboxylate group. The thermal denaturation temperature of the HLA-A2/V9-Cdel complex was measured as T_m = ∼44.0 °C (Table I), a value similar to that of HLA-A2/A9-Cdel, T_m = 42.8 °C (Table I). The strategy was thus successful; the bound conformation of the C-terminal of V9-Cdel is much more similar to that of the matrix peptide than

A9-Cdel was, except that a valine side chain occupies the P9 pocket (Figs. 2d and 4). Also, the positions of the MHC side chains, which normally form hydrogen bonds with the terminal carboxylate group of the peptide, are conserved except for a small deviation in the orientation of the solvent exposed Lys146. This residue has previously been observed to occupy different positions in different HLA/peptide complexes.[27,30]

Unexpectedly, a crystal lattice contact, unique to the V9-Cdel crystal (Table II), occurs between the C-terminal of the HLA-A2 heavy chain (Glu275) and positions P6 and P7 of the V9-Cdel peptide. The contact appears to have the effect of "pressing" the peptide further down into the peptide binding site from P6 to P8 by about a few Å (Fig. 4). There is no difference in structure at the N-terminal of the peptide. There is no connected (or interpretable) electron density at the two peptide positions P4-Gly and P5-Phe preceding this lattice contact (Figs. 2d and 4). The observation that a crystal lattice contact can alter the conformation of the central, exposed region of a bound peptide is consistent with previous crystallographic observations on the conformations of a collection of peptides bound to HLA-A2[18] and a change in the conformation of the same region of a peptide bound to HLA-A2 caused by interaction with a T-cell receptor.

MHC side chains Arg97 and Tyr116 point towards the carboxyl-terminal end of the binding site in the HLA-A2/V9-Cdel complex consistent with the presence of a P9 Val side chain and an upward-pointing P7-Phe side chain.[18]

DISCUSSION

Previous experiments showed a decrease in the thermal denaturation temperature of −21 °C and −23 °C for HLA-A2 complexed with the G1-Ndel and A9-Cdel, peptides which had methyl substitutions at the amino- and carboxyl-termini, respectively (Table I). Using a number of assumptions frequently made in thermal denaturation studies of proteins,[9,31] this difference represents a loss of free energy of stabilization of about $\Delta\Delta G = -4.6$ kcal/mole. A peptide with both termini modified (G1-Ndel+A9-Cdel) failed to promote the folding of stable class I MHC complexes. By contrast, the decrease in thermal denaturation temperature for a peptide with both anchor residues P2 and P9 substituted with alanine (L2A+L9A) was only −5.5 °C or about $\Delta\Delta G = -1.2$ kcal/mole. These thermodynamic measurements were interpreted as indicating that the loss of hydrogen bonds at either end of the peptide binding site to the charged peptide termini (Fig. 1), a peptide-sequence independent component of peptide binding, was energetically more important than the loss of interactions between both the peptide anchor residues and the major specificity pockets, P2 and P9, of HLA-A2. This suggests that class I MHC molecules have evolved conserved binding sites to recognize the termini of short peptides, as suggested by Madden et al. 1991, with anchor pockets to provide specificity. Because different MHC molecules have different anchor pockets, yet antigenic class I MHC/peptide complexes all form extremely long-lived complexes, it also seems likely that the conserved peptide termini binding sites may contribute substantially to the conserved property of long half-lives.

Interpretation of the thermodynamic measurements for the terminally modified peptides (Table I) was based on the assumption that the structures of HLA-A2/peptide complexes had been modified only by the loss of the hydrogen bonds from the charged terminal groups. The synthetic strategy of making minimal steric alterations to the peptide termini by replacing the $^{+}NH_3$- with a CH_3- at one end and the COO^{-} with a CH_3- at the other, was designed to allow the modified peptides to still fit into the binding site, in contrast to acetylation at the N-terminus, for example, where the added acetyl group would prevent a normal mode of binding into the sterically restricted N-terminal pocket.[24,32] The finding of conformational changes in three of the peptides (G1-Ndel, A9-Cdel, V9-Cdel) in the crystal structures of the peptide HLA-A2 complexes determined here illustrates the danger in interpreting thermodynamic data based even on the simplest modifications without three-dimensional structural information. In each of these cases, although sterically comparable substitutions were made to ensure that peptide-protein contacts are unperturbed, the modified peptides bound to HLA-A2 with unforeseen conformational changes to both ligand and MHC residues.

The interpretation of the thermodynamic measurements can now be extended from knowledge of the crystal structures of the HLA-A2 complexed with the terminally modified peptides. The observations that MHC-peptide contacts other than the conserved hydrogen bonds (bold dashed lines in Figure 1) were missing in the crystal structures of G1-Ndel and A9-Cdel, as the result of unanticipated peptide conformational changes (Figs. 2e and 3), suggests that the measured thermodynamic destabilization is only a lower limit for the energetic contribution of the hydrogen bonds to the stability of the complexes. Because no external factors such as lattice contacts appear to contribute to the structural alterations seen in the crystals of either of these class I MHC/peptide complexes, it seems appropriate to make the usual assumption that these structures represent the lowest free-energy- (or solution-) state of the HLA-A2/G1-Ndel and HLA-A2/A9-Cdel complexes. Therefore, the conformational state where only the hydrogen bonds are lost may have a higher free energy so that our estimate of the energetic consequences of the stabilization provided by those hydrogen bonds should increase. The increase may be zero or larger, we have only measured the lower limit.

Because no single state of the bound peptide was observed at the disordered C-terminus in HLA-A2/A9-Cdel, it is possible that many states, including that missing only the hydrogen bonds, have approximately the same stability, and that the T_m measured indicates the stability of that hypothetical state.

The observation that the thermal denaturation temperatures of HLA-A2/G1A+L9A and HLA-A2/L9A are about the same (Table I and Reference 9) indicates that the presence or absence of a methyl group in the P1 side chain pocket is of almost no overall thermodynamic consequence. Therefore, the rotation of the P1 methyl group into the P1 side chain pocket observed in HLA-A2/G1-Ndel (Fig. 3) is also probably of negligible thermodynamic importance. This argument suggests that the consequences of having the charged amino-terminus replaced by an apparent water molecule in HLA-A2/G1-Ndel are the source of the difference in stability, and not the unanticipated structural change.

A second indication of the importance of interactions at the peptide termini for stabilization of peptide complexes, is the observation of a lattice-contact-induced disorder in the center of the peptide in the HLA-A2/V9-Cdel complex crystal structure (Fig. 4). This observation emphasizes the relative conformational flexibility of the central section of peptides bound to HLA-A2. In an earlier study of the crystal structures of 5 different short peptides bound to HLA-A2, a remarkable array of different main chain conformations and side chain positioning was observed for residues between the P2 and P9 anchors.[18]

The same amino acid residue, valine, at P7 in the context of two different peptide sequences bound completely differently to HLA-A2; in one case extending down into the binding site and in the other case pointing out of the site in position to be contacted by a TCR.[18] These observations suggested that the class I MHC molecule had no fixed binding sites for either the main chain or side chain atoms, except those at the termini and the two or three specific anchor pockets, and that each peptide adopted a conformation dependent on its sequence (a marked contrast to peptides bound to class II MHC molecules where every bound peptide observed to date adopts the same polyproline type-II main chain conformation, with side chains disposed almost identically (summarized in Fig. 6b of Reference 33). Similarly, the crystal structure of HLA-Aw68, with a collection of endogenous peptides in the binding site, suggested the same idea since only the ends of the peptides were visible, thus indicating that peptides could adopt a number of conformations at their centers including looping-out off the binding site.[17]

The crystal structure of a TCR complex with HLA-A2 and the Tax peptide also revealed a conformational change in the bound peptide at P6 and P7,[18] induced by contacts with the TCR.[5] The HLA-A2/V9-Cdel crystal structure determined here showed that a crystal lattice contact is enough to disorder two residues (at P4 and P5) that are not contacted at the center of the peptide. The path of the peptide at P6-P8, near the contacts (at P6 and P7), also changes slightly, but the position of P9 is unaffected even though the C-terminal carboxylate is substituted for by a methyl group. These observations again suggest that the central parts of bound peptides are not as well stabilized upon binding to class I MHC molecules, in comparison to the anchors and to the termini.

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