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Binding of Rationally Designed Non-natural Peptides to the Human Leukocyte Antigen HLA-B*2705

STEFAN KREBS, GERD FOLKERS and DIDIER ROGNAN*

Department of Pharmacy, Swiss Federal Institute of Technology, CH-8057 Zurich, Switzerland

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Abstract: High-affinity ligands of non-peptidic nature, binding to the class I major histocompatibility complex protein HLA B*2705 whose expression is strongly linked to the pathogenesis of the autoimmune disease ankylosing spondylitis, should give way to a selective immunotherapy by blocking or antagonising the interaction with autoreactive T cell clones. Here we present experimental data on the binding of modified peptides, designed to optimally bind to HLA-B*2705 by filling a hydrophobic binding pocket (pocket D) with nonencoded aromatic amino acids. Three peptides with altered side chains (alpha-naphthylalanine, beta-naphthylalanine and homophenylalanine) in position 3 were synthesised. The thermal denaturation profiles of the HLA protein in complex with the modified peptides, monitored by circular dichroism spectroscopy, showed a significant shift towards higher melting temperatures with respect to the parent T cell epitope. The proposed binding mode of the nonnatural peptides was checked by site-directed mutagenesis of the pocket D, hypothesised to accommodate the large hydrophobic side chains. Reducing the size and depth of the pocket by mutating Leu l56 into Trp only affects the binding of the non-natural ligands, thus providing experimental evidence that the nonnatural peptide amino acids bind as predicted to the host MHC protein.

Keywords: HLA-B27; circular dichroism; thermal denaturation; computer-aided ligand design

INTRODUCTION

Presentation of antigenic peptides by class I major histocompatibility complex (MHC)-encoded proteins and their recognition by the $\alpha\beta$ T cell receptor (TCR) of $CD8^+$ T lymphocytes is a crucial event in the generation of a cellular immune response [1]. As MHC molecules are unable to distinguish the source of immunogenic peptides it is likely that recognition of self-derived peptides by alloreactive T cells is responsible for the chronic aspect of autoimmune diseases to which many HLA alleles are associated [2,3]. With the well-established linkage between certain B27 subtypes and the occurrence of ankylosing spondylitis, an inflammatory rheumatic disorder of the joints [4], it should be possible to stop the propagation of this disease by either selectively blocking the presentation of the putative arthritogenic peptide by a high-affinity peptide analogue [5], or antagonising the T cell recognition by a peptide of slightly altered geometry [6]. Due to unfavourable pharmacokinetics of small peptides, a candidate for selective immunotherapy should rather be of non-peptidic nature [7].

The binding of peptides to class I MHC molecules is now known in great detail (see [8] for review). Proteins are first degraded by cytoplasmic proteasomes to shorter fragments [9], transported to the endoplasmic reticulum via the TAP pathway [10] and loaded to the HLA heavy chain-β2 microglobulin heterodimer [11]. The peptides are generally bound to the binding groove at both termini, via a network of hydrogen bonds [12]. While a substantial amount of the total binding free energy originates

Abbreviations: DIC, *N*,*N*-diisopropylcarbodiimide; IPTG, isopropyl, β-D-thiogalactopyranoside; Mtt, 4-methyltrityl; TIS, triisopropylsilane; Tmd-Phe, 4′-(3-trifluoromethyl)-3H-diazirin-3-yl-phenylalanine.

* Correspondence to: Department Pharmazie ETH, Winterthurerstr. 190, CH-8057 Zurich, Switzerland.

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from these electrostatic interactions [13], selectivity is usually ensured by the binding of peptide side-chain in allele-specific pockets [14] at the so-called anchor positions [15], generally found at peptide positions 2 and 9 [8]. With both ends tightly bound to MHC, the middle part of the bound peptide (between positions 4 and 8) bulges out of the binding groove and serves as the TCR contact area [16]. Although there are additional binding pockets, they play no stringent role in peptide selection, but positional preferences for certain amino acids can be part of the binding motif and fine-tune the allele specificity [17,18]. Furthermore, auxiliary anchors allow to design peptides that have a higher affinity than the naturally selected antigens [19,20].

For HLA-B27, the binding motif comprises an invariant Arg at position 2 and positively-charged or hydrophobic residues at position 9. Positions 1 and 3 provide secondary anchors, important for the determination of peptide affinity [21,22].

For inhibiting the HLA-B27-restricted T cell immune response, the strategy we chose was: (i) to enhance the binding to the restricting MHC molecule in order to successfully compete with natural T cell epitopes; and (ii) to alter the T-cell contact area by reducing the interaction possibilities of the bulging middle part. In a first step, we have designed peptides with an altered side-chain in position 3 or with non-peptidic spacers replacing peptide amino acids 4 to 8 [19]. Their binding to HLA-B27, predicted on the basis of molecular dynamics simulation was assessed by an in vitro semi-quantitative reconstitution assay [19]. Here we present experimental data indicating the enhanced binding of the designed peptide analogues and validating their binding mode, by monitoring the thermal stability of two HLA-B*2705 molecules, wild type and binding pocket D mutant L156W, complexed with natural and designed peptides.

MATERIALS AND METHODS

Expression and Reconstitution of HLA-B*2705

Using standard molecular biology techniques, a cDNA encoding for the human β-2-microglobulin (gift from C. Vilches, Madrid) was cloned into a pGex vector (Pharmacia), yielding a fusion protein with glutathion-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 IPTG. The cells were frozen at − 70°C, thawed, suspended in TBS (20 mM Tris/150 mM NaCl/pH 8.0) and lysed by addition of lysozyme and brief sonication. The crude extract was passed over a glutathion agarose column (Sigma) and after excessive washing with TBS, β-2-microglobulin was eluted by thrombin cleavage as a single band at 11 kDa (SDS-PAGE).

HLA B*2705 was affinity-purified as a His_6 fusion protein. The expression vector was obtained by subcloning the DNA encoding for the first 274 amino acids (domains $\alpha 1-\alpha 3$; gift of K.C. Parker, Bethesda) into the polycloning site of the oligohistidine vector pQE 30 (Qiagen) with the restriction endonucleases Bam HI and Hind III. The protein was expressed in *E. coli* at 37°C for 2 h after induction with IPTG. Longer expression time led to an increase of immature or degraded heavy chains that co-purified with the desired product. Inclusion bodies were prepared following a standard procedure [23] and solubilised in 8 M urea /20 mM Tris/150 mM NaCl at pH 8.0. Purification on a Ni-nitrilotriacetate agarose column led to a product free of *E. coli* proteins showing only two minor impurities of lower molecular weight consisting of truncated heavy chains.

Reconstitution of the 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 β-2-microglobulin and 0.1 mg/ml peptide and 5 mM glutathion to establish reducing conditions in 6 M urea against TBS. To prevent premature formation of disulphide bridges and oxidation of unpaired Cys 67 in the B*2705 heavy chain, the solution was sparged with nitrogen. After 36 to 48 h at 10°C the mixture was concentrated to 500, µl in a Centriprep 30 ultrafiltration unit (Amicon-Grace). This sometimes resulted in notable amounts of precipitate. The folded complex was purified by gel filtration on a Superdex 75 (Pharmacia) column run on a preparative HPLC with UV detection at 280 nm. The chromatogram showed three major peaks at 9, 11.5 and 14 ml elusion volume corresponding to heavy-chain aggregates, refolded heterotrimer (with an average yield of 5%) and excess β_2m, respectively. The heterotrimer peak was collected, concentrated in a Centricon 30 ultrafiltration unit (Amicon-Grace Ltd.) and immediately subjected to thermal denaturation.

Site-Directed Mutagenesis

The phosphothioate method of Eckstein [24] was used to obtain mutants for the B*2705 heavy chain.

The required single stranded DNA was generated by subcloning the 203 bp Kpn I/Pst I fragment into the M13 mp l8 filamentous phage polycloning site. For the formation of the Leu 156 Trp mutant, the synthetic oligonucleotide 3′-CGGAGCAGTTGGAGAGC-CTAC-5′ was used for priming the polymerisation of the mutant DNA by application of the Sculptor™ *in vitro* mutagenesis kit (Amersham). Mutant strains were identified by DNA-sequencing and after cloning back into the expression vector the sequence of the whole fragment was checked for the absence of random mutations.

Peptide Synthesis

The peptides were synthesised by automated, multiple solid-phase peptide synthesis using the Fmoc/*t*Bu strategy, based on the principles of the original Merrifield method. Chain elongation was performed by a robot system (Syro, Multi-Syn-Tech, Bochum, Germany), subsequent deprotection and analysis was done manually.

The Fmoc protected amino acids were coupled to the DIC activated C-terminus in ten-fold excess with HOBt as coupling reagent. The completed peptide was cleaved from the resin and deprotected by addition of trifluoro acetic acid with thiocresol and thioanisol as scavengers. The peptides were precipitated and washed with ice-cold ether and lyophilised from water.

Peptides were analysed by reversed-phase HPLC (Merck-Hitachi, Darmstadt, Germany) on a nucleosil 5 μ, C-18 column (125 × 3 mm) at a flow rate of 600 μl/min. The absorbance was measured at 220 nm. The solvent system used consisted of 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). A linear gradient from, 0–70% B in 40 min was applied. Furthermore the peptides were analysed by ion spray mass spectrometry on a triple-quadrupole mass spectrometer API III with a mass range of m/z 10–2400 equipped with an ion spray interface (Sciex, Thornhill, Ontario, Canada). The mass spectrometer was operated in positive ion mode under conditions of unit mass resolution for all determinations. For the synthesis of the tracer peptide the photo-labile amino acid Tmd-Phe (provided by P. Brunner, ETH Zurich) was coupled over night in only twofold excess and in a second cycle for completion with TBTU as coupling reagent. Tritium was incorporated by acetylation of the ω-amino group of lysine 8 with ^{3}H-acetic anhydride after selective cleavage of the lysine side-chain protection group Mtt (1% TFA, 5% TIS in CH_2Cl_2 4 × 5 min). The rest of the sequence was built by automated synthesis as described above. All operations involving Tmd-Phe were performed under strongly dimmed light.

Thermal Denaturation

All CD measurements were performed on a Jasco J-720 spectropolarimeter 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. Sample concentration was determined photometrically and held at 0.2 mg/ml. General reversibility of the process was checked by recording spectra at 25°C, at a temperature well above T_m and after cooling again at 25°C. Different scan rates (0.7°C and 0.5°C/min) led to similar T_m values for a reference peptide (data not shown). Thermal denaturation profiles were recorded with the Jasco TEMPSCAN software with 0.1°C increments at a heating rate of 30°C/h. Three denaturation curves from independent refolding preparations were averaged after converting to molar ellipticity values. The curves were reduced to 70 data points by replacing each ten neighbouring points by their mean value.

By assuming a two state equilibrium process the data were fitted by a non-linear least-squares routine with the program Origin 2.90 (MicroCal Software, Inc.) to the following equation:

$$\Theta = \Theta_u + [\Theta_f - \Theta_u / 1 + \exp x] \quad (1)$$

$$x = (-\Delta H_m/R)\,(1/T - 1/T_m) + \Delta C_p/R[(T_m/T - 1) + \ln(T/T_m)] \quad (2)$$

It describes the measured ellipticity Θ as a function of temperature, with enthalpy ΔH_m, heat capacity upon unfolding ΔC_p and the midpoint temperature of unfolding T_m being the fitting parameters. Initial estimates for ΔH_m were obtained by plotting ln K versus $1/T$ (van't Hoff plot) in the transition region. ΔC_p was assumed to be temperature-independent [25] and initial values were estimated from the primary sequence [26]. The linear baseline functions of the unfolded and folded states Θ_u and Θ_f were determined as linear regressions of the pre- and post-transitional regions. Gibbs free energy ($\Delta\Delta G$) values were calculated, where appropriate, relative to reference peptide 1 (KRGIDKAAK) using a previously described method [27], based on the equation:

$$\Delta\Delta G = \Delta G_{peptide} - \Delta G_{ref} = \Delta T_m[\Delta H_{m(ref)}/T_{m(ref)}] \quad (3)$$

This approximation has been used only when the entropy of unfolding ΔS was very similar for a pair of MHC-peptide complexes (similar steepness of the transition region). The enthalpy change at the midpoint of unfolding ΔH_m was determined by the least-squares fit of the unfolding curve to Equation (1). Because ΔC_p estimates obtained by this approach are not very accurate and the ΔH_m values are largely influenced by the observed deviations of the two-state model, no direct extrapolation from the midpoint of unfolding to obtain $\Delta G^{25°C}$ was made.

Competitive Binding Assay

Samples containing 10 pmol of heavy chain in 8 M urea were diluted into a solution containing 20 pmol of β_2m, 10 nmol of tracer peptide and varying concentration of the test-peptide to a total volume of 50 µl. After incubating over night at room temperature the tracer was covalently linked to the heavy chain by irradiation with ultraviolet light (10 min at 180 W, 366 nm). The samples were concentrated in a vacuum centrifuge (Speedvac), mixed with 20 µl of SDS-sample buffer and subjected to SDS-PAGE in a 12% Gel with *N*,*N*′-diallyltartardiamide as cross-linker. The gel was stained with Coomassie Blue and the band corresponding to the HLA heavy chain was cut out with a scalpel. The gel slices were solubilised by a 10 min incubation at 37°C in 2% periodic acid, which cleaves the diol function of the gel-crosslinker *N*,*N*′-diallyltartardiamide, allowing quantitative measurement of protein mass and radioactivity [28]. The obtained solution was thoroughly mixed with 3 ml scintillation cocktail and measured in a liquid-scintillation counter.

RESULTS AND DISCUSSION

The non-natural ligands reported in Table 1 have been designed to exhibit an enhanced affinity to HLA-B*2705 through optimised hydrophobic interactions between the side-chain of the peptide amino acid 3 and the HLA binding pocket D. By calculating the optimum interaction energy between a neutral methyl probe and the amino acids of pocket D, it could be shown that natural amino acid side-chains could not completely fill the most favoured interaction area, whereas the aromatic side chains of homophenylalanine and naphthylalanine optimally fitted the cavity. Molecular dynamics simulations suggested that the additional buried surface area should result in a higher free energy of binding, confirmed by a semi-quantitative radioimmuno assay [19]. To quantify the binding differences between natural and nonnatural ligands, the circular dichroism spectra (CD) of every MHC–ligand complex was monitored as a function of temperature. If the thermal unfolding follows a two-state equilibrium mechanism, the transition midpoint or melting temperature T_m is linked to the free energy of folding by the van't Hoff and Gibbs-Helmholtz equations [25]. Therefore differences in ΔG can be determined by measuring the thermal unfolding transition, e.g. as a change in circular dichroism. If the same protein with different ligands is tested, differences in T_m should be related only to different intermolecular interaction energies, provided that all tested ligands have the same binding mode and do not alter the three-dimensional structure of the protein. It has been shown previously that the thermal stability of class I MHC molecules depends on the sequence of the bound peptide [13,29] and T_m correlates with equilibrium dissociation constants for a set of related peptides [30].

We investigated the thermal stability of MHC heterodimers in complex with single peptides, isolated by gel-filtration chromatography (Figure 1). Assembly of MHC heterodimers from denatured components occurs only upon peptide binding [31]. As a prerequisite for the denaturation experiments, the reversibility of unfolding/folding was investigated. For this purpose the circular dichroism spectrum of a HLA-peptide complex was measured consecutively at 25°C, after heating with the normal scan rate of 30°C/h at 70°C and then, after cooling, again at 25°C. The native fold of the MHC-peptide pair was easily identified by a strong negative dichroic signal at about 220 nm typical of an α-helix (Figure 2). The unfolding occurring at high temperature, characterised by a spectrum typical for an unordered conformation, was found to be reversible, as shown by the parallelism of the pre- and post-heating spectra (Figure 2).

However, as the spectrum at high temperature was recorded with the same sample, the native spectrum could only be restored to 75% of the starting signal after recooling, due to kinetically driven, irreversible formation of aggregates, only occurring at longer exposure to temperatures well above T_m [13,32]. The denaturation curve of HLA-B*2705 in complex with the bacterial epitope KRGIDKAAK (peptide 1, Table 1), recorded at 218 nm, shows a well-defined single transition with a midpoint of unfolding at 47.1°C (Figure 3A). In accordance to our initial predictions, the designed

Table 1 Sequence and Analytical Data of the Investigated Peptide Analogues and Melting Temperatures (T_m, °C) of the HLA-Peptide Complexes

Number	Peptide sequence									Purity[a]	HPLC[b]	$Mass_{calc}$	$Mass_{exp}$	T_m	
										[%]	[min]	[Da]	[Da]	B*2705 wildtype	L156W
1	Lys	Arg	Gly	Ile	Asp	Lys	Ala	Ala	Lys	85	10.36	986.2	986.0	47.1	37.6
2	–	–	Phe	–	–	–	–	–	–	85	12.26	1076.3	1076.0	57.2	n.d.[c]
3	–	–	Ana	–	–	–	–	–	–	>90	13.92	1126.4	1124.8	62.3	35.2
4	–	–	Bna	–	–	–	–	–	–	>90	13.89	1126.4	1125.0	51.9	41.5
5	–	–	Hpa	–	–	–	–	–	–	>90	12.56	1090.3	1088.8	62.0	37.5

Ana, α-naphthylalanine; Bna, β-naphthylalanine; Hpa, homophenylalanine.
[a] According to the HPLC (220 nm).
[b] retention time detected at 220 nm. The solvent system used consisted of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A linear gradient from 0–70% B in 40 min was applied.
[c] non determined

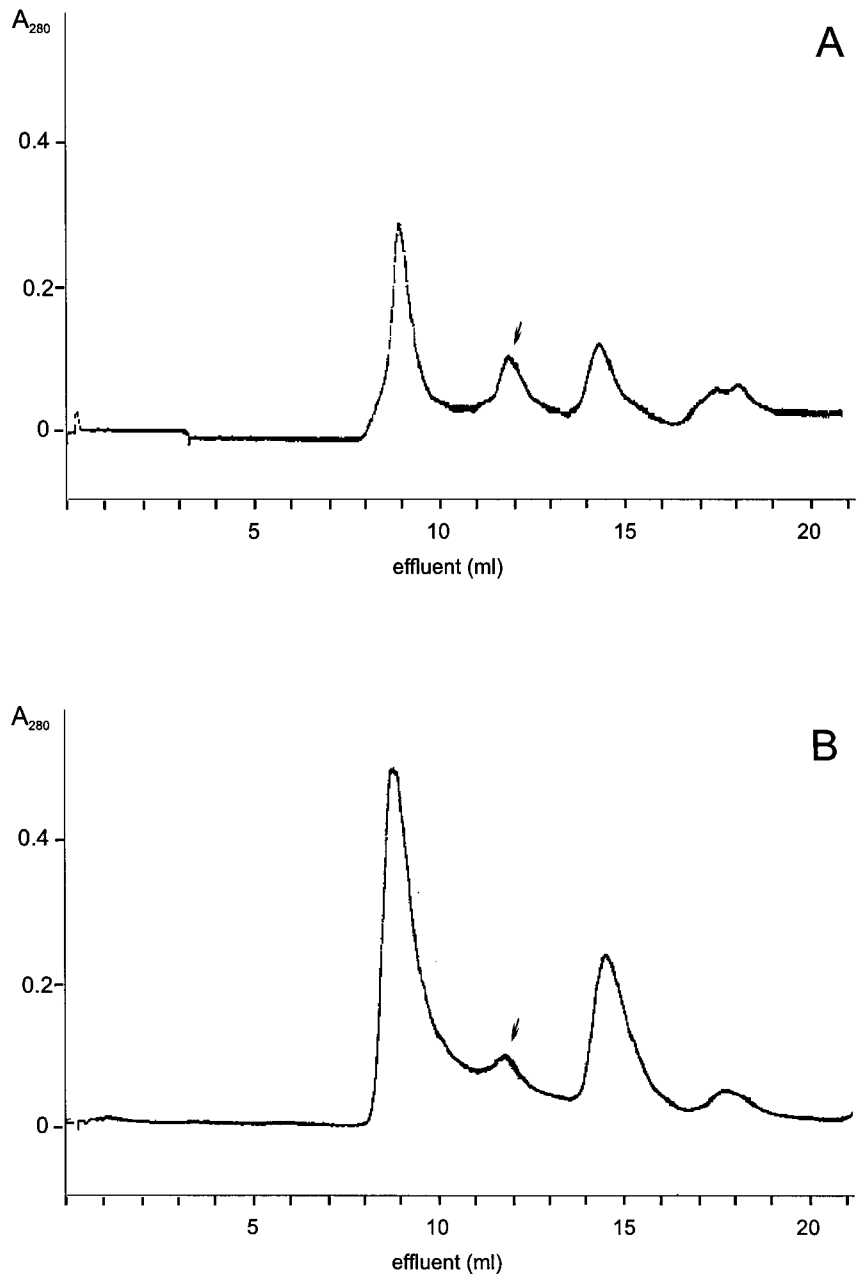

Figure 1 Purification of the reconstituted heterotrimer with peptide 1 (KRGIDKAAK) by gel-filtration on a Superdex 75 16/60 column (Pharmacia) at a flow rate of 0.7 ml/min. A total amount of 2 mg protein was purified. The peak at 12 ml elusion volume (arrow) corresponds to the heterotrimer. (A) Reconstitution with wildtype B27 heavy chain. (B) Reconstitution with heavy chain mutant L156W.

non-natural peptides (3–5, Table 1) lead to a higher stability of the resulting complexes. All analogues of the chlamydial peptide 1, in complex with HLA-B*2705, show a higher T_m than the parent peptide already described as a medium-affinity binder [33,34]. Not surprisingly, the substitution of Phe for Gly at P3 also gives a substantial temperature shift towards a melting temperature of 57.2°C, but the methylene homologue homophenylalanine 5, designed to fill the bottom of pocket D results in a further increase in stability, of the same magnitude as that observed for the α-naphthylalanine analogue 3 (Figure 3A, Table 1). In contrast to α-naphthylalanine, a β-naphthylalanine (peptide 4) with a

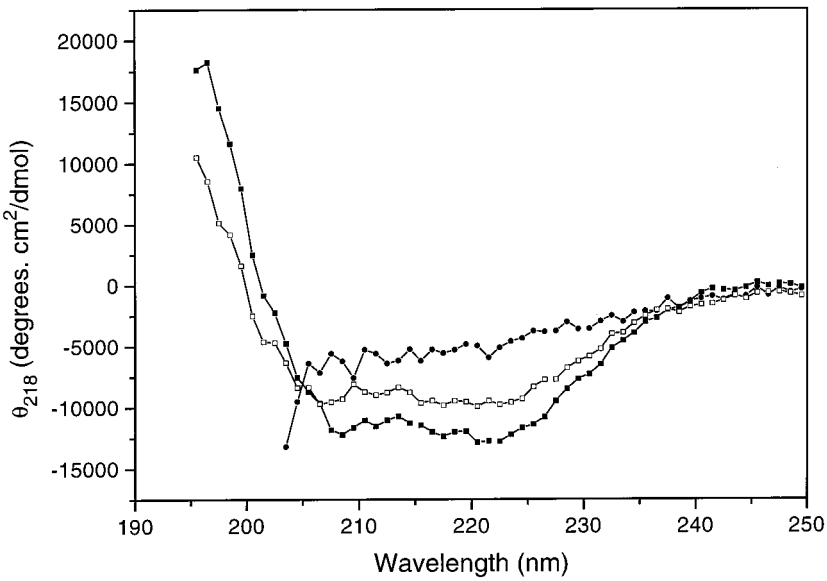

Figure 2 CD spectra of HLA B*2705 in complex with the peptide KRGIDKAAK (*Chlamydia trachomatis* GroEL 117–125) at different temperatures (squares: 25°C, diamonds: 70°C, open squares: 25°C after heating to 70°C).

T_m of 51.9°C leads to a slightly decreased melting temperature with respect to phenylalanine. This might be due to the different orientation of the naphthyl moiety and a lower steric complementarily to pocket D. Apparently, the CD curves do not follow the same unfolding pathway, as indicated by the different slopes of the transition regions. The non-natural analogues show a broader transition, commonly attributed to low cooperativity of unfolding or a low apparent enthalpy of unfolding. This is illustrated by the ΔH_m values obtained by van't Hoff analyses or the model fit that yield to a ΔH_m value of 45 kcal/mol for the phenylalanine peptide, but an apparent ΔH_m of 24 kcal/mol for the α-naphthylylanine analogue.

To validate the binding hypothesis, the pocket D was mutated at its bottom (Leu 156 Trp) in order to fulfil the most unfavourable conditions for the placement of a bulky hydrophobic side chain (Figure 4). Either the non-natural analogues deeply bind to pocket D and their binding to the protein mutant should be dramatically reduced, or they bind non specifically to the protein surface near the rim of the pocket and their affinity for the Leu 156 Trp mutant should not be altered. Heterotrimers with the mutant heavy chain, β_2m, and the *Chlamydia* peptide as well as its nonnatural analogues were assembled and isolated by gel-filtration chromatography (Figure 1B). Reassembly of denatured heavy chain and β-2-microglobulin has been shown to occur only in the presence of a MHC-binding peptide [31]. Therefore, the mutant is able to bind the natural peptide. The denaturation profiles of the Leu 156 Trp mutant in complex with our set of peptides are clearly in favour of a specific pocket-mediated binding-mode. The mutant shows a reduced stability even with the parent chlamydial peptide, but more important, there are nearly no differences in the melting temperatures of the natural and nonnatural peptides (Figure 3B).

The stabilisation of the MHC heavy chain upon peptide binding, and the resulting stability differences, observed as the change of the circular dichroism signal upon thermal unfolding is a good indicator of MHC binding properties for a set of related peptides [29,13]. The present data are in agreement with the melting temperatures reported for HLA-B27 peptide complexes by other groups [32,35]. The melting temperature for the chlamydial peptide 1 ($T_m = 47.1$°C) is comparatively low, as expected for a medium-affinity ligand. The substitution of hydrophobic residues for Gly3 brings the peptide into the range of known high-affinity ligands. The free energy contribution ($\Delta\Delta G$) of the Phe side chain to the stabilisation of the MHC heavy chain was estimated, from the corresponding ΔT_m value, to be 1.2 kcal/mol. This observation is in agreement with a previous report, quantifying the substitution of alanine for P2 and P9 anchor positions of a HLA-A2-binding peptide, yielding to ΔG stabilisation values of -1.2 and -3.0 kcal/mol, respectively [13]. Consistently, the total contribu-

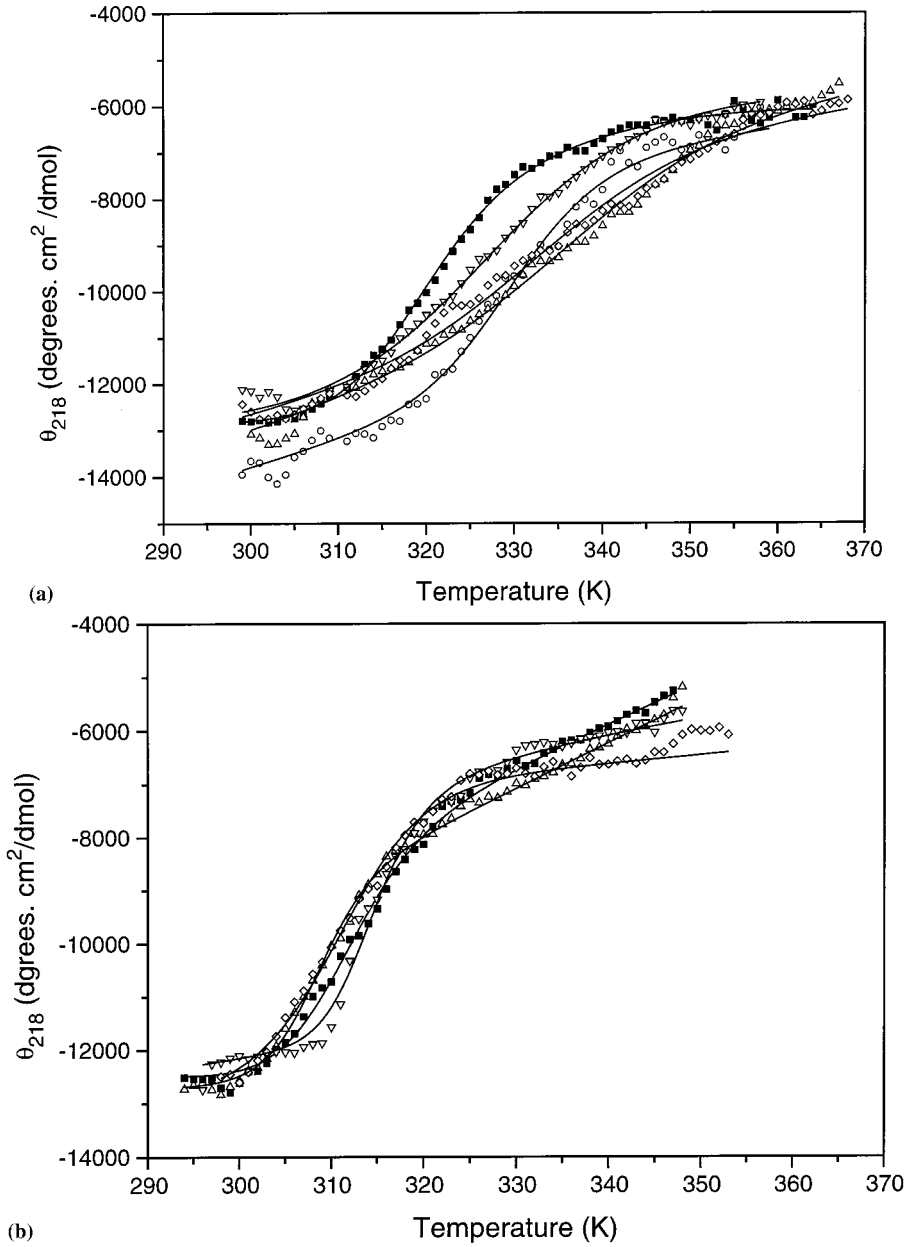

Figure 3 Thermal denaturation of HLA-B*2705 in complex with different peptides. The peptides differ only in position 3. The reference peptide KRGIDKAAK (*Chlamydia trachomatis* GroEL 117–125) is represented by solid squares, respectively, by a solid line for the least squares fit to Equation (1). (circle) Phe substituted analogue 2, (triangle) alpha-naphthylalanine derivative 3, (inverted triangle) β-naphthylalanine compound 4, (diamond) homophenylalanine peptide 5 (see Table 1). (A) Denaturation of wildtype HLA-B*2705. (B) Denaturation of the L156W mutant. The melting temperatures T_m are listed in Table 1.

tion of the peptide to stabilisation of the MHC molecule is significantly higher, with 4.2 kcal/mol for murine H-2 K_d [29] and higher than 5.8 kcal/mol for HLA-A2 [13]. As T_m is further increased for peptides 3 and 5, their free energy contribution to the heterotrimer stabilisation should also be higher. However, due to the observed deviations in the co-operativity of unfolding, no quantitative estimates for $\Delta\Delta G$ can be given. The determination of the transition midpoint, in contrast to the other thermodynamic parameters such as ΔH that depends largely on the cooperativity, is hardly affected by

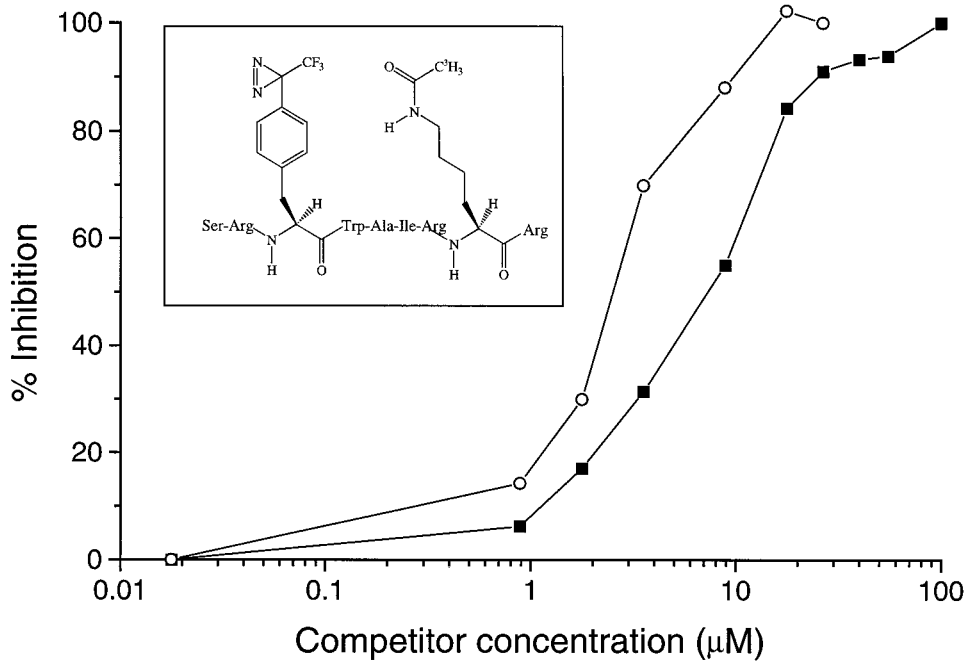

Figure 5 Competitive binding of a photoactivatable ^{3}H-acetylated tracer with peptides KRGIDKAAK (squares) and KR-Ana-IDKAAK (open circles). Replacement of the tracer was measured after photocrosslinking, by liquid-scintillation counting of the SDS-PAGE purified heavy-chain band. Coomassie stained gel slices (polyacrylamide gel with diallyltartardiamide as crosslinker) were solubilised in 2% periodic acid. The inset shows the structure formula of the tracer.

these deviations [36]. Our data also show a general agreement between $\Delta\Delta G$ values and the IC_{50} score of an epitope stabilisation assay, provided that the compared ligands are derived from the same sequence (Krebs *et al.*, manuscript in preparation).

The increased binding affinity of peptide 3, demonstrated by a T_m shift of 15°C, but somewhat uncertain due to the change in cooperativity, receives further corroboration by the results of a competition assay. We tested the best binder (peptide 3) and the natural peptide for their ability to compete with a tritiated photoactivatable control peptide. The tracer peptide (see inset Figure 5) contains a diazirine moiety (Tmd-Phe) at position 3 that allows covalent binding to the heavy chain via the transient carbene functionality generated by N_2 elimination upon UV-irradiation. It shows a specific saturable binding to HLA-B*2705 and a low unspecific linear binding to the unrelated class I MHC protein HLA-A2 (data not shown). The IC_{50} value for the *Chlamydia* peptide is reduced from 9 to 2 μM upon substitution of α-naphthylalanine for Gly at the P3 position.

The large shift in T_m observed for the hydrophobic peptide variants and the absence of this shift in the pocket D mutant strongly suggests the validity of the predicted binding-mode for the non-natural aromatic side-chains (Figure 3). As, β-naphthylalanine differs from the other analogues in binding to the wildtype protein, as reflected by a substantially smaller temperature shift with respect to the parent peptide 1, as well as in binding to the mutant heavy chain where it induces a T_m shift of 4°C, this P3 side chain possibly exhibits an alternative local binding-mode.

CONCLUSIONS

Based on this encouraging results, further cycles of rational design for MHC binding molecules are possible. As shown, the prediction of the binding properties of modified peptides is in good agreement with experimental data. In synopsis of the modification that have been made so far to class I MHC-associated peptides it should be possible to diverge from the consensus sequence in nearly every peptide positions. Relatively few successful peptide modifications have been reported in the literature, notably in altering MHC-anchor positions [35,37–39]. The combination of these modifications with variations of the TCR-contact area [19,40,41]

should enable a stepwise development of a nonpeptidic ligand.

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REFERENCES

1. M.T. Heemels and H.L. Ploegh (1995). Generation, translocation and presentation of MHC class I-restricted peptides. *Ann. Rev. Biochem. 64*, 643–691.
2. F. Garcia, A. Marina and J.A. López de Castro (1997). Lack of carboxy-terminal tyrosines distinguishes the B*2706-bound peptide repertoire from those of B*2704 and other HLA-B27 subtypes associated to ankylosing spondylitis. *Tissue Antigens 49*, 215–221.
3. M.T. Fiorillo, L. Meadows, M. D'Amato, J. Shabanowitz, D.F. Hunt, E. Apella and R. Sorrentino (1997). Susceptibility to ankylosing spondylitis correlates with the C-terminal residue of peptides presented by various HLA-B27 subtypes. *Eur. J. Immunol. 27*, 368–373.
4. R. Benjamin and P. Parham (1990). Guilt by association: HLA-B27 and ankylosing spondylitis. *Immunol. Today 11*, 137–142.
5. L. Adorini, S. Muller, F. Cardinaux, P.V. Lehmann, F. Falcioni and Z.A. Nagy. (1988). *In vivo* competition between self peptides. *Nature 334*, 623–625.
6. M.T. De Magistris, J. Alexander, M. Coggeshall, A. Altman, F.C.A Gaeta., H.M. Grey and A. Sette (1992). Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell 68*, 625–634.
7. G.Y. Ishioka, L. Adorini, J.C. Guery, F.C.A. Gaeta, R. LaFond, J. Alexander, M.F. Powell, A. Sette and H.M. Grey (1994). *J. Immunol. 152*, 4311–4319.
8. H.G. Rammensee (1995). Chemistry of peptides associated with MHC class I molecules. *Curr. Opin. Immunol. 7*, 85–96.
9. H.J. Fehling, W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Muller and H. von Boehmer (1994). MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science 265*, 1234–1237.
10. T. Spies, M. Bresnahan, S. Bahram, D. Arnold, G. Blank, E. Mellins, D. Pious and R. DeMars (1990). A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature 348*, 744–748.
11. A. Townsend, C. Ohlen, J. Bastin, H.G. Ljunggren, L. Foster and K. Karre (1989). Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature 340*, 443–448.
12. D.R. Madden, J.C. Gorga, J.L. Strominger and D.C. Wiley (1992). The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell 70*, 1035–1048.
13. M. Bouvier and D.C. Wiley (1994). Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science 265*, 398-402.
14. M.A. Saper, PJ. Bjorkman and D.C. Wiley (1991). Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol. 219*, 277–319.
15. K. Falk, O. Rotzschke, S. Stevanovic, G. Jung and H.G. Rammensee (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature 351*, 290–296.
16. D.N. Garboczi, P. Ghosh, U. Utz, Q.R. Fan, W.E. Biddison and D.C. Wiley (1996). Structure of the complex between human T- cell receptor, viral peptide and HLA-A2. *Nature 384*, 134–141.
17. J. Ruppert, J. Sidney, E. Celis, R.T. Kubo, H.M. Grey and A. Sette (1993). Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell 74*, 929–937.
18. B. Galocha, J.R. Lamas, J.A. Villadangos, J.P. Albar and J.A. Lopez de Castro (1996). Binding of peptides naturally presented by HLA-B27 to the differentially disease-associated B*2704 and B*2706 subtypes, and to mutants mimicking their polymorphism. *Tissue Antigens 48*, 509–518.
19. D. Rognan, L. Scapozza, G. Folkers and A. Daser (1995). Rational design of nonnatural peptides as high-affinity ligands for the HLA-B*2705 human leucocyte antigen. *Proc. Natl. Acad. Sci. 92*, 753–757.
20. P. Rovero, S. Vigano and S. Pegorado (1995). Augmentation of the affinity of HLA class I-binding peptides lacking primary anchor residues by manipulation of the secondary anchor residues. *J. Peptide Sci. 1*, 266–273.
21. T.S. Jardetzky, W.S. Lane, R.A. Robinson, D.R. Madden and D.C. Wiley (1991). Identification of self-peptides bound to purified HLA-B27. *Nature 353*, 326–329.
22. O. Rotzschke, K. Falk, S. Stevanovic, V. Gnau, G. Jung and H.G. Rammensee (1994). Dominant aromatic/aliphatic C-terminal anchor in HLA-B*2702 and B*2705 peptide motifs. *Immunogenetics 39*, 74–77.
23. K. Nagai and H.C. Thogersen (1987). Synthesis and sequence-specific proteolysis of hybrid proteins produced in *Escherichia coli*. *Methods Enzymol. 153*, 461–481.
24. J.W. Taylor, J. Ott and F. Eckstein (1985). The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioatemodified DNA. *Nucl. Acids Res. 13*, 8764–8785.
25. P.L. Privalov and S.J. Gill (1988). Stability of protein structure and hydrophobic interaction. *Adv. Prot. Chem. 39*, 191–234.

26. J.K. Myers, C.N. Pace, J.M. Scholtz (1995). Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci. 4*, 2138–2148.
27. W.J. Becktel and J.A. Schellman (1987). Protein stability curves. *Biopolymers 26*, 549–559.
28. R.B. Young, M. Orcutt and P.B. Blauwiekel (1980). Quantitative measurement of protein mass and radioactivity in *N,N′*-diallyltartardiamide crosslinked polyacrylamide slab gels. *Anal. Biochem. 108*, 202–206.
29. M.L. Fahnestock, I. Tamir, L. Narhi and P.J. Bjorkman (1992). Thermal stability comparison of purified empty and peptide-filled forms of a MHC class I molecule. *Science 258*, 1658–1662.
30. C.S. Morgan, J.M. Holton, B.D. Olafson, P.J. Bjorkman and S.L. Mayo (1997). Circular dichroism determination of class I MHC-peptide equilibrium dissociation constants. *Protein Sci. 6*, 1771–1773.
31. D.N. Garboczi, D.T. Hung and D.C. Wiley (1992). HLA-A2-peptide complexes: refolding and crystallisation of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc. Natl. Acad. Sci. USA 89*, 3429–3433.
32. Z. Reich, J.D. Altman, J.J. Boniface, D.S. Lyons, H. Kozono, G. Ogg,, C. Morgan and M.M. Davis (1997). Stability of empty and peptide-loaded class II major histocomapibility complex molecules at neutral and endosomal pH: Comparison to class I proteins. *Proc. Natl. Acad. Sci. 94*, 2495–2500.
33. A. Daser, U. Henning and P. Henklein (1994). HLA-B27 binding peptides derived from the 57 kDa heat schock protein of *Chlamydia trachomatis*: novel insights into the peptide binding rules. *Mol. Immunol. 31*, 331–336.
34. P.J. Goodford (1985). A computational procedure for determining energetically favorable binding sites on biologically important macromolecules. *J. Med. Chem. 28*, 849–857.
35. G.A. Weiss, R.J. Valentekovich, E.J. Collins, D.N. Garboczi, W.S. Lane, S.L. Schreiber and D.C. Wiley (1996). Covalent HLA–B27/peptide complex induced by specific recognition of an aziridine mimic of arginine. *Proc. Natl. Acad. Sci. 93*, 10945–10948.
36. C.N. Pace (1986). Determination and analysis of urea and guanidinium hydrochloride denaturation curves. *Methods Enzymol. 131*, 266–280.
37. G. Guichard, S. Calbo, S. Muller, P. Kourilsky, J.P. Briand and J.P. Abastado (1995). Efficient binding of reduced peptide bond pseudopeptides to major histocompatibility complex class I molecule. *J. Biol. Chem. 270*, 26057–26059.
38. G. Guichard, F. Connan, R. Graff, M. Ostankovitch, S. Muller, J.G. Guillet, F. Choppin and J.P. Briand (1996). Partially modified retro-inverso pseudopeptides as non-natural ligands for the class I histocompatibility molecule HLA-A2. *J. Med. Chem. 39*, 2030–2039.
39. D. Rognan, S. Krebs, O. Kuonen, J.R. Lamas, J.A. Lopez de Castro and G. Folkers (1997). Fine specificity of antigen binding to two class I major histocompatibility proteins (B*2705 and B*2703) differing in a single amino acid residue. *J. Comp. Aided Mol. Des. 11*, 463–478.
40. G.A. Weiss, E.J. Collins, D.N. Garboczi, D.C. Wiley and S.L. Schreiber (1995). A tricyclic ring system replaces the variable regions of peptides presented by three alleles of human MHC class I molecules. *Chem. Biol. 2*, 401–407.
41. M. Bouvier and D.C. Wiley (1996). Antigenic peptides containing large PEG loops designed to extend out of the HLA-A2 binding site form stable complexes with class I major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA 93*, 4583–4588.

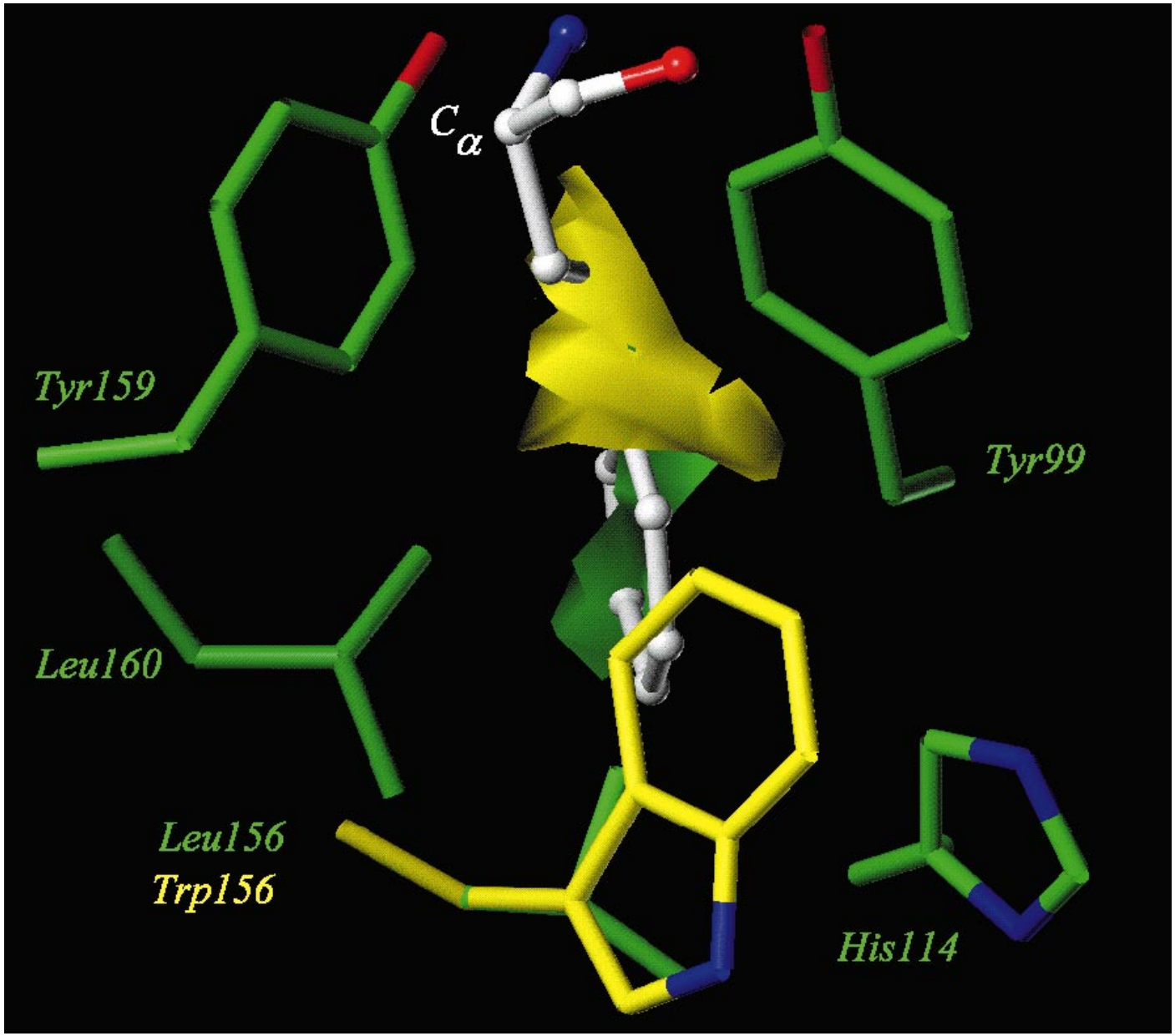

Figure 4. Energy contours (at -3 kcal/mol) indicating the most favourable hydrophobic interactions between a neutral methyl probe, and pocket D of two HLA-B27 heavy chains (HLA-B*2705 wild type, L156W mutant). Interaction energies were calculated using the GRID v.14 program [33] as previously described [19]. The green and yellow solid surfaces represent the most favourable interaction areas for the wild type and L156W mutant, respectively. An homophenylanine side chain, labeled at the Ca atom, corresponding to position 3 (P3) of a bound peptide was fitted *a posteriori* to the energy contour maps, from the crystal structure of a HLA-B27 in complex with a peptide model [12]. The following atom colour coding was used: white, carbon atom of the peptide; green, carbon atom of the B*2705 wild type protein; yellow, carbon atom of the L156W mutant; blue, nitrogen; red, oxygen.