Efficient Binding of Reduced Peptide Bond Pseudopeptides to Major Histocompatibility Complex Class I Molecule*

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Reduced peptide bond pseudopeptide analogues have been examined for their ability to bind murine class I molecules of the major histocompatibility complex (MHC). Eight pseudopeptide analogues of an antigenic peptide derived from Plasmodium berghei (H-Ser252-Tyr-Ile-Pro-Ser-Ala-Glu-Lys-Ile260-OH) were obtained by systematically replacing one peptide bond at a time by a reduced peptide bond ψ(CH2-NH). The resulting analogues were then tested for their binding to a recombinant single chain SC-Kd class I molecule. The comparative results show that five analogues can efficiently mimic the parent peptide while the introduction of the reduced bond between P3-P4, P7-P8, and P8-P9 is deleterious for SC-Kd binding. The fact that more stable pseudopeptides containing reduced peptide bonds can bind major histocompatibility complex I molecules is of great interest for the design of peptidomimetics with potential therapeutic properties. Such peptide analogues may prove useful for the development of peptide-based cytotoxic T lymphocyte vaccines.

MHC class I molecules bind short peptides derived from the cytosol, which are then presented at the cell surface to CD8-expressing T lymphocytes (1). MHC molecules are highly polymorphic. Each MHC allele selects a unique subset of peptides (2), but while a single MHC allele can bind up to 104 different polymorphic. Each MHC allele selects a unique subset of peptides expressing T lymphocytes (1). MHC molecules are highly polymorphic. Each MHC allele selects a unique subset of peptides (2), but while a single MHC allele can bind up to 104 different polymorphic residues of the MHC and common features of the peptide were revealed by crystallographic analysis of MHC molecules complexed with a single peptide. On the other hand, polymorphic residues with a bulky side chain, which are rather frequent in the peptide binding site, restrict the size of the repertoire of bound peptides for example by steric hindrance. In this model, conserved interactions would guarantee high affinity binding with every peptide of correct length while polymorphic negative interactions would minimize peptide repertoire overlapping between the different MHC alleles. This view stems from crystallographic studies of MHC molecules bound to single peptides (4, 6–10) and has only been partially confirmed by mutagenesis of the MHC molecule and by amino acid substitution in the peptide. These studies did not directly examine the peptide backbone for which a role has been postulated from the MHC crystal structure.

In order to directly test the contribution of atoms from the peptide backbone in the binding affinity, we decided to introduce sequential chemical changes at each peptide bond (CO-NH) of the peptide. Starting from a 9-residue-long antigenic peptide derived from Plasmodium berghei and known to bind to SC-Kd (11), we systematically replaced one peptide bond at a time by a reduced peptide bond ψ(CH2-NH). The eight resulting analogues were then tested in vitro for their binding to a recombinant, single chain SC-Kd molecule. We found that out of the eight peptide bonds, five can be reduced with only a modest effect on the binding affinity. Our results specify the contribution of each peptide bond and the importance of the peptide backbone as postulated by crystallographers (4, 6, 8–10).

**MATERIALS AND METHODS**

Peptide Synthesis—Reduced peptide bond pseudopeptide analogues of the parent peptide 252–260 of circumsporozoite P. berghei (PiC5) were synthesized in t-butyloxycarbonyl chemistry by the stepwise solid-phase methodology on a multichannel peptide synthesizer (12). Protected amino acids were from NeoSystem (Strasbourg, France), and Boc-Ile-phenylacetonitromethyl resin was from Perkin-Elmer (Saint Quentin en Yvelines, France). Assembly of the protected peptide chains was carried out on a 200-μmol scale using the in situ neutralization protocol described previously (12). The reduced peptide bond was formed on the resin by reductive amination of N-Boc-ω-aminooxyaldehydes (2.5-fold excess) in dimethylformamide containing 1% acetic acid as described previously (13). Typically, the reaction was complete within 1 h as determined by the ninhydrin test. Protected aldehydes were synthesized by reductive synthesis of their corresponding O,N-dimethyloxamides with LiAlH4 in tetrahydrofuran (14, 15) with the exception of N′-Boc-glutam-ω-al-β-cylohexyl ester, which was obtained after reduction of the corresponding S-benzyl thiourea with triethylsilane and 10% Pd/C as a catalyst (15, 16).

All the coupling procedures following the reduced peptide bond formation were performed using a 2-fold excess of N-Boc-ω-amino acid and activation reagents and were monitored with the ninhydrin test. At the end of the synthesis and after the last deprotection step, the peptide resin was washed twice with ether and dried under vacuum in a desiccator. The peptides were cleaved from the resin by treatment with anhydrous hydrogen fluoride (HF) containing 10% (v/v) anisole and 1% (v/v) 1,2-ethanediol. After removal of HF in vacuo, the peptides were extracted from the resin and lyophilized. The crude peptides were purified by reverse phase HPLC using a Perkin-Elmer preparative HPLC system on an Aquapore ODS 20-μm column (100 × 10 mm) by elution with a linear gradient of aqueous 0.05% trifluoroacetic acid (A) and 80% acetonitrile, 20% A (B) at a flow rate of 6 ml/min with UV detection at 220 nm.

Analytical HPLC was run on a Beckman instrument (Gagny, France) with a Nucleosil C18 S-μm column (3.9 × 150 mm) using a linear gradient of 0.1% trifluoroacetic acid and acetonitrile containing 0.08% trifluoroacetic acid at a flow rate of 1.2 ml/min. Mass spectra were

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1 The abbreviations used are: MHC, major histocompatibility complex; Boc, t-butoxycarbonyl; HPLC, high pressure liquid chromatography; NP, nucleotide.

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obtained by fast atom bombardment-mass spectrometry in the positive mode on a VG analytical ZAB-2E double focusing instrument and recorded on a VG 11-250 data system (VG Analytical, Manchester, UK). Proton spectroscopy was acquired at 300 K on a Bruker ASP200 spectrometer (Bruker Spectrospin, Wissembourg, France).

SC-Kd Purification—SC-Kd was purified by affinity chromatography from the culture supernatant of Chinese hamster ovary cells transfected with a plasmid containing the SC-Kd sequence donated into P Raf53 as described previously (17).

Peptide Binding Assay—Peptide analogue binding to SC-Kd was determined in a previously described competition assay (18). Briefly, since iodination of the Tyr residue at position 2 prevents peptide binding to Kd, a derivative of the PbCS peptide containing a second Tyr residue at the non-anchor position was synthesized. This peptide called S9I and displaying the sequence SYIPSAEYI was shown to bind to SC-Kd (19). It was radiolabeled using chloramine T-catalyzed iodination (specific activity, 100 ± 50 × 10^6 dpm/μg). With this specific activity, only 1% of the tyrosine residues are iodinated, and most labeled peptide molecules display a single iodinated tyrosine residue. As peptides labeled at position 2 do not bind to SC-Kd, only molecules labeled at position 8 are active in the binding test. Labeled peptide was incubated for 1 h at room temperature in phosphate-buffered saline (1.5 mM KH2PO4, 8 mM Na2HPO4, 2.5 mM KCl, 140 mM NaCl, pH 7.4) with SC-Kd (10 μg/ml) and increasing concentrations of PbCS peptide or pseudopeptides. Unbound peptide was eliminated by immunoprecipitation with an H-2d specific monoclonal antibody SF1-11.1 (ATCC, Rockville, MD, HB159).

RESULTS AND DISCUSSION

Table I shows the sequences of the antigenic peptide 252–260 of P. berghei circumsporozoite (PbCS) and of eight pseudopeptides (V1 to V8), which were used in this study. The reduced peptide bond ψ(CH2–NH) was formed by reductive amination of N-Boc-glycine aldehydes in acidic dichloromethane as described previously (13). All the glycine aldehydes and intermediates were characterized by H1 NMR spectroscopy and showed expected features. Stepwise elongation of peptide chains using t-butyloxy carbonyl chemistry led to the fully protected peptide resin. HF cleavage and HPLC purification on a C18 column yielded the final products. All the compounds were identified by fast atom bombardment mass spectrometry, and their purity was assessed by analytical HPLC. HPLC retention times and fast atom bombardment-mass spectroscopy values of the peptides are reported in Table I.

The eight different analogues were then tested for their binding to purified soluble, single-chain SC-Kd produced by transfected Chinese hamster ovary cells. This recombinant protein contains the three external domains of the mouse MHC class I molecule H-2Kd connected through a 15-amino-acid-long spacer to the N terminus of the β2-microglobulin (20). The resulting fusion protein is soluble in the absence of detergent (21). It contains β2-microglobulin of mouse origin exclusively, unlike heterodimeric MHC class I molecules purified from tissue cultures, which include bovine β2-microglobulin that may alter the heavy chain conformation (22). It selects a peptide repertoire indistinguishable from that of cell surface-associated SC-Kd (19).

Parent peptide or pseudopeptide binding to SC-Kd was determined using a competition assay with the 125I-labeled peptide S9I of known equilibrium affinity value for SC-Kd (Kd = 30 × 10−10 M) (19). This assay was previously used to identify antigenic epitopes derived from mouse mammary tumor virus sequences (18). Fig. 1 shows the competitive binding activity of PbCS peptide and of six analogues, V1–V6. With increasing concentrations of the Ψ1, Ψ2, Ψ4, Ψ5, and Ψ6 competitors, the binding of labeled peptide was reduced. For the parent peptide (PbCS) and each analogue, the concentration leading to 50% inhibition binding of S9I (IC50) was determined and used to calculate the relative affinity following the Cheng and Prussoff (23) relationship, Kref = 1/Cref(1 + [S9I]Ks9I), where [S9I] is the concentration of the labeled peptide and Ks9I is its equilibrium affinity value for SC-Kd (30 nM). Table II shows the effect of introducing reduced peptide bonds in the parent peptide on its binding to SC-Kd. While every PbCS analogue consistently displayed a reduced binding to SC-Kd, broad differences were observed. Out of eight pseudopeptides tested, five (Ψ1, Ψ2, Ψ4, Ψ5, and Ψ6) demonstrated strong binding (Kref between 150 and 280 nM as compared with 50 nM measured with PbCS peptide). In contrast, Ψ7 and Ψ8 showed weak activity and Ψ3 did not compete at all for SC-Kd. Thus, introduction of a reduced amide bond between Ile254 and Pro255 (P3–P4), Glu258 and Ly259 (P7–P8), and Ly259 and Ile260 (P8–P9) causes deleterious for binding to SC-Kd while modifications between P1–P2, P2–P3, P4–P5, P5–P6, and P6–P7 had only a minor effect.

The tendency for a reduced affinity of pseudopeptides in binding SC-Kd may be related to the loss of a potential intermolecular hydrogen bonding at the point of replacement of the 2-microglobulin moiety by a methylene group. This observation is consistent with crystallographic studies showing the importance of extensive hydrogen bonding between the MHC residues and 2-microglobulin of mouse origin exclusively, which contains a potential intermolecular hydrogen bond between the 2-microglobulin and a potential intermolecular hydrogen bond between the N and C termini of the peptide, nine direct hydrogen bonds are formed between main chain atoms of the peptide and side chain atoms of cleft residues (6). The structure of H-2Kd with a peptide from vesicular stomatitis virus reveals that, in addition to hydrogen bonds involving the carbonyl moiety of the C-terminal cysteine of the peptide backbone (6). The structure of H-2Kd with a peptide from vesicular stomatitis virus reveals that, in addition to hydrogen bonds involving the carbonyl moiety of the C-terminal cysteine of the peptide backbone (6). The structure of H-2Kd with a peptide from vesicular stomatitis virus reveals that, in addition to hydrogen bonds involving the carbonyl moiety of the C-terminal cysteine of the peptide backbone (6). The structure of H-2Kd with a peptide from vesicular stomatitis virus reveals that, in addition to hydrogen bonds involving the carbonyl moiety of the C-terminal cysteine of the peptide backbone (6).
Tyr^{156}, and Trp^{47} form a ridge across the cleft of H-2D^{b} (10). This peculiar structural feature is shared by the H-2K^{d} molecule (24), and one should expect to see a similar hydrogen bonding pattern for Trp^{73} in the SC-K^{d}-PbCS complex and hence a decrease in affinity binding of \( \psi^6 \) and \( \psi^7 \) to SC-K^{d}. On the other hand, the hydroxyl group of the conserved Tyr^{159} was proposed to be hydrogen bonded to the carbonyl of the first peptide bond (6–8, 10). This conserved hydrogen bond may not be as critical as suggested by these previous studies, since affinity of \( \psi^1 \) is only reduced by a factor of 6 as compared with the parent peptide PbCS.

It should be kept in mind that, in addition to preventing a particular carbonyl from forming a hydrogen bond, the presence of a secondary amine has conformational effects on reduced peptide bond pseudopeptide analogues. The neutral amine form introduces an increased flexibility due to the higher rotation around the C-N bond. However, it is known that the reduced amide bond is protonated at physiological pH, and it has been shown (25) using pseudopeptide units that the resulting ammonium link \( \psi(CH2-N^2-H2) \) could stabilize a folded structure by acting as a strong proton donor. Therefore, the total lack of reactivity of the \( \psi^3 \) peptide toward the SC-K^{d} molecule may be related to a major conformational change of the analogue, which is no longer able to mimic the extended bioactive conformation of the parent PbCS peptide. Alternatively, the MHC may not be able to adapt to the \( \psi^3 \) peptide.

**TABLE II**

Relative affinity of pseudopeptides for SC-K^{d}

| Peptide | \( K_{\text{m}} \) |
|---------|------------------|
| PbCS (parent) | 10^{-9} M |
| \( \psi^1 \) | 50 ± 20 |
| \( \psi^2 \) | 280 ± 90 |
| \( \psi^3 \) | 150 ± 70 |
| \( \psi^4 \) | 100,000 |
| \( \psi^5 \) | 150 ± 100 |
| \( \psi^6 \) | 240 ± 30 |
| \( \psi^7 \) | 230 ± 20 |
| \( \psi^8 \) | 480 ± 50 |
| >3,000 | |

**FIG. 1. Binding of analogues to K^{d}**. Peptide binding was measured as described under “Materials and Methods” with different concentrations of test peptides. Peptide analogues were compared with unlabeled positive control peptide PbCS (open circle). The means and standard deviations of three independent experiments are presented. \( \bullet \), \( \psi^1 \); \( \psi^2 \); \( \bullet \), \( \psi^3 \); \( \psi^4 \); \( \psi^5 \); \( \psi^6 \).

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Under certain conditions, synthetic peptides are highly immunogenic in animals and might constitute chemically defined, safe, and cheap vaccines. Recently, tumor- and virus-specific cytolytic T lymphocytes have been generated using appropriate peptide immunization protocols. However, the rapid clearance of the peptide in the blood circulation may represent a severe limitation to human applications since high doses of peptide would be required. Pseudopeptides with altered peptide bond are less susceptible to natural proteases. Thus it would be tempting to use such pseudopeptides to induce cytolytic effector cells in vivo. But it was not obvious that such analogues could bind to MHC class I molecules, particularly given the postulated crucial role of the backbone in binding to the MHC molecules. The reduced amide bond has been used for the design of various analogues of bioactive peptides, but only two recent investigations have dealt with the immunological impact of this modification in the context of peptide-antibody (15) and peptide-class II HLA molecule interaction (26). We report here that certain reduced peptide bond pseudopeptide analogues are bound almost as well as the parent peptide by MHC class I molecule and thus that reduced bond pseudopeptide analogues can mimic the parent peptide. Our results confirm the major role of the peptide backbone in the binding of peptide to MHC molecule but reveal a differential contribution for each carbonyl. By selectively introducing reduced peptide bonds in pseudopeptides, it might be possible to produce peptidomimetics with full biological activity but reduced sensitivity to proteases. It should be pointed out that the effects of such backbone modifications on the MHC-ligand recognition by the T cell receptor still need to be addressed. This study presents the first unequivocal data for the potent applicability of reduced peptide bond pseudopeptides in the context of peptide-MHC class I recognition.