Isomeric Complexes of Peptides with Class II Proteins of the Major Histocompatibility Complex

Craig Beeson, Thomas G. Anderson, Chris Lee, and Harden M. McConnell*

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305

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Abstract: An important event in the generation of an immune response is the activation of T cells by peptides bound to the class II proteins of the major histocompatiblity complex (MHC). Binding of a peptide to an MHC protein is stabilized by multiple interactions between the protein, peptide side-chains, and peptide backbone. Unstable protein−peptide complexes that precede formation of long-lived complexes are presumably engaged in a smaller number of these binding interactions. To investigate the effect of peptide structure on the formation of unstable complexes, we have strategically modified a peptide that forms only long-lived complexes (dissociation rate constant $k_{off} = 2.5 \times 10^{-6}$ s^{-1}). Dissociation of the modified peptide from an MHC protein is biphasic with dissociation rate constants $k_{off} = 5.3 \times 10^{-4}$ and 2.6×10^{-6} s^{-1}. Thus, at least two detectable complexes are formed. These results demonstrate that changes in peptide structure alone are sufficient to result in the formation of isomeric structures of MHC protein−peptide complexes.

Introduction

The activation of T cells by peptides bound to the class II proteins of the major histocompatibility complex (MHC) is important to immune function.[1] The MHC proteins have a unique binding motif that enables them to bind a wide variety of peptides with lifetimes sufficient to ensure T cell surveillance.[2] The peptides are held in an extended conformation by a series of hydrogen bond between the protein side-chains and the peptide amide backbone.[3] Selectivity is achieved through interactions between peptide side-chains and unique protein binding pockets determined by the MHC polymorphism. The complexity of these interactions results in complicated reaction kinetics which are described by several related mechanisms.[2b,4] Structural investigations of these mechanisms have been partly limited by the apparent insensitivity of the reaction kinetics to peptide structure.[5] However, the recent identification of peptides that form unstable complexes now provides an opportunity to evaluate the effect of peptide structure on the kinetics of these reactions.[4b,6−8]

Several studies have described the biphasic dissociation of a peptide from an MHC protein.[6,7] Thus, at least two detectable peptide−MHC complexes can be formed in these reactions. In the first report of this kind, it was proposed that the short-lived complex was an intermediate that preceded formation of a long-lived, terminal complex.[6a] Similar observations were reported for the reactions of the myoglobin 110−121 peptide with the murine class II MHC protein I-E^d.[7] For these reactions it was demonstrated that, after a short incubation of peptide with protein, only a short-lived complex could be detected in the dissociation kinetics (Figure 1 inset). After longer incubations, a long-lived complex was also detected in the dissociation kinetics (Figure 1). This induction phase in the reactions of the myoglobin peptide supports the suggestion that the short-lived complex is an intermediate in the formation of the terminal complex.

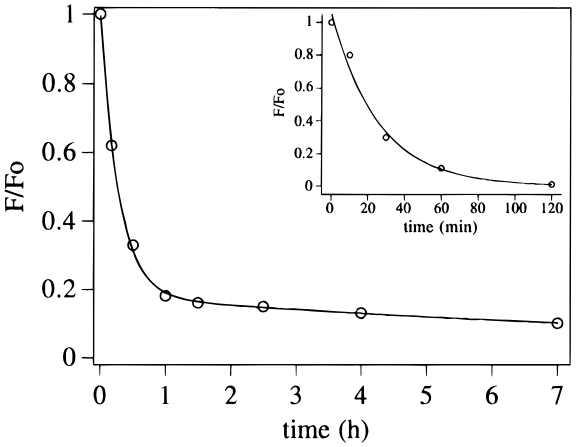

Figure 1. Dissociation of fluorescein labeled myo(110−121) peptide bound to the class II MHC protein I-E^d isolated after 4 h (inset) and 14 h of incubation at 37 °C. Fluorescence of the peptide−MHC complexes was normalized to the fluorescence of the initially isolated complexes. A single exponential function fit to the data for 4 h (inset) gave the rate constant $k_{off} = 6.4 \times 10^{-4}$ s^{-1}. A double exponential function fit to the data for 14 h gave the rate constants $k_{off} = 8.7 \times 10^{-4}$ s^{-1} (82%) and 2.3×10^{-5} s^{-1} (18%).

We reasoned that a peptide might initially engage part of the peptide binding site of an MHC protein and then "zip"[9] into the partly twisted conformation observed in a crystal structure.[3] Steric congestion between peptide and protein side-chains might

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temporarily halt this process and generate an intermediate. To test this concept further, we have strategically modified one residue in an antigenic peptide and have thereby brought about biphasic dissociation kinetics. It is proposed that the multiplicity of structures of the peptide−MHC complex is related to steric properties of the peptide. Our results represent the first case in which the kinetics of the reactions of MHC proteins with peptides have been altered by a rational modification of peptide structure.

Experimental Section

Kinetics. The murine class II MHC protein I-A^d was isolated by affinity chromatography from the A20 murine B cell lymphoma cell line. Cells were grown in RPMI with 3% fetal calf serum to a density of 2×10^6 cells/mL. The cells were lysed in 0.75% NP-40 detergent in tris buffered saline (TBS) pH 8.3 and the lysate (1 mL/10^8 cells) was passed over a lentil lectin column. The lentil lectin column was eluted onto an affinity column (10 mg MK.D6 on 10 mL Sepharose CL-4B) with 10% α-methyl mannoside in TBS pH 8.3. The affinity column was washed with 1 mM dodecyl maltoside in TBS pH 8.3 and eluted with 100 mM Na_2CO_3 in 1 mM dodecyl maltoside/0.5 M NaCl pH 11.5. Fractions (2 mL) eluted from the affinity column were immediately neutralized with 200 μL 1 M tris pH 6.8. Fractions containing protein were dialyzed into 1 mM dodecyl maltoside in phosphate buffered saline (PBS) pH 7.0. Purity of the protein was assayed with SDS-PAGE and protein concentration was determined with a Micro BCA assay (Pierce, IL).

The peptides Ova(325−336) with either the L- and D-isomer of His-328 (sequence: QAVHAAHAEINE) were synthesized with standard fmoc chemistry on an Applied Biosystems 431A peptide synthesizer and cleaved in a solution of ethanedithiol (5%) and thioanisole (5%) in trifluoroacetic acid. Peptides were labeled at the N-terminus with the N-hydroxysuccinimidyl ester of 5(6)-carboxyfluorescein. The D-His-328 peptide was labeled in 0.2 M $NaHCO_3$ pH 8.3 after cleavage and the native Ova(325−336) peptide was labeled on the resin in a solution of diethylisopropylamine (4 equiv) in dimethyl sulfoxide. After reverse phase HPLC purification (Vydac C18, CH_3CN/water gradient), the identities of the peptides were confirmed with mass spectrometry.

Solutions of protein (0.2 μM) and labeled peptide (10−100 μM) in 1 mM dodecyl maltoside in PBS/100 mM sodium citrate pH 5.3 were incubated at 37 °C. Peptide-MHC complexes were isolated from excess peptide at 4 °C by size exclusion (Sephadex G50-SF) and then incubated at 37 °C to initiate dissociation. Isolations of the peptide-MHC complexes and dissociation reactions were also carried out in 1 mM dodecyl maltoside in PBS/100 mM sodium citrate. Periodically, during the dissociation reactions, the amount of fluorescein labeled peptide bound to the protein was determined by high performance size exclusion chromatography (Toso Haas TSK3000SW, 7.5 mm × 60 cm) with a fluorescence detector. Addition of excess unlabeled peptide does not affect the dissociation kinetics.

Molecular Modeling. The structure of the Ova(325−336) peptide bound to I-A^d was modeled using self-consistent ensemble optimization (SCEO).[10] Backbone coordinates of the crystal structure of hemagglutinin peptide complexed with HLA-DR1[3] were superimposed upon our existing model of I-A^d,[11] by least-squares fit of the peptide-binding domain backbones of HLA-DR1 and I-A^d. The side-chains of Ova(325−336) were modeled on the HA peptide backbone by SCEO, using the program CARA (Molecular Applications Group, CA), with Val-327 placed in the P1 position. This placement was chosen based on available TCR and MHC binding data for mutant Ova peptides,[12] and graphical examination of possible complexes. Modeling of the D-His-328 peptide−MHC complex was performed by using the CARA program option to reverse α-carbon chirality.

Kinetic Simulations. Simulations of the possible mechanisms for the reactions of peptides with MHC proteins have been discussed extensively.[4a] The reactions of the D-His-328 Ova peptide with I-A^d were simulated with a mechanism that includes a kinetic intermediate:

$$mP_e \overset{k_e}{\rightleftharpoons} m + P_e \quad (1)$$

$$m + P^* \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \{mP^*\}_i \quad (2)$$

$$\{mP^*\}_i \overset{k_f}{\underset{k_r}{\rightleftharpoons}} mP^* \quad (3)$$

$$m \xrightarrow{k_i} \underline{m} \quad (4)$$

The protein m used in these experiments is occupied with a heterogeneous population of endogenous peptides P_e which must dissociate prior to binding of labeled peptide (eq 1).[13] A double exponential function was used to describe the dissociation of the heterogeneous population of endogenous peptides. After dissociation of these self-peptides, the protein either binds labeled peptide P* (eq 2) or it is inactivated (eq 4).[4] Formation of the short-lived complex $\{mP^*\}_i$ precedes formation of the long-lived complex mP* (eq 3). Differential equations describing eqs 1−4 were solved numerically with Mathematica V 2.2 (Wolfram Research, IL) using the default step sizes, precision, and accuracy goals. To generate the curve fit illustrated in Figure 4, the relative amount of long-lived complex was increased by the amount formed from the conversion of the short-lived complex during the initial phase of dissociation.

Results

Peptides bound to the MHC proteins are in an extended conformation within a cleft formed by two α-helices on top of a β-sheet platform. Some of the peptide side-chains make contacts within the cleft while others point away from the cleft.[3] Some of the side-chains directed out of the cleft make contact with the T cell receptor. We chose to modify one of these T cell contact residues to avoid a negative interaction with the protein. The peptide corresponding to residues 323−339 of the chicken ovalbumin, Ova(323−339), is an immunogenic peptide restricted to the murine class II MHC protein I-A^d.[14] The shorter Ova(325−336) peptide also binds I−A^d and triggers T cells specific to the longer peptide.[12] The reactions of Ova(325−336) with I-A^d have been well characterized[12] *and show no evidence for a detectable kinetic intermediate*.[15]

Mutations of the Ova(325−336) peptide have identified residues that are putatively either MHC binding contacts or T cell receptor recognition elements.[12] A computer model (Figure 2) of Ova(325−336) was generated from an alignment of the Ova peptide backbone to that of an influenza hemagglutinin peptide in the crystal structure of a human class II MHC protein.[3] The peptide register was based on the relative disposition of MHC and T cell contacts as determined from the reactions of peptide mutants.[12] We chose to mutate the Ova(325−336) peptide with an inversion of chirality at the α-carbon of the His-328 residue. This mutation was not expected to significantly affect the stability of the fully bound peptide since the His-328 residue is a putative T cell receptor contact. In the computer

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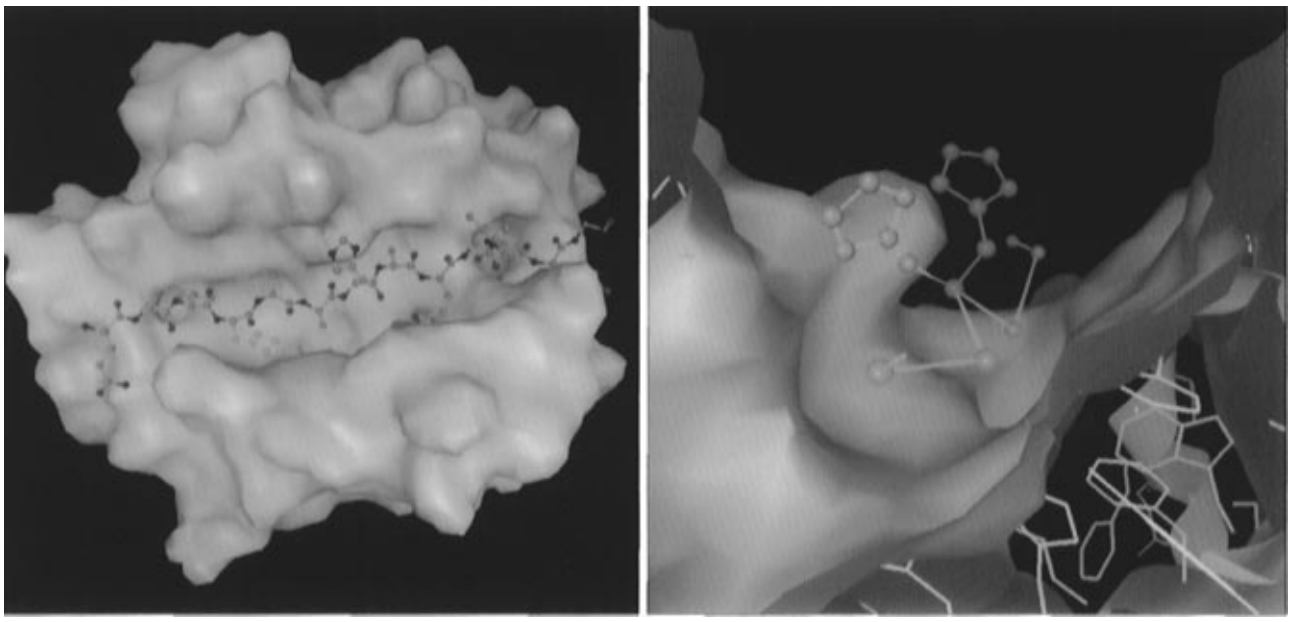

Figure 2. A computer model of the Ova(325−336) peptide bound to the class II MHC protein I-A^d. The peptide is illustrated with a ball and stick representation and the protein is illustrated with a surface representation. The peptide lies in an extended conformation within the binding cleft (left). The N-terminus of the peptide is to the left and the His-328 side-chain is highlighted in yellow. The enlarged representation (right) illustrates the relative side-chain disposition for the L-isomer (yellow) and D-isomer (green) of the His-328 residue. This perspective is a view from the C-terminus of the peptide along the peptide backbone.

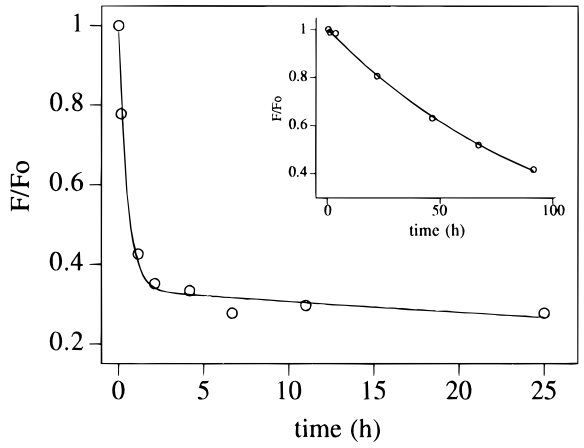

Figure 3. Dissociation of fluorescein labeled D-His-328 Ova(325−336) peptide bound to the class II MHC protein I-A^d isolated after 12 h of incubation at 37 °C and (inset) dissociation of the native Ova-(325−336) peptide bound to I-A^d isolated after 29 h of incubation at 37 °C. Fluorescence of the protein−peptide complexes was normalized to the fluorescence of the initially isolated complexes. A double exponential function fit to the data for the D-His peptide gave the rate constants $k_{off} = 5.3 \times 10^{-4}$ s^{-1} (65%) and 2.6×10^{-6} s^{-1} (35%). A single exponential function fit to the data for the native peptide (inset) gave the rate constant 2.5×10^{-6} s^{-1}.

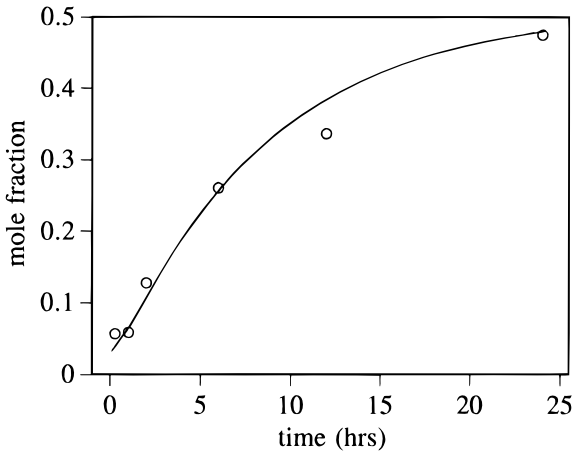

Figure 4. The mole fraction of long-lived complex formed during the incubation of D-His-328 Ova(325−336) with the class II MHC protein I-A^d at 37 °C. Mole fractions were obtained from the relative amplitudes of a double exponential function fit to the dissociation data for complexes isolated after different incubation times. The solid line is a simulation in which formation of the short-lived complex precedes formation of the long-lived complex. The simulation also includes conversion of the short- to long-lived complex during the dissociation experiments. The rate constants used in the simulation are the following: $k_e = 5.6 \times 10^{-4}$ s^{-1} (35%), 6.9×10^{-6} s^{-1} (65%), $k_{on} = 100$ M^{-1} s^{-1}, $k_{off} = 5.6 \times 10^{-4}$ s^{-1}, $k_f = 1.9 \times 10^{-5}$ s^{-1}, $k_r = 1.7 \times 10^{-5}$ s^{-1}, $k_i = 1.4 \times 10^{-5}$ s^{-1}.

model, the His-328 side-chain is directed out of the cleft flanking one of the two helices (Figure 2). Inversion of the α-carbon at this position moves the side-chain away from the helix and centers it in the cleft pointing away from the protein surface.

The dissociation of the native Ova(325−336) peptide from the MHC protein is monophasic with a rate constant of $k_{off} = 2.5 \times 10^{-6}$ s^{-1} (Figure 3, inset). In contrast, the dissociation of the mutated D-His-328 peptide is biphasic (Figure 3). The D-His-328 peptide forms both short- and long-lived complexes that dissociate with rates of $k_{off} = 5.3 \times 10^{-4}$ and 2.6×10^{-6} s^{-1}, respectively. The relative amount of long-lived complex increases during the incubation of peptide with protein (Figure 4). The absence of an induction phase in the kinetics of formation of the long-lived complex indicates either the absence of an observable kinetic intermediate or that, during the peptide dissociation reaction, some of the short-lived complex converts to the long-lived complex. The solid line in Figure 4 is a simulation in which there is a conversion of the short-lived complex during the dissociation experiment.[16] These results may be contrasted with those involving the myoglobin peptide

(16) Simulation of a mechanism in which both the short- and long-lived complexes are formed independently from binding of P* to the protein produces a curve similar to that illustrated in Figure 4. The apparent plateau at about 0.5 mol fraction in Figure 4 represents a quasi steady state determined by competition between the peptide binding, peptide dissociation, and protein inactivation reactions.[4a]

(Figure 1). Conversion of the myoglobin peptide intermediate to the long-lived complex is slow and, thus, an induction phase is observed in the kinetics of formation of the long-lived complex.[7]

Discussion

Many of the complexes of antigenic peptides with MHC proteins are extraordinarily stable, with lifetimes on the order of 10−100 h.[2] In addition, the dissociation rates are often insensitive to peptide sequence.[5] The work described here was prompted by a desire to understand how the recently reported[4b,6−8] unstable peptide−MHC complexes might differ from the long-lived complexes. The generation of an unstable complex via a single mutation in the sequence of the Ova peptide confirms the role of peptide structure in determining the kinetic stability of these complexes. It is notable that unstable complexes have not been previously reported for the MHC protein I-A^d. Evidently unstable complexes are not restricted to specific MHC alleles.

Residue 328 of the Ova(325−336) peptide was chosen for a mutation because the side-chain was predicted to contribute little to the stability of the peptide−MHC complex (Figure 2). The insertion of a D-amino acid into the sequence of a peptide composed of L-amino acids biases the peptide conformation toward a bent structure.[17] Thus, His-328 in the Ova peptide was replaced with the D-isomer to determine whether peptide conformational bias might affect the reaction kinetics without adversely affecting the stability of the bound complex. We have demonstrated that this mutated peptide forms a complex that dissociates with a rate 200-fold faster than the native peptide (Figures 3). It is suggested that, in this complex, steric interactions between the D-His-328 side-chain and other peptide/protein side-chains retards the complete formation of binding contacts at the N-terminus of the peptide.

The similar dissociation rate of the long-lived D-His Ova peptide complex relative to the native peptide confirms that the His-328 side-chain contributes little to the stability of the fully bound state. Due to the absence of an induction phase in the formation of the long-lived complex (Figure 4), we were unable to determine if it is formed directly from the short-lived complex. Irrespective of whether the short-lived isomeric state reported here corresponds to a kinetic intermediate, its lifetime is sufficiently long to potentially have biological activity. Thus, in addition to the relevance to kinetic reaction mechanisms and the problems of protein folding,[18] isomeric peptide−MHC complex structures are of potential interest in connection with immune surveillance and peptide antagonism of T cell response.

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