X-ray diffraction studies as well as structure-activity relationships indicate that the central part of class I major histocompatibility complex (MHC)-binding nonpeptides represents the main interaction site for a T cell receptor. In order to rationally manipulate T cell epitopes, three nonpeptidic spacers have been designed from the x-ray structure of a MHC-peptide complex and substituted for the T cell receptor-binding part of several antigenic peptides. The binding of the modified epitopes to the human leukocyte antigen-B*2705 protein was studied by an in vitro stabilization assay, and the thermal stability of all complexes was examined by circular dichroism spectroscopy. Depending on their chemical nature and length, the introduced spacers may be classified into two categories. Monofunctional spacers (11-amino undecanoate, (R)-3-hydroxybutyrate trimer) simply link two anchoring peptide positions (P3 and P9) but loosely contact the MHC binding groove and thus decrease more or less the affinity of the altered epitopes to human leukocyte antigen-B*2705. A bifunctional spacer ((R)-3-hydroxybutyrate tetramer) not only bridges the two distant anchoring amino acids but also strongly interacts with the binding cleft and leads to a 5-fold increase in binding to the MHC protein. To our knowledge, this is the first report of a nonpeptidic modification of T-cell receptor binding residues that significantly enhances the binding of altered peptide ligands to their host MHC protein. The presented modified ligands constitute interesting tools for perturbing the T cell response to the parent antigenic peptide.

Class I MHC molecules are highly polymorphic proteins that play a key role in immune surveillance by presenting foreign peptides to cytotoxic T lymphocytes (1). The molecular mechanisms of peptide selection have been characterized by x-ray diffraction studies of several MHC proteins in complex with either a peptide pool or single ligands (2). Peptides, generally nonamers, tightly bind to conserved MHC residues in a sequence-independent manner at their N and C termini (3), whereas the central part of the bound peptide bulges out of the binding groove (4). Peptide specificity is governed by the position and chemical nature of some anchoring side chains (often P2, P3, and P9) that bind to MHC polymorphic pockets (5, 6). Complementary to x-ray structure determinations, sequencing self-peptides naturally bound to MHC proteins allows the determination of peptide binding motifs (7, 8) and thus the identification of conserved amino acids responsible for MHC binding (named dominant anchors, generally at positions P2 and P9) and more variable residues hypothesized to account for TcR recognition (usually in the central part of the peptide sequence, from P4 to P8). Peptide mutation (9, 10) as well as recently determined x-ray structures of αβ TcRs in complex with a MHC-peptide (11, 12) unambiguously support this assumption. Since some class I MHC alleles are associated with either susceptibility or resistance to human diseases (13–15), altering TcR contact residues of T cell epitopes has been proposed for designing altered peptide ligands with TcR antagonist properties (16), leading to in vivo T cell anergy (17). However, natural peptides cannot be easily used as immunosuppressors because of poor enzymatic stability and pharmacokinetic properties (18). Herewith, we describe the substitution of nonpeptidic moieties for the TcR contact amino acids of several T cell epitopes naturally presented by the class I MHC protein B*2705, which is strongly linked to severe inflammatory diseases like ankylosing spondylitis (13) or reactive arthritis (19). Some reports in which a similar strategy has been followed (20–22) show that the altered peptide ligands still form stable complexes with their host MHC protein but often present a reduced affinity relative to the parent peptide. The present study describes a novel oligomeric spacer able not only to link two MHC anchoring positions (P3 and P9) but also to significantly improve binding to the restricting class I MHC protein.

**EXPERIMENTAL PROCEDURES**

**Computer-assisted Ligand Design—**Molecular mechanics and dynamics calculations were carried out using the AMBER 4.1 package (23), using the parm94 parameter set (24) and an all-atom force field representation. Force field parameters for the ester group were taken from the literature (25). Atomic charges for the Aua and HB monomers were calculated using the GAUSSIAN 94 package (26) and the HF/6–31G* basis set by fitting atom-centered charges to an ab initio electrostatic potential, using the RESP method (27) according to a previously described procedure (28). Atomic charges for both new monomers are listed in Table I.

Initial coordinates for the MHC-ligand complexes were obtained from the x-ray structure of HLA-B*2705 (3) as described previously (20, 29). The spacers were substituted for the natural pentapeptide sequence using the SYBYL modeling package (TRIPOS Association, Inc., St.

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¶ The abbreviations used are: MHC, major histocompatibility complex; HLA, human leukocyte antigen; TcR, T cell receptor; P1, peptide position n; Fmoc, N-(9-fluorenyl)methoxycarbonyl; Aua, 11-amino undecanoate; HB, (R)-3-hydroxybutyrate.
Restrained electrostatic potential-derived point charges, calculated using the RESP method (26) from GAUSSIAN 94 HF/6–31G* electrostatic potentials

| Monomer | Atomic charges$^a$ |
|---------|--------------------|
| Aua$^b$ | ![Atomic Charges Diagram](attachment:atomic_charges.png) |
| HB$^c$  | ![Atomic Charges Diagram](attachment:atomic_charges.png) |

$^a$ Ab initio derived electrostatic potentials have been calculated for protected monomers (Ac-Aua-NMe, Ac-HB-OMe) and atomic charges of the isolated monomers (Aua and HB) adjusted to neutrality using Lagrange constraints, as previously described (28). Charges for equivalent hydrogen atoms are only mentioned once.

$^b$ 11-aminoundecanoate.

$^c$ (R)-3-hydroxybutyrate.

Louis, MO). From a starting fully extended conformation, dihedral angles of the main chain between P3 and P9 were modified by hand in order to reproduce a correct trans geometry for the newly introduced amide or ester bonds. The ligand was first relaxed by 500 steps of conjugate gradient energy minimization while maintaining the protein fixed. It was then submitted to a 100-ps simulated annealing protocol in order to sample the broadest conformational space accessible. Starting with random velocities assigned at a temperature of 1000 K, the ligand was first coupled for 50 ps to a heat bath at 1000 K using a relatively weak temperature coupling constant $\tau$ (0.2 ps) and then linearly cooled down to 50 K for the next 50 ps while $\tau$ was strengthened to a value of 0.05 ps. During these 100 ps, no protein atoms were allowed to move. The last conformer was then solvated in a 10-Å-thick TIP3P water shell. Energy minimization of the ligand, of the MHC-ligand complex, followed by 200-ps molecular dynamics simulation of the fully solvated MHC-ligand pair was performed as previously reported (20).

**Synthesis of the Modified Peptides**—Ligands 1–8 (Table II) were obtained by automated solid-phase peptide synthesis using a Fmoc/tBu protecting strategy. Chain elongation was performed by a robot system (Syro Multi-Syn-Tech, Bochum, Germany) with a subsequent manual deprotection and analysis. Fmoc-protected amino acids were coupled to the diisopropylcarbodiimide-activated carboxyl terminus in a nonnatural peptide coupling strategy. Chain elongation was performed by a robot system (Syro Multi-Syn-Tech, Bochum, Germany) with a subsequent manual deprotection and analysis. Fmoc-protected amino acids were coupled to the diisopropylcarbodiimide-activated carboxyl terminus in a 10-Å-thick TIP3P water shell. Energy minimization of the ligand, of the MHC-ligand complex, followed by 200-ps molecular dynamics simulation of the fully solvated MHC-ligand pair was performed as previously reported (20).

**Epitope Stabilization Assay**—The quantitative assay used was described previously (30). Briefly, RMA-S transfectants expressing B*2705 or B*2704 were used. These are murine cells with impaired TAP-mediated peptide transport and low surface expression of (empty) class I MHC molecules, which can be induced at 26 °C (31) and stabilized at the cell surface through binding of exogenously added ligands.

These cells were incubated at 26 °C for 24 h. After this, they were incubated 1 h at 26 °C with $10^{-4}$ to $10^{-8}$ M peptides, transferred to 37 °C, and collected for flow microcytometry analysis with the ME1 monoclonal antibody (IgG1, specific for HLA-B27, -B7, and -B22) (32) after 4 h for B*2705 or after 2 h for B*2704. The determinant recognized by ME1 is not affected by bound peptides or by polymorphism in these two subtypes (data not shown). Binding of a given ligand was measured as its C50. This is its molar concentration at 50% of the fluorescence obtained with that ligand at 10^-4 M. Ligands with C50 ≤ 5 μM were considered to bind with high affinity, since these were the values obtained for most of the natural B27-bound peptides. C50 values between 5 and 50 μM were considered to reflect intermediate affinity. C50 > 50 μM indicated low affinity. Binding of peptide analogs was measured as the concentration of the peptide analog required to obtain the fluorescence value at the C50 of the unchanged peptide. This was designated as EC50. Relative binding was the ratio between the EC50 of the peptide analog and the C50 of the corresponding unchanged peptide.

**HLA-B*2705 Expression and Purification**—A cDNA encoding for human β2-microglobulin (gift of Dr. C. Vilches, Clinica Puerta de Hierro, Madrid) was cloned into a pGex vector (Amersham Pharmacia Biotech), yielding a fusion protein with glutathione S-transferase. Escherichia coli cells transformed with this pGex vector were grown under vigorous shaking in LB broth for 24 h at 25 °C after induction with isopropyl-1-thio-β-D-galactopyranoside. Cells were frozen at −70 °C, thawed, and suspended in TBS (20 mM Tris, 150 mM NaCl, pH 8.0), and lysed by the addition of lysozyme and brief sonication. The crude extract was passed over a glutathione-agarose column (Sigma), and after extensive wash
TABLE II

| Ligand | Sequence | P3 | Spacer | P9 | B*2705 | B*2704 | Tm °C |
|--------|----------|----|--------|----|--------|--------|-------|
| 1      | R R R    |    |        |    | 1.2    | 0.8    | 52.8 ± 0.7 |
| 2      | Aua℃    |    |        |    | 4.0    | 1.0    | 42.9 ± 0.3 |
| 3      | S R Y    |    |        |    | 3.0    | 5.4    | 46.3 ± 0.5 |
| 4      | G R A    |    |        |    | 8.6    | 100.0  | 39.5 ± 0.2 |
| 5      | A FVTIG  |    |        |    | 1.8    | 6.4    | 61.9 ± 0.3 |
| 6      | Aa       |    |        |    | 7.0    | >100.0 | 48.1 ± 0.4 |
| 7      | Q R L    |    | KEAAE  |    | 10.0   | 5.3    | 62.8 ± 0.7 |
| 8      | Aua      |    |        |    | 46.5 ± 0.2 |
| 9      | (HB) 4   |    |        |    | 40.0   |        |       |
| 10     | (HB) 7   |    |        |    | 2.5    |        |       |
| 11     | A        |    |        |    | 20.6   | 63.7 ± 0.6 |
| 12     | (HB) 8   |    |        |    | 1.6    | 62.1 ± 0.7 |

* Concentration of ligand at which HLA-B27 fluorescence (measured by flow microfluorometry analysis with an anti-B27 monoclonal antibody) on RMA-S cells was half the maximum obtained with the wild type peptide (30).

a Melting temperature: midpoint of the thermal unfolding of the B*2705 heavy chain. Tm values are means of three denaturation experiments performed on independently reconstituted heavy chain-β2-microglobulin-ligand heterotrimers. S.D. values have been obtained by fitting the obtained curves to a two-state model as previously described (35).

b Epstein-Barr virus latent membrane protein (236–244) (49).

c Influenza A nucleoprotein (383–391) (50).

d Human immunodeficiency virus 1 glycoprotein 120 (314–322) (39).

e E. coli DnaK protein (260–268) (20).

With TBS, the β2-microglobulin was eluted by thrombin cleavage as a single band at 11 kDa (SDS-polyacrylamide gel electrophoresis).

The HLA-B*2705 heavy chain was affinity-purified under denaturing conditions as a His6 fusion protein. The expression vector was obtained by subcloning the cDNA encoding for the first extracellular 274 amino acids (gift of Dr. K. C. Parker, National Institutes of Health, Bethesda) into the polycloning site of the oligohistidine vector pQE30 (Quiagen) with the restriction endonucleases BamHI and HindIII. The heavy chain was expressed in E. coli at 35 °C for 2 h after induction with isopropyl-1-thio-β-D-galactopyranoside. Longer expression times led to an increase of immature or degraded heavy chains. Inclusion bodies were prepared using a standard procedure (33) and solubilized in 8 M urea, 20 mM Tris, 150 mM NaCl at pH 8.0. Purification on a nickel-nitritocaceta-garose column led to the HLA-B*2705 heavy chain with two minor impurities of lower molecular weights consisting of truncated heavy chains.

Folding of the MHC Protein upon Ligand Binding—Reconstitution of the heavy chain-β2-microglobulin-ligand heterotrimer was achieved by dialysis (cellulose ester tubings, 500-Da cut-off) of a solution containing 0.15 mg/ml heavy chain, 0.1 mg/ml β2-microglobulin, and 0.1 mg/ml peptide ligand, using 5 mM glutathione to establish reducing conditions in 6 M urea against TBS. The solution was sparged with nitrogen to prevent premature formation of disulfide bridges and oxidation of free Cys residues in the B*2705 heavy chain. After 36–48 h at 10 °C, the mixture was concentrated to 500 μl in a Centricon ultrafiltration unit (Amicon-Grace Ltd.). The folded heterotrimer was purified by gel filtration on a Superdex 75 column (Amersham Pharmacia Biotech) with UV detection at 280 nm. The chromatogram showed three major peaks at 9, 11.5, and 14-ml elution volume corresponding to heavy chain aggregates, refolded complex, and excess β2-microglobulin, respectively. The overall yield of the fully reconstituted heterotrimer varied around 5%. The heterotrimer peak was collected, concentrated in a Centricon 30 ultrafiltration unit (Amicon-Grace), and immediately subjected to thermal denaturation.

Monitoring the Thermal Stability of MHC-Ligand Complexes by CD Spectroscopy—All CD measurements were done on a Jasco J-720 polarimeter with a water-jacketed 1-mm sample cell connected to a computer-interfaced Neslab 111 circulating water bath. Temperature control was achieved by measuring the circulating water immediately after the sample cell. The thermal denaturation profiles were recorded at 218 nm in 10 mM Tris, 150 mM NaCl (pH 8.0) with the Jasco TEMPCSCAN software using 0.1 °C increments at a heating rate of 30 °C/h. Sample concentrations were determined photometrically and held at 0.2 mg/ml. Different scan rates did not affect the Tm value of B*2705 in complex with a reference peptide (GRAFVTIGK; compare Ref. 34 and Table II). Three denaturation curves from independent refolding preparations were averaged, after conversion to molar ellipticity values. The curves were reduced to 70 data points by replacing each of the 10 neighboring points with their mean value. By assuming a two-state equilibrium (35), data were fitted by a nonlinear least-squares routine with the program Origin 2.9 (MicroCal Software, Inc.) to the following equations.

\[
\Theta(T) = \Theta_0 + (\Theta_1 - \Theta_0)/1 + e^{\Delta H_m/Tm/RT_m} 
\]

\[
x = (-\Delta H_m/R)(1/T - 1/T_m) + (\Delta C_p/R)(1/T_m - 1) - \ln(T/T_m) \]  

The measured ellipticity (\(\Theta\)) is given as a function of the temperature (T) with the enthalpy (\(\Delta H_m\)) heat capacity upon unfolding (\(\Delta C_p\)), and the midpoint temperature of unfolding (Tm) being the fitting parameters. Initial estimates for \(\Delta H_m\) were obtained by plotting ln K versus 1/T (van’t Hoff plot) in the transition region. \(\Delta C_p\) was assumed to be temperature-independent (36), and initial values were estimated from the primary sequence (37). The linear base-line functions of the unfolded and folded states \(\Theta_0\) and \(\Theta_1\) were determined as linear regressions of the pre- and post-transitional regions. The enthalpy change at the midpoint of unfolding (\(\Delta H_m\)) was determined by the least-squares fit of the unfolding curve to Equation 1. Because \(\Delta C_p\) estimates obtained by this approach are not very accurate and the \(\Delta H_m\) values are largely influenced by the observed deviations from a two-state model, a direct extrapolation from the midpoint of unfolding to obtain \(\Delta G_{\text{unfolding}}\) at 25 °C was not taken into consideration.

RESULTS

Replacing a Pentapeptide with a Polymethylene Spacer in Four Unrelated Natural Epitopes—For mimicking the sequence of the central pentapeptide part (P4–P8) of MHC-bound nonpeptides, any nonpeptidic fragment needs first to reproducibly link to the neighboring positions (P3 and P9) that are linked by the new spacer. The key distance between C-terminal anchors (P3 and P9) is 16.6 Å in the x-ray structure of HLA-B*2705 complexed by a nonpeptide model (3). The same distance can be easily obtained after linking a polymethylene chain (Aua, Fig. 1) to P3 and P9 residues by simple amide bonds. The 11-amino undecanoate fragment was then chosen...
for its optimal length in an extended conformation and the absence of any substituents, which should allow a conformational flexibility sufficient for a proper fit into the binding groove. To check the independence of the proposed modification on the parent epitope sequence, the Aua spacer was introduced in four unrelated sequences of natural epitopes, known to bind to B*2705 (Table II). The question of whether the new ligands were able to remain tightly bound in the peptide binding cleft like the natural nonapeptides was addressed by molecular dynamics simulations of the solvated complexes (Table II). The computational protocol used has been previously shown to explain the binding potency of several HLA-B27-binding peptides (38) and to predict the high affinity of designed peptide analogues (20, 29). Energy-minimized conformations show that the proposed bridging has modified neither the overall conformation of the bound ligands nor the intermolecular distance between P3 and P9 C-atoms (Fig. 2). Moreover, the chemical substitutions were compatible with the conservation of the main interactions between the altered peptides and B*2705, especially the electrostatic interactions provided by the two charged termini and the arginine found at position 2 of B*2705-binding peptides (39, 40).

In order to experimentally validate the proposed model, the four B*2705-restricted T cell epitopes and their modified analogues (Table II) were synthesized and then tested for their binding to B*2705, in an in vitro epitope stabilization assay (30). Replacing the central pentapeptide sequence by the unsubstituted Aua fragment led in all cases to a slight decrease in B*2705 stabilization (Table II), which was also reflected by a lesser thermal stability of the resulting complexes monitored by CD spectroscopy (Fig. 3, A–D). The temperature shift in the midpoint of unfolding depends on the sequence of the reference peptide but varies from −7 to −14 °C (Table II). Whereas the effect of the Aua spacer is similar in both assays, there seems to be no clear correlation between the EC_{50} scores obtained from the epitope stabilization assay and the T_m values calculated from the thermal denaturation experiments. The T_m values reported here are fairly similar to those found for B*2705-binding peptides by other groups (21, 34, 41). The highest stability against temperature was found for complexes with peptides 5 and 7, which all present a lysine at P9. Less favored amino acids at P9 are Val (peptides 1 and 2) and especially Arg (peptides 3 and 4), which gives by far the least stable complexes with B*2705 (peptides 1 and 3, respectively).

Binding of peptides 1–6 to a closely related HLA-B27 subtype (B*2704) was also examined by the same in vitro stabilization assay. B*2704 differs from B*2705 by two amino acid changes in the peptide binding groove (Asp77 to Ser; Val156 to Glu), which influence its peptide specificity, relative to B*2705 (42). In contrast to B*2705, substitution of Aua spacers for P4–P8 dramatically decreases binding to B*2704 in our epitope stabilization assay (Table II) when the last anchoring position (P9) is a basic amino acid (Lys, Arg). If P9 is an apolar residue (Val, peptide 2), no real change in B*2704 binding was noticed.

Substituting 3-Hydroxybutyrate Oligomers for the P4–P8 Sequence of a Natural Peptide—The decreased binding of the Aua analogues to B*2705 is probably due to the nonfunctionalized nature of the introduced spacer and the lack of interactions between the unsubstituted Aua moiety and the central part of the binding groove. Thus, a rational improvement in terms of binding affinity would be to ramify the spacing moiety in order to reach one of the two central pockets (pockets C and E) of the peptide binding groove that face the spacer fragment. The (R)-3-hydroxybutyrate (HB) monomer was selected for three main reasons: (i) polymers of HB are chemically stable (43); (ii) they adopt conformations whose folding in the free state resembles that found for peptides (44); and (iii) the methyl substituent is large enough to fit into pockets C and E. Thus, a trimer (three units) and a tetramer (four units) of HB were substituted for the P4–P8 sequence of one natural peptide (polyesterpeptides 9 and 10; Table II), since they should optimally span the key distance between the two anchor positions (P3 and P9) to bridge (Fig. 1).

In order to circumvent cyclization of the N-terminal glutamine (45) that would prevent binding of the peptidic N terminus in the A pocket of B*2705, the Ala1 analogue was also synthesized in the nonnatural series (polyesterpeptides 11 and 12; Table II).

The modified ligands 9–12 have totally different binding affinities in the in vitro stabilization assay (Table II), the tetramer-containing compounds (ligands 10 and 12) being about 15 times more potent that the trimeric analogues (ligands 9 and 11). Furthermore, a HB tetramer segment leads to a significant enhancement of the binding to B*2705 relative to the natural pentapeptide sequence. Again, the differences observed between natural and polyesterpeptides in the in vitro stabilization assay are not reflected by the thermal denaturation experiments, performed only for ligands 11 and 12. Both compounds promote a similarly high stability of the resulting MHC-ligand pair with T_m values of 62–63 °C (Fig. 4, Table II) analogous to that found for the parent peptide 7, and characteristic of high affinity ligands (41).

**Molecular Modeling of the Altered Peptides in Complex with B*2705**—A rationale for the (de)stabilizing effects of the three spacers presently studied is proposed by the molecular dynamics time-averaged conformations of a reference peptide (QR-LKEAAEK; peptide 7) and its analogues (peptides 8–10). By looking at all close nonbonded contacts between any peptide residue and its protein neighboring atoms, the three spacers (Aua, HB trimer, and HB tetramer) can be easily distinguished (Fig. 5). The Aua spacer provides fewer contacts to the MHC binding groove than the pentameric P4–P8 sequence of the parent peptide 7. This could explain the decreased binding
affinity of Aua-containing peptides to B*2705. The detrimental effect of the HB trimer can be explained by the weakening of the interactions between both terminal residues (PN and PC) and their respective pockets (A and F). The better complementarity of the HB tetramer to the B*2705 binding cleft is probably related to the following factors: (i) the additional interactions provided by two methyl groups of the tetrameric spacer itself and (ii) a higher number of nonbonded contacts of all other MHC anchors (PN, P2, P3, and PC).

The total buried surface area of the modified ligands 8–10 has been maintained when compared with that of the parent peptide 7 (about 650 Å²; data not shown). However, the total accessibility of the ligands in their bound state is different. It is reduced by 20% for HB analogues with respect to the natural epitope 7 (from 500 to 400 Å²). The Aua compound 8, although slightly less potent, has a much lower accessible surface area (250 Å²) due to the lack of substituents in the spacing area.

**DISCUSSION**

Replacing the central TcR-binding residues of MHC class I-bound peptides (P4–P8) by nonpeptidic moieties has been reported previously (20, 21). Herewith, we propose to rationalize the effect of three novel spacers on binding to the HLA-B*2705 protein. The simplest spacer (Aua) is a single polymethylene chain linking the P3 and P9 positions by amide bonds. In accordance with a previous report studying the effect of non-α amino acids (20), the Aua spacer does not impair binding to B*2705. Only a moderate decrease in relative binding to B*2705 was observed in an epitope stabilization assay, performed for four unrelated modified peptides (Table II). However, the effect of this modification on the thermal stability of the resulting MHC-ligand pair was more significant (Fig. 3, A–D). Depending on the peptide in which the Aua moiety was introduced, the midpoint of unfolding of the B*2705 heavy chain (T_m) was lowered by 7–14 °C. The corresponding free

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**Fig. 2.** Close-up into the binding groove of HLA-B*2705 (orange surface) in complex with peptides 7–10 (Table II). These structures represent energy-minimized conformations obtained from the x-ray structure of HLA-B*2705 in complex with a model peptide (Protein Data Bank entry 1hsa) under a previously described protocol (20, 29). The heavy chain backbone atoms of HLA-B*2705 have first been fitted together in the four complexes, and the protein atoms are not shown for the sake of clarity. Since protein distortion upon energy minimization of the resulting complexes is minimal, the MHC protein is here represented by a unique molecular surface independent of the bound ligand. The color coding is as follows: blue, nitrogen; red, oxygen; white, carbon atoms of ligand 7; cyan, carbon atoms of ligand 8; green, carbon atoms of ligand 9; yellow, carbon atoms of ligand 10. The arrows indicate two methyl substituents of the HB tetramer interacting with the central pockets C/E of the protein. The figure has been prepared using the program GRASP (51).
energy change in unfolding $\Delta \Delta G^{\text{unfolding}}$ at the midpoint of unfolding, derived from the CD spectra (22), varies from $-0.8$ to $-1.3$ kcal/mol. Since unfolding of the heavy chain should follow release of the ligand, this observation supports a faster dissociation of the modified peptides with respect to the parent epitope, as recently illustrated in a homogeneous series of H-2K$^d$-binding nonapeptides (46). However, the present study suggests that extrapolating peptide binding differences from $T_m$ values is not allowed for unrelated sequences. For the set of 4 T cell epitopes presently studied, EC$_{50}$ values cannot be related to melting temperatures calculated by CD spectroscopy. A likely explanation for this is that binding, as measured in epitope stabilization assays, is significantly influenced by the association rate of the peptide, whereas CD measurements relate only to the dissociation rates. The highest thermal stabilities were obtained for the B$^*2705$ protein in complex with
peptide ligands bearing a Lys at P9. This makes sense, since Lys is the P9 residue most complementary to its binding pocket F. Its side chain forms a buried salt bridge with Asp located at the bottom of the pocket. The predominance of the enthalpic contribution to peptide dissociation would thus be compatible with the lower $T_m$ values observed with peptides having an amino acid (Val, Arg) for which the interaction with pocket F is weaker. It also corroborates previous computational simulations, suggesting that peptide dissociation first occurs at the C terminus (20, 29, 38).

Interestingly, the effect of the Aua spacer is subtype-dependent, since differences between the natural and the Aua peptides in binding to B*2704 were much more significant (Table II). B*2704 basically differs from the B*2705 allele by its weak propensity to present peptides with basic P9 amino acids and its improved suitability for nonpolar P9 residues (42). Thus, the deleterious effect of the Aua spacer is amplified for peptides bearing a weak anchoring amino acid at P9 (peptides 4 and 6; Table II) and decreased for peptides with nonpolar P9 residues (peptide 2). The Aua group can be considered as a monofunctional spacer, since it simply provides the covalent linkage between two neighboring anchor positions (P3 and P9). Therefore, it has the same effect on HLA binding as previously reported spacing moieties like oligomers of 4-aminobutyrate or the tetramer of HB, introduced between P3 and P9, since it simply provides the covalent linkage that provides additional interactions to the binding groove. The tetramer of HB, introduced between P3 and P9, significantly enhances binding to B*2705 (Table II). The beneficial effect of the HB$_4$ spacer is attributed to two of its methyl substituents that reach the central pockets C/E of the binding cleft (Fig. 2). Since the global binding mode of the modified peptide has not been altered, the direct consequence of this replacement is an enhanced number of nonbonded contacts with the protein (Fig. 5). Again, discrepancies are observed for that series of compounds (ligands 7–12) between EC$_{50}$ values and melting temperatures derived from CD experiments on the reconstructed complexes (Fig. 4). $T_m$ values calculated for the tetrameric and trimeric HB analogues are nearly identical, whereas a 12–16-fold decreased binding was observed after shortening the length of the spacing area by one HB unit. The $T_m$ values of a series of MHC-peptide complexes have recently been directly related to experimental equilibrium dissociation constants, $K_D$ (46). Thus, the higher affinity observed for the (HB)$_4$ compounds relative to the parent peptide and the trimeric analogues could be due to faster on-rate kinetics. Alternatively, since the correlation proposed by Morgan et al. (46) takes into account a series of highly related nonapeptides, it may not be valid for altered ligands lacking a canonical nonapeptide structure. Importantly, the present study demonstrates that CD denaturing curves cannot be used alone to explain differences in binding of altered peptide ligands to a class I MHC protein. This is of crucial importance in any design effort aimed at enhancing binding affinities by increasing the on-rate kinetics of the designed molecule. It should be noted that two CD denaturation curves (peptides 7 and 12, Fig. 4) slightly deviate from the expected two-state model by presenting an additional transition at a temperature (45 °C) corresponding to the unfolding of peptide-free heavy chain (47). Such deviations from an ideal two-state model have already been observed (34) but remain difficult to explain at the molecular level.

Our data demonstrate that B*2705-restricted epitopes may be easily modified by introducing simple nonpeptidic elements in their central part without drastic changes in binding to their restriction MHC proteins. Two conditions seem to be necessary for these modifications: (i) the last amino acid (PC) should be a strong anchor, and (ii) the parent epitope should not contain a dominant anchor position between the P4 and P9 positions. Since this is the case for a majority of class I MHC peptide binding motifs (8), such chemical manipulations should be feasible for many antigenic peptides binding to class I MHC proteins. Class II MHC-binding peptides that utilize nearly all peptidic bonds to interact with their host MHC protein (48) must be excluded from these epitope modifications. The altered ligands reported in this study constitute a further step toward obtaining full nonpeptide ligands for class I MHC proteins. They represent interesting tools for altering the response of B*2705-restricted T cells to naturally occurring antigenic peptides and for designing novel synthetic vaccines.

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