Selected HLA-B27 subtypes are associated with spondyloarthropathies, but the underlying mechanism is not understood. To explain this association in molecular terms, a comparison of peptide-dependent dynamic and structural properties of the differentially disease-associated subtypes HLA-B*2705 and HLA-B*2709 was carried out. These molecules differ only by a single amino acid at the floor of the peptide binding groove. The thermostabilities of a series of HLA-B27 molecules complexed with nonameric and decameric peptides were determined and revealed substantial differences depending on the subtype as well as the residues at the termini of the peptides. In addition we present the crystal structure of the B*2709 subtype complexed with a decameric peptide. This structure provides an explanation for the preference of HLA-B27 for a peptide with an N-terminal arginine as secondary anchor and the lack of preference for tyrosine as peptide C terminus in B*2709. The data show that differences in thermostinic properties between peptide-complexed HLA-B27 subtypes are correlated with a variety of structural properties.

Major histocompatibility complex (MHC) class I molecules consist of two non-covalently associated proteins, the heavy chain (HC) and β2-microglobulin (β2m). Their function is to present peptides produced during intracellular protein degradation to cytotoxic T cells (1). The peptides typically exhibit lengths of 8–10 amino acid residues. X-ray crystallographic studies (Ref. 2; for review, see Ref. 3) and determination of the allele-specific binding motifs of peptides eluted from purified MHC molecules (4) have served to establish the rules governing peptide binding. Peptide side chains can insert into a series of pockets (termed A to F) that exhibit highly variable properties, thereby assuring allele specificity (5, 6). Characteristic amino acids, usually near the N and C termini (positions p2 and p9), anchor a peptide within the binding groove of an MHC class I molecule (7). The affinity between peptide and MHC class I molecule is further enhanced by amino acids serving as secondary anchors, often found at p3 and p4 (8).

The human MHC class I molecule HLA-B27 is very strongly associated to spondyloarthropathies, in particular ankylosing spondylitis (AS) (9, 10). A clue to the understanding of the disease association might come from the finding that not all HLA-B27 subtypes are associated to AS. HLA-B27 subtypes known today differ in only very few amino acids, most of which affect the peptide binding properties of the molecule (10). Two subtypes, B*2706 and B*2709, are at best very weakly associated with disease. The only difference between the prototypical, strongly AS-associated B*2705 and the non-associated B*2709 subtypes is the exchange of position 116 from aspartate to histidine (11). This exchange is located within the peptide binding groove at the floor of the F pocket and plays a pivotal role in anchoring the C-terminal peptide residue, as demonstrated by differences in the repertoires of peptides eluted from B*2705 and B*2709 molecules (12, 13). In particular, in B*2709-bound peptides, basic amino acids are not observed at the p9 position, and Tyr is clearly underrepresented, whereas these amino acids are frequently found as p9 in B*2705-bound peptides. The role of HLA-B27 in disease development may, therefore, be related to the repertoire of the presented peptides, the exact conformation that these peptides adopt when bound to the molecule, the stability of the complexes, or their folding behavior, although other factors, including genetic and environmental influences, cannot be neglected either (10, 14–16).

We have previously addressed these issues by determining the crystal structures of the subtypes B*2705 and B*2709 complexed with the model nona-peptide GRFAAAAAK (abbreviated as m9) (17).

Although it is obvious that subtype-specific binding properties could be connected to differential disease association as well (18, 19), dynamic properties of MHC molecules have so far received comparatively little attention. A variety of different
methods have been reported for the biophysical characterization of equilibrium and kinetic parameters of the interaction between MHC molecules and their respective peptide ligands.

Thermodynamic properties of B*2705 molecules were investigated by monitoring the thermal unfolding using circular dichroism (CD) spectroscopy, demonstrating the importance of peptide residues at p3 and p1 as well as the B-pocket residue Cys-67 (20). Using an human immunodeficiency virus 1-derived peptide, Dedier et al. (8) emphasized the importance of secondary anchors of the peptide for the stability of the entire complex. Furthermore, peptide affinity and the kinetics of the formation or unfolding of various MHC molecules (HLA-A2, HLA-E, H-2K\(^\alpha\), H-2K\(^\beta\)) were investigated by fluorescence depolarization, steady state fluorescence resonance energy transfer, and CD spectroscopy, revealing the interdependence of HC, \(\beta_m\), and peptide during folding and unfolding of these molecules (21–25). However, comparative measurements employing disease-associated and non-associated subtypes binding the same peptide have not been carried out so far, although they can be expected to shed light on the contribution of individual HC residues to peptide binding.

To this end we have now carried out for the first time experiments with the B\(^*\)2705 as well as the B\(^*\)2709 subtype complexed with the m9 peptide and with two decameric peptides (termed s10R and s10G), employing both CD and differential scanning calorimetry (DSC). We also present the crystal structure of B\(^*\)2709 complexed with s10R. Our results demonstrate that the binding of a single amino acid buried within the binding groove can have pronounced effects on the stability of differentially AS-associated HLA-B27 subtypes.

**MATERIALS AND METHODS**

**Protein Preparation**—The peptides m9 (GRFAAAIAK), s10R (RBLRLGHQNY), and s10G (GRLLRGHNQY) were synthesized by standard solid phase methods and purified by HPLC (m9, Alta Bioscience, Birmingham, UK; s10G, Biosyntan, Berlin, Germany). The extracellular domains of B\(^*\)2705 and B\(^*\)2709 as well as \(\beta_m\) were expressed separately in *Escherichia coli* as inclusion bodies. These were solubilized with urea, and the proteins were refolded by rapid dilution in the presence of the respective peptide and purified as described previously (17). Free \(\beta_m\) was identified as a refolding byproduct and was subsequently purified by gel filtration. The final buffer for CD and DSC experiments was 10 mM sodium phosphate, pH 7.5, 150 mM NaCl (pH-adjusted with NaOH), whereas for crystallization 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (Tris-buffered saline) was used. N-terminal sequencing revealed the loss of the N-terminal Met-0 from the HC of both HLA-B27 subtypes, whereas 80% of the \(\beta_m\) molecules still possessed Met-0.

**Circular Dichroism and Differential Scanning Calorimetry**—All samples of HLA-B27, \(\beta_m\), and buffers were filtered through 0.2-\(\mu\)m HPLC membrane filters (Roth, Karlsruhe, Germany). The protein solutions were in the concentration range 0.06–0.15 mg/ml as determined by UV absorption at 280 nm. Absorption coefficients \(\varepsilon_{280}\) at 1 cm were 1.62; B\(^*\)2705:m9 and B\(^*\)2709:m9, 2.00; B\(^*\)2705:s10R and B\(^*\)2709:s10R, 1.98; B\(^*\)2705:s10G and B\(^*\)2709:s10G, 1.59) and molecular masses were calculated from the amino acid composition using the ProtParam tool on the Exasy-server (expasy.ch/tools/protparam.html). Thermal unfolding was measured by CD and DSC. CD measurements were performed with aliquots of the samples employed for the DSC measurements. Changes of the ellipticity \(\theta\) were monitored at 218 nm with a Jasco J-720 spectropolarimeter equipped with a temperature-controlled cell holder and a thermostat system (ThermoNeslab, Portsmouth, NH) at a heating rate of 20 °C/h. The negative molar ellipticity of the half-transition temperature of one well-defined “two-state” process. Rather they indicate the temperature where just one-half was observed of the total ellipticity change.

For DSC, excessive heat capacity curves were recorded using an ultrasensitive scanning microcalorimeter (VP-DSC, MicroCal Inc., Northampton, MA) at a heating rate of 1 K min\(^{-1}\) and a sample cell volume of ~0.5 ml. To examine the reversibility of folding the samples were cooled to 20 °C after the first run and heated up a second time. The experimental data were base-line-corrected and analyzed using the “ORIGIN for DSC” software package supplied by the manufacturer. The classical two-state model provided the best fits with a minimum of parameters and was applied for the deconvolution of all experimental curves. The model is based on the assumption that the macromolecule is composed of a number of domains, each of which is involved independently in a transition between the folded and unfolded states. Each transition is characterized by two parameters, \(T_m\) and \(\Delta H_m\). \(T_m\) is the thermal midpoint of a transition. \(\Delta H_m\) is the calorimetric heat change and is calculated from the area under the transition peak. The basic equations used to deconvolute the DSC data are described by Privolov and Potekhin (27).

**Cryocooled crystals**—X-ray Diffraction, Structure Collection, and Structure Determination of B\(^*\)2709:s10R—All crystallization trials with the B\(^*\)2709:s10R complex were performed by vapor diffusion at 18 °C using the hanging drop technique. Initial conditions were identified using a PEG 8000–pH screen. Clusters of thin plates grew at 24–27% PEG and pH 6.5–8.0 (protein at 14 mg/ml). Streak seeding in drops made from 0.8 M PEG 8000, 2% glycerol as cryoprotectant allowed collection of a complete data set to 1.9 Å of resolution from 1 crystal at 100 K on the MPG/GBF wiggen beamer BW6/DORIS at DESY, Hamburg, Germany using a MAR CCD detector. Data were integrated and scaled using DENZO and SCALEPACK (28). The crys-
The structure of B*2709/H18528s10R was determined by molecular replacement using peptide-stripped B*2705/H18528m9 (PDB entry 1jge) as search model and AmoRe (29) from the CCP4 program suite (30). Refinement was carried out as described for B*2705/H18528m9 (17) using, first, CNS (31) and, subsequently, Refmac 5.0 (32) with TLS refinement (33). The R factor was 0.182 (R_free 0.220) after CNS and finally converged at 0.169 (R_free 0.194). Table I gives a summary of the data collection and refinement statistics.

The final model comprises all 276 residues of the HC ectodomain, 100 residues of B*2709/H18528s10m (all 99 residues plus N-terminal Met), all atoms of peptide s10R, 459 water molecules, and 1 glycerol molecule. All polypeptide φ/ψ angles (analyzed using PROCHECK (34)) are in the allowed regions of the Ramachandran plot. Superimpositions were performed using the McLachlan algorithm (35) as implemented in Profit (A. C. R. Martin, www.bioinf.org.uk/software/profit). Figures were generated using Molscript (36) and Raster3D (37) together with a graphical interface (Moldraw) developed by N. Sträter. Molecular surfaces were calculated with the MSMS computer program (38).

RESULTS

Peptide Choice—In addition to the model peptide m9 (sequence GRFAAAIAK) described recently (17), we employed the

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2 Institut für Kristallographie, Freie Universität Berlin, unpublished information.
peptides s10G (GRLLRGNHQY) and s10R (RRLLRGNQY), which supported in vitro refolding of both subtypes, B*2705 and B*2709. s10G is a decameric peptide derived from the sequence 107GRLLRGNQY116 of subtype B*2707, whereas in s10R, pGly1 is exchanged to Arg to compensate for suboptimal anchoring of pTyr1 in the F pocket. pArg1 is a preferred secondary anchor residue that was expected to stabilize the complex (39–41).

Circular Dichroism and Differential Scanning Calorimetry—The thermostabilities of the different HLA-B27-peptide complexes and of free β2m were studied by CD and DSC measurements. A comparison of the CD melting profiles revealed that the subtype has a larger influence on the thermostability than the peptides tested here; the B*2709 complexes were considerably less stable than the B*2705 complexes (Fig. 1, A and B, and Table II). However, the peptides contributed to the distinct thermostabilities of the complexes as well. For both subtypes s10G resulted in the least stable complexes, s10R showed intermediate stability, whereas the complexes formed with m9 were the most stable ones. β2m melted with a Tm(CD) of 64 °C (Fig. 1A, Table II), a value that is similar to the peak position of the DSC excessive heat capacity curve (63.8 °C, see later in text) and also agrees with data from Bouvier and Wiley (7).

The CD measurements (Fig. 1, C and D, and Table II) confirmed the CD observations with regard to the different thermostabilities of the HLA-B27 complexes. More importantly, they suggest the presence of distinct unfolding pathways for the complexes formed by B*2705 and B*2709. The DSC melting profiles of B*2705-s10R and B*2705-m9 showed only one peak (Fig. 1D), whereas those of all three B*2709-peptide complexes and B2705-s10G showed two peaks (Fig. 1C). Therefore, the s10G peptide appears to change the unfolding behavior of the B*2705 subtype in such a way that it becomes similar to that of the three B*2709 complexes but is different from that of the other two B*2705 complexes.

For β2m, a simple unfolding behavior was expected. β2m used in this study has a molecular mass of 11,862 Da, is composed of 100 amino acids, and folds into a stable domain containing exclusively β strands, which should unfold at a high temperature in a single two-state transition. For two-state transitions, the quotient κ of the experimental, calorimetric enthalpy change ΔHcal and the model-dependent, calculated van’t Hoff enthalpy change ΔHvH = ΔHvH(κ = ΔHvH/ΔHelcal) should be close to 1 (42), which in fact was confirmed for β2m (data not shown). To prove the reversibility of folding, β2m samples were cooled to 20 °C after the first run and heated again to 90 °C. About 70% of the initial enthalpy change was found in the second run, demonstrating refolding of a significant portion of β2m. In contrast to β2m, all HLA complexes investigated did not refold when cooled after the first run. The melting curves obtained in the second run showed the transition peak of β2m but no features of the complexes.

In comparison to β2m, more complex patterns were expected for the thermally induced unfolding of the HLA-B27 molecules that consist of three chains (β2m, HC, and peptide), with the HC folded into two structural domains containing α helices and β strands. Dissociation of a given complex and melting of HC and β2m may occur simultaneously or sequentially, and HC domains could unfold in separate or interdependent transitions. Therefore, the observed excessive heat capacity profiles may result from several overlapping contributions that require deconvolution of experimental heat capacity profiles in order to extract more detailed information about the transitions occurring during thermal unfolding.

The experimental excessive heat capacity curves of B*2709-s10G, B*2709-m9, and B2705-s10G were characterized by two partly overlapping peaks (Fig. 1C) with the melting temperature of the first peak significantly below that of β2m. Unfolding of B*2709-s10G started at an even lower temperature, resulting in two distant peaks separated by a pronounced shoulder at the high temperature side of the first peak. Deconvolution of the experimental profiles by applying the two-state routine of the ORIGIN software yielded two partly overlapping transitions for the low temperature peak (Tm1, ΔHm1) and Tm2, ΔHm2 (see Table II) and one transition for the high temperature peak (Tm3, ΔHm3) (Fig. 1E). The single transition of the high temperature peak correlated with the melting of β2m as indicated by a comparison of the Tm3 values and ΔHm3 values of B*2709-s10G, B*2709-s10R, B*2709-m9, and B*2705-s10G with that of β2m (Table II). The data evaluation suggests that melting of these complexes started by unfolding of two energetic domains of the HC (characterized by Tm1, ΔHm1 and Tm2, ΔHm2) and concomitant release of folded β2m, which melted at a significantly higher temperature (Tm3, ΔHm3). All three B*2709-peptide complexes as well as B*2705-s10G exhibited this unfolding behavior (Fig. 1C), with peptide- and subtype-dependent differences. Melting of B*2709-m9 did not significantly change (increase of Tm for −0.6 °C) in the presence of a 3-fold excess of free β2m (data not shown).

In contrast, the melting profiles of B*2705-s10R and B2705-m9 were composed of single peaks (Fig. 1D) with a slight asymmetry toward the low temperature side. Deconvolution with the two-state ORIGIN routine yielded only two partly overlapping transitions, characterized by Tm1, ΔHm1 and Tm2, ΔHm2 (Fig. 1F). A correlation of these transitions with the melting of structural domains (HC and β2m) was not justified by the data. B*2705-s10R and B*2705-m9 melted at temperatures equal to (B*2705-s10R) or even higher (B*2705-m9) than that of β2m, indicating that dissociation of the complexes would inevitably be coupled to the unfolding of both HC and β2m. The total enthalpy changes ΔHm connected with the melting of B*2705-m9 and B*2705-s10R were 316 and 259 kcal mol−1, respectively, for the sum of the two transitions (Table II). They were only marginally larger than those obtained for the sums of the three transitions of B*2709-m9 and B*2709-s10R (303 and 246 kcal mol−1, respectively). The low thermostability of

| Sample | n | Tm1 | ΔHm1 | Tm2 | ΔHm2 | Tm3 | ΔHm3 | Tm1 | ΔHm1 | Tm2 | ΔHm2 | Tm3 | ΔHm3 | Tm(1) |
|--------|---|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-------|
| B*2705-s10G (3) | 63.8 ± 0.5 | 75 ± 9 |
| B*2705-s10R (4) | 49.4 ± 2.0 | 74 ± 7 |
| B*2705-m9 (2) | 52.0 ± 0.8 | 96 ± 15 |
| B*2705-s10G (2) | 53.3 ± 0.1 | 96 ± 7 |
| B*2705-s10R (3) | 58.9 ± 3 | 105 ± 3 |
| B*2705-m9 (1) | 67.0 | 134 |

Tm is in °C, ΔH is in kcal mol−1; PBS buffer. The number of DSC experiments is in parentheses.
thermostability and crystal structure of HLA-B27

B*2709 correlates with a low enthalpy change (211 kcal/mol). For B*2705 (326 kcal/mol), this was not the case, suggesting that the HLA melting transition is too complex to allow a direct comparison between the overall melting temperature and the overall enthalpy change. However, it must be stated that all three B*2705 complexes were significantly more thermostable than their B*2709 counterparts. Even the least stable B*2705 complex (B*2705-s10G) was slightly more stable than the most stable B*2709 complex (B*2709-m9).

Structure Determination of B*2709-s10R—For a detailed understanding of the results from the CD and DSC measurements, it was important to determine the three-dimensional structure of at least one of the HLA-B27 subtypes in complex with a peptide featuring pArg1 and pTyr10. A data set to 1.9Å resolution was collected from B*2709-s10R using synchrotron radiation. The structure was determined by molecular replacement and refined to a crystallographic R/ Rfree factor of 0.169/0.194 (Table I). It shows the typical properties of an MHC class I molecule with a peptide binding groove built of two helices and a floor formed by an eight-stranded anti-parallel β sheet (Fig. 2), very similar to those of the previously reported HLA-B27-m9 complexes (Ca root mean square deviation of 0.86 and 0.88 Å2 compared with B*2709-m9 and B*2705-m9, respectively) with larger conformational variations restricted to exposed loop regions (e.g. HC residues 14–20 and 40–45).

Peptide Coordination in Structures of HLA-B27—After re-bulging of the molecular replacement model and refinement, difference electron density maps revealed well defined density in the peptide binding groove (Fig. 2A). s10R binds in an extended conformation, bulging out in the middle section as observed with other deca-peptides presented by MHC class 1 molecules and even with an HLA-B35-bound nona-peptide (43, 44). Figs. 2 and 3 show the accommodation of s10R and compare it to m9 and ARA7. The first four residues of all three peptides bind in essentially identical conformations and exhibit the established coordination features for HLA-B27 (41); that is, the pentagonal hydrogen bond network for p1, p2 inserting into the deep B pocket, which is sterically and electrostatically ideally suited to bind an arginine, and p3 accommodated in the hydrophobic D pocket followed by an outward directed kink (ϕ, −74.0°; ψ, 131.8°) of the backbone that leads to a solvent-exposed p4 residue. This conformation of pLeu4, which is stabilized by hydrophobic interactions with Ile-66, is the beginning of the exposed bulge that extends from the binding groove and is a key feature of the T cell receptor contact region (Fig. 2, Table III). The unoccupied space between the floor of the binding groove and the peptide is filled with a water network (Fig. 3B, compare with Ref. 17), which compensates for suboptimal fit to the groove but also provides an unfavorable entropic contribution that will influence the stability of the complexes.

The residues p4 to p9 (in s10R) and p4 to p8 (nonamers), respectively, show only very few or no (p6) direct interactions to the binding groove. Some indirect contacts to the HC are observed with other deca-peptides presented by MHC class 1 molecules (Ref. 45 and references therein). pArg2 of both s10R and m9 and ARA7 peptides reveals that the additional residue in s10R is inserted at position p7 (Fig. 3C). pAsn8 of s10R and pLeu7 of m9 share several interactions and both occupy the E pocket formed by HC residues Trp-147 and Val-152.

The pTyr residues of s10R and pLys9 of m9 both insert deeply into the F pocket (see below in “Results” and “Discussion”). Although the overall extension of the s10R peptide within the binding groove is 1.5 Å longer than that of m9, many side chain conformations and contacts are maintained among these HLA-B27 structures, in particular the deep insertion of the pTyl side chain into the F pocket. This is possible as the HLA binding groove shows a marked plasticity around the C-terminal end of the peptide (see below in “Results” and “Discussion”).

When comparing the average B factors, those of the m9 peptide are comparable (for B*2705 and B*2709) to the average B values for the HC and βm, whereas the values for s10R are higher (compare Table I and Ref. 17). This suggests that s10R is less stably coordinated within the B*2709 peptide binding groove than m9.

Variability of Contacts Formed by B Pocket Residues—The coordination of the primary anchor pArg2 in the B pocket shows an unexpected variability (Fig. 4). This is of interest as several B pocket residues, in particular Glu-45, have been shown to influence the in vivo folding properties of B*2705 molecules (Ref. 45 and references therein). pArg2 of both s10R

Fig. 2. Peptide electron density and differential conformations in the HLA-B27 binding groove. A, electron density map for the s10R peptide. B, electron density map for the s10R peptide. C, electron density map for the s10R peptide.
and m9 and all B pocket residues apart from Glu-45 and Glu-63 occupy identical positions. From the side chain of Glu-45 in B*2709-s10R and in B*2705-m9 (Figs. 4, A and B), one carboxyl oxygen forms a bifurcated salt bridge to the guanidinium head group of pArg2, whereas the second carboxyl oxygen is at very short distance (2.5 and 2.4 Å, respectively) to the proximal...
carboxyl group of Glu-63, suggesting that one of the carboxylates of Glu-45 or Glu-63 is protonated (crystallized at pH 8.0). By contrast, in B*2705-ARA2 (Fig. 4C, crystallized at pH 8.5), Glu-63 does not directly interact with Glu-45 because its side chain is rotated to form a salt bridge with Arg-62 at the entrance of the B pocket. A comparable geometry is also found in B*2709-m9 (Fig. 4D, crystallized at pH 9.0) where in addition both carboxyl oxygens of Glu-45 are involved in a bidentate salt bridge with pArg2.

Coordination of Peptide C Termini in the F Pocket—The coordination of the C-terminal residue within the F pocket is of particular interest as the single point mutation (D116H) that distinguishes B*2709 from the disease-associated subtype B*2705 is located at the floor of this pocket. This particular property may be related to the ability to bind specific disease-associated foreign or self-peptides, and it was shown recently that the two subtypes indeed exhibit differential peptide presentation capabilities (12, 13). In the B*2709-s10R complex, the side chain of pTyr10 inserts into the F pocket (Fig. 5), forming hydrophobic contacts with several HC residues, in particular Leu-81, Trp-147, Tyr-123, and Asp-77. At the floor of the pocket, the pTyr10 hydroxyl group forms water-bridged hydrogen bonds to Lys-70 and Asp-77. However, due to unfavorable geometry, it cannot form a direct hydrogen bond to His-116 but interacts instead with this residue via an OH–π bond that is considered to be weak (46, 47). Comparison with the F pocket coordination of pLys101 of m9 in B*2709 shows that all hydrophobic interactions to F pocket residues are maintained. This is remarkable since the length of s10R forces the HC side chains and the terminal carboxyl group of the peptide to become disordered (cf. Tyr-84, Thr-143, and Lys-146) follow with a similar movement (Fig. 6).

pArg1 as Secondary Anchor—The importance of the peptide N terminus for the stability of the entire HLA-B27-peptide complex has already been pointed out (3, 40), and the CD and DSC measurements described above demonstrate the superiority of pArg1 over pGly1 as a secondary anchor (Fig. 1, A–D; Table II). The structural analysis of the B*2709-s10R complex now provides an explanation for this finding (Fig. 7). The side chain of pArg1 is directed toward the solvent, allowing the formation of a unique and tightly packed hydrophobic sandwich coordination between the guanidinium group of Arg-62 (helix α1) and the aromatic side chain of Trp167 (helix α2) (Fig. 7A). Although pArg1 and Arg-62 interact via π–π stacking of their head groups, the hydrophobic indole ring of Trp-167 contacts the aliphatic section of the side chain of pArg1 by van der Waals interactions. The positive charges of the two guanidinium groups of pArg1 and Arg-62 are partly compensated by the adjacent Glu-163 on the helix α2. Glu-163 features two alternative conformations and forms either a direct salt bridge with pArg1 and a water-bridged salt bridge with Arg-62 or one bidentate salt bridge with pArg1 (Glu163DEG–pArg1H9252) (Table III). Arg-62 and Glu-163 form a clamp over the peptide, strongly suggesting a considerable contribution to the binding of the peptide and the stability of the complex. For comparison, Fig. 7B shows the situation in B*2709-m9, where p1 is a glycine. Here, the indole ring of Trp-167 folds down onto the peptide N terminus, shielding it completely from the solvent. Because the Arg-62–pArg1–Trp-167 clamp stack cannot form in this configuration, the side chain of Arg-62 adopts a different conformation as well.

Among the 101 MHC class I structures found in the PDB (analyzed using the SCOP database, version 1.63, May 2003, scop.mrc-lmb.cam.ac.uk/scop/index.html), there are seven structures of murine H-2Kb (PDB entry 2ava (48), 1fzm (49), 1fjz (49), 1kg (50), 1oxz (51), 1bqh (52), and 2mha (53)) and three structures of murine H-2Dd molecules (1q03 (54), 1bii (55), and 1dhh (56)) that also feature a peptide with pArg1. Despite the fact that Trp-167 and Arg-62 are conserved among B*2709, H-2Kb, and H-2Dd, the H-2Kb structures feature only partial stacking either to Arg-62 or to Trp-167 (Fig. 7C). Among the H-2Dd structures, only one shows a sandwich-like topology, but the arrangement is not as even as the one observed in B*2709-s10R (Fig. 7D). Local differences like the E163T exchange in H-2Kb or variations in the overall orientation of helix α1 in H-2Dd appear to be responsible for these discrepancies.

**DISCUSSION**

**Influence of Subtype and Peptide on Thermal Stability of HLA-B27 Molecules**—It has already been pointed out that a significant contribution to an understanding of the molecular basis of the disease association of certain HLA-B27 subtypes will come from a combination of structural and dynamic studies of these molecules (18, 19). The comparative thermodynamic studies of the B*2705 and B*2709 peptide complexes described here can be regarded as a first step in this direction. When loaded with the peptides m9, s10R, and s10G employed in this study, the B*2709 and B*2705 complexes differ significantly in their thermostabilities. This is in agreement with results described for HLA-B*2705 complexes with nonapeptide GRAFVTTIGK and p3 and p9 variants of this peptide, which showed a wide thermostability range between about 38 and 60 °C (8). However, although in those cases the thermostability differences of the studied complexes were caused by the peptides in the binding grooves of the HLA molecules, we additionally observed a pronounced influence of the subtype-specific H116D exchange in the HC on the thermostability of the trimERIC HC/β2m-peptide complexes. Alterations of the thermostability of HLA B*2705-peptide complexes by changes of HC residues were described recently for a point mutation of a B pocket residue (20). We consider these findings less relevant to explain the disease association of HLA-B27 since the disease-associated subtype B*2705 and the non-associated subtype B*2709 do not differ in their B pocket residues (discussed in Ref. 15).

Asp-116 of the B*2705 HC markedly contributes to the high thermostability of complexes with m9 and s10R when compared with His-116 of B*2709. The DSC curves of these B*2705 complexes show a single peak at high temperature (Fig. 1D). Deconvolution results in two two-state transitions and points to the melting of two energetic folding units (Fig. 1F) with transition characteristics $T_m^1$, $ΔH_m^1$ and $T_m^2$, $ΔH_m^2$. Neither corresponds to the melting characteristics $T_m^3$, $ΔH_m^3$ of β2m nor to the other two transitions, $T_m^1$, $ΔH_m^1$ and $T_m^2$, $ΔH_m^2$ of the HLA complexes with double-peak melting characteristics (Table II). The data do not allow correlation of these energetic units to structural units such as HC-peptide complexes, or any of the structural domains of the HC. Also, there is no direct evidence either for the exact time point of the release of the peptides during the thermal unfolding or for the existence of stable HC-peptide complexes as intermediates of the unfolding reaction. However, the data do not exclude the possibility that one of the cooperative two-state transitions that is not correlated to the unfolding of β2m corresponds to the unfolding of a HC-peptide complex.

In contrast to the complexes of m9 and s10R with B*2705, the melting behavior of B*2709-m9, B*2709-s10R, B*2709-s10G, and B*2709-s10G suggests a triphasic mechanism with melting of the HC and separation of all components in two transitions followed by the unfolding of β2m in a third transition (Fig. 1, C and E). Of course, the precise sequence of probably mutually dependent melt-
**TABLE III**

**Detailed comparison of the peptide coordination in B*2709:s10R and B*2709:m9**

Intrapeptide contacts are listed in the HC column in bold letters. AC1/2 = alternative conformation 1 and 2, respectively.

| Peptide s10R: B*2709 (this work) | Peptide m9:B*2709 (Ref. 17 PDB entry ljge) |
|----------------------------------|------------------------------------------|
| s10 HC Distance Interaction      | s10 HC Distance Interaction              |
| pArg1                            | pGly1                                    |
| pArg1N                           | Gly-N                                    |
| pArg1O                           | Gly-O                                    |
| pArg1H                           | Gly-H                                    |
| pArg1X                           | ArgNH2                                   |
| pArg2                            | Arg2                                      |
| pArg2N                           | Arg2N                                     |
| pArg2O                           | Arg2O                                     |
| pArg2H                           | Arg2H                                     |
| pArg2X                           | Arg2X                                     |
| pLeu3                            | pPhe3                                     |
| pLeu3N                           | pPhe3N                                    |
| pLeu3O                           | pPhe3O                                    |
| pLeu3H                           | pPhe3H                                    |
| pLeu4                            | pAla4                                     |
| pLeu4N                           | pAla4N                                    |
| pLeu4O                           | pAla4O                                    |
| pLeu4H                           | pAla4H                                    |
| pLeu4X                           | pAla4X                                    |
| pGly6                            | pGly6                                     |
| pHis7                            | pHis7                                     |
| pHis7N                           | pHis7N                                    |
| pHis7O                           | pHis7O                                    |
| pAsn8                            | pPhe3                                     |
| pAsn8N                           | pPhe3N                                    |
| pAsn8O                           | pPhe3O                                    |
| pAsn8H                           | pPhe3H                                    |
| pAsn8X                           | pPhe3X                                    |
| pTyr10                           | pTyr10                                    |
| pTyr10N                          | pTyr10N                                   |
| pTyr10O                          | pTyr10O                                   |
| pTyr10H                          | pTyr10H                                   |
| pTyr10X                          | pTyr10X                                   |
| pTyr1N                           | pTyr1N                                    |
| pTyr1O                           | pTyr1O                                    |
| pTyr1H                           | pTyr1H                                    |
| pTyr1X                           | pTyr1X                                    |
| pTyr2                            | pTyr2                                    |
| pTyr2N                           | pTyr2N                                    |
| pTyr2O                           | pTyr2O                                    |
| pTyr2H                           | pTyr2H                                    |
| pTyr2X                           | pTyr2X                                    |
| pTyr3                            | pTyr3                                    |
| pTyr3N                           | pTyr3N                                    |
| pTyr3O                           | pTyr3O                                    |
| pTyr3H                           | pTyr3H                                    |
| pTyr3X                           | pTyr3X                                    |
| pTyr4                            | pTyr4                                    |
| pTyr4N                           | pTyr4N                                    |
| pTyr4O                           | pTyr4O                                    |
| pTyr4H                           | pTyr4H                                    |
| pTyr4X                           | pTyr4X                                    |
| pTyr5                            | pTyr5                                    |
| pTyr5N                           | pTyr5N                                    |
| pTyr5O                           | pTyr5O                                    |
| pTyr5H                           | pTyr5H                                    |
| pTyr5X                           | pTyr5X                                    |
| pTyr6                            | pTyr6                                    |
| pTyr6N                           | pTyr6N                                    |
| pTyr6O                           | pTyr6O                                    |
| pTyr6H                           | pTyr6H                                    |
| pTyr6X                           | pTyr6X                                    |
| pTyr7                            | pTyr7                                    |
| pTyr7N                           | pTyr7N                                    |
| pTyr7O                           | pTyr7O                                    |
| pTyr7H                           | pTyr7H                                    |
| pTyr7X                           | pTyr7X                                    |
| pTyr8                            | pTyr8                                    |
| pTyr8N                           | pTyr8N                                    |
| pTyr8O                           | pTyr8O                                    |
| pTyr8H                           | pTyr8H                                    |
| pTyr8X                           | pTyr8X                                    |
| pTyr9                            | pTyr9                                    |
| pTyr9N                           | pTyr9N                                    |
| pTyr9O                           | pTyr9O                                    |
| pTyr9H                           | pTyr9H                                    |
| pTyr9X                           | pTyr9X                                    |
| pTyr10                           | pTyr10                                   |
| pTyr10N                          | pTyr10N                                   |
| pTyr10O                          | pTyr10O                                   |
| pTyr10H                          | pTyr10H                                   |
| pTyr10X                          | pTyr10X                                   |

*a Interacting atoms are specified for polar interactions only. For water-mediated hydrogen bonds, the first distance listed is for the peptide-water interaction, and the second is for the water-HC interaction.*
ing events cannot be deduced from the DSC curves, e.g., the release of peptide, unfolding of HC domains, dissociation of \( \beta_m \). However, the analysis of the melting curves clearly indicates the presence of folded \( \beta_m \) at temperatures where the HC has lost its binding site for \( \beta_m \) because it is largely unfolded. Compared with the B*2705 complexes with m9 and s10R, B*2709-s10G and the three B*2709 complexes reveal lower thermostability with weaker interactions between HC, \( \beta_m \), and peptides. Because the subtypes differ only in residue 116 in the F pocket, this residue must be responsible for the observed strong differences in thermostability of the B*2705 and B*2709 complexes. Although at the level of the HLA molecule, B*2705 forms the more stable complexes, and at the peptide level it

**Fig. 4. Differences in the coordination of B pocket residues.** Cross-section through the B pocket showing selected residues. Glu-45 and Glu-63 adopt different positions in the B pocket of HLA-B27, which are probably triggered by the pH of the crystallization buffer. The varying conformations do not appear to influence the strength of pArg2 binding as it uniformly shows the lowest flexibility (smallest B-factor) among all residues in the respective peptides.

**Fig. 5. Hydrogen bonds and salt bridges within the F pocket.** Shown are hydrogen bonds (black) and salt bridges (green) within the F pocket between selected residues and water molecules (red spheres). The dependence of the water network on the peptide residue present in the F pocket becomes obvious when the known HLA-B27 structures with peptide m9 (PDB entries 1jge and 1k5n) are compared with B*2709-s10R. pTyr10 (s10R) forms no direct hydrogen bonds to the B*2709-specific His-116 but only a weak OH–π bond. This indicates that a C-terminal tyrosine cannot be optimally accommodated in the F pocket of B*2709. By contrast, in B*2705, pLys9 of m9 forms a salt bridge with Asp-116, whereas in B*2709 it hydrogen bonds with His-116 and forms a salt bridge with Asp-77. The black arrows designated A and B indicate the two conformations of His-116 found in B*2709-m9.
is the m9 peptide that stabilizes both HLA subtypes more than s10R and s10G do (Table II). These results are in good agreement with the crystal structure, as s10R shows less tight contacts to the binding groove and a poorer fit to the important F pocket as compared with the m9 peptide, even when taking pLys into consideration, which is unfavorable for peptides binding to B*2709 (13). In conclusion, the experimental differential heat capacity curves clearly demonstrate major differences in the melting behavior of HLA-B27-peptide complexes. Deconvolution into three and two two-state transitions for the double-peak and single-peak melters, respectively, extends the information that is deducible from CD melting.

**Differential Coordination of the Primary Anchor pArg2**—
Glu-45 and Glu-63 are instrumental in shaping the B pocket, one of the most highly conserved features of HLA-B27 molecules. Nevertheless, they show a surprising conformational variability among the available HLA-B27 structures. The short distance between the two carboxyl oxygens of these glutamates (2.5 and 2.4 Å, respectively) in the structures of B*2709-s10R and B*2705-m9 (Fig. 4, A and B) can only be explained by assuming that one of the two glutamate residues is protonated and serves as a hydrogen bond donor. For Glu-45, the proximal positively charged guanidinium group of pArg2 renders protonation improbable (pArg2N2H2/NR—Glu-45Oε1, 2.7/2.9 Å), leaving Glu-63 as the likely candidate for protonation. All four available HLA-B27 structures were crystallized at pH 8.0—9.0. Because of these high pH values, protonation of Glu-63 requires a major shift of the pH₄ value. Such a shift is, however, not unusual for adjacent charged residues buried in the hydrophobic interior of a protein. Here, protonation helps to minimize the electrostatic repulsion between the buried anionic groups, and indeed, Glu-45 is completely buried, and Glu-63 is 90% buried from the solvent. Also, except for the equally buried head group of pArg2, the protein environments of these two residues are hydrophobic. The observed differences in pH further support the hypothesis of a protonated Glu-63; the short Glu-45—Glu-63 distances (B*2709-s10R and B*2705-m9) are found at the lower pH 8.0, whereas the larger Glu-Glu distance as well as the bidentate salt bridge are found at pH 9.0 (B*2709-m9, Fig. 4D). B*2705-ARA₁₇ was crystallized at an intermediate pH value, pH 8.5) and already shows a larger Glu-Glu distance, suggesting deprotonation (Fig. 4C). Here, the formation of a salt bridge between Glu-63 and Arg-62 may favor this conformation. Following this reasoning, the short Glu-45—Glu-63 distance and the monodentate bifurcated salt bridge between Glu-45 and pArg2 would be the conformation present at physiological pH, whereas the bidentate salt bridge of B*2709-m9 would represent a crystallization artifact. A very similar protonation of a buried glutamate residue at pH 8.0 has been experimentally observed in the ultrahigh resolution x-ray structure of TEM-1 β-lactamase (57). Similarly short distances between buried carboxyl groups have also been found in the peptide binding groove of several class II HLA-DR molecules (2.4—2.5 Å interaction Glu-11Oε1—Asp-66Oε2 (58—62)).

**Coordination of the Peptide Termini**—The B*2709-s10R structure described here is the first structure with a tyrosine residue at the p1 position bound to the F pocket of an HLA-B27 molecule. The tyrosine residue interacts mainly via hydrophobic interactions with the inner walls of the pocket, but there are no direct hydrogen bonding contacts with the subtype-specific His-116 at the floor of the F pocket. With regard to B*2705, we predict that a tyrosine should interact more favorably here because Asp-116 would be expected to allow the formation of a direct hydrogen bond, suggesting that both s10R and s10G should form more stable complexes with B*2705 than with B*2709. This corresponds well with the thermodynamic data where these peptides formed more stable complexes in the case of B*2705 than with B*2709. A recent study of the peptide repertoires of B*2705 and B*2709 (13) confirmed that a C-terminal tyrosine was almost exclusively identified in peptides that were specifically eluted from B*2705 and not from B*2709.

Various studies investigating the peptide repertoire bound by HLA-B27 molecules have shown a preference for pArg1 (39, 41), and it was suggested that pArg1 could compensate for suboptimal binding of other secondary anchors at p3 and/or p1 (39). The HLA-B27 structures with ARA₁ and m9 determined earlier (41, 17) only featured an alanine or a glycine, respectively, at p1. The structure of the B*2709-s10R complex presented here now provides a structural explanation for the Arg preference. The long side chain was found in a tight sandwich formed with Arg-62 (via π-π stacking) and Trp-167 (via hydrophobic interaction). To our knowledge this favorable coordination has not yet been described for any other MHC class I molecule. A number of murine H-2Kb and H-2D⁴ structures contain peptides with pArg1, and the residues Trp-167 and Asp-62 are conserved among B*2709, H-2Kb, and H-2D⁴. However, the favorable sandwich interaction observed in B*2709-s10R is not found in H-2Kb or in H-2D⁴ (see “Results” and Fig. 7). A series of six homology models for HLA-B27-peptide complexes reported by Rognan et al. (63) also contained three peptides with a pArg1, but the sandwich found in B*2709-s10R was not predicted for any of these structures. Interestingly, the clamp-stack conformation of Arg-62 and Glu-163 is also present in B*2705-ARA₁₇ (Fig. 3A, lower panel), and electron density shown in Madden et al. (41) and Fig. 2A suggests the presence of pArg1 (albeit not included in the final model submitted to the Protein Data Bank), underscoring the pref-
Fig. 7. HLA-B27-specific sandwich coordination of the secondary anchor pArg1. A. A stereo figure showing the clamp stack involving pArg1 of the s10R peptide (red) and Arg-62, Glu-163, and Trp167 from the HC (green). The alternative conformation of Glu-163 is indicated in light green. Hydrogen bonds between Arg-62, Glu-163, pArg1, and Wat58 are indicated with dotted lines, and π-π-stacking and hydrophobic interactions are indicated by stripes. B, the same region in the B2709:m9 complex (PDB entry 1bii). Because the N-terminal pArg1 is missing in m9, no stacking is present. Consequently, Trp-167 folds onto pGly1, and the Arg-62 side chain adopts a conformation parallel to helix c1. C and D, for comparison two murine MHC molecules are shown. Both feature a pArg1 but do not adopt the Arg-62–pArg1–Trp167 sandwich conformation found in B2709:s10R. C. H-2Kb:SVS8, PDB entry 1bii, molecule 2. D. H-2Dd:HIV-1 (human immunodeficiency virus 1), PDB entry 1bji.

ference of HLA-B27 for pArg1. Our comparison of the thermosta-

bilities of the s10R and s10G peptide complexes demonstrates the stabilizing effect of pArg1; independent of the subtypes, the CD and

DSC melting temperatures of the s10G complexes are significantly lower than those of the respective s10R complexes (Table II).

With regard to the disease association of HLA-B27, it has been suggested that unconventional properties of the B pocket of the molecule may be related to peculiarities in the folding behavior (16). However, the differentially AS-associated subtypes are distinguished only by a change in position 116 (B2705 and B2709) or positions 114 and 116 (B2704 and B2706) close to p1 (10, 11). Furthermore, the Arg-62–Glu-163–Trp167 constellation, which may be thought to contribute to T cell interaction unique to HLA-B27, is absent from at least one HLA-B subtype that is clearly disease-associated; Lopez-Larrea et al. (64) show that B*1403 (Arg-62–Thr-163–Trp-167) is AS-associated, whereas B*1402 (differing only in residue 156 from B*1403) is not. Therefore, it appears unlikely that residues of the B pocket or those responsible for “locking” the peptide near the N terminus like Arg-62, Glu-163, and Trp167 play a role in AS pathogenesis (see also Ref 15 for a discussion).

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