

Conformational Isomers of a Class II MHC-Peptide Complex in Solution

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A number of kinetic measurements of peptide dissociation from class II MHC-peptide complexes provide compelling evidence for the existence of conformational isomers in solution. There is evidence that T-lymphocytes can distinguish such isomers. However, virtually nothing is known about the structure of these isomers. Accordingly, we have investigated a water-soluble version of the murine class II MHC molecule I-E^k complexed with an antigenic peptide derived from pigeon cytochrome *c* residues 89-104 (PCC) by ¹⁹F-NMR. Two fluorine labels were placed on the PCC peptide; one fluorine label was placed at a MHC contact site, the other at a position involved in T-cell receptor (TCR) recognition. Introduction of these labels did not alter the observed kinetics of the PCC/I-E^k complex. The NMR data show two conformational isomers of this immunogenic complex. The presence of conformational isomers at a TCR contact site suggests that these structures may be recognized differently by the TCR. The agreement between the dissociation kinetics and the ¹⁹F-NMR data demonstrate that kinetic heterogeneity is correlated with structural counterparts observed by NMR. Dissociations in the presence of dimethyl sulfoxide were used to show that the rate of interconversion of these conformational isomers at pH 7.0 is low, with a lifetime on the order of hours or more. Modification of a peptide residue of PCC occupying the minor MHC binding pocket P6 alters the ¹⁹F-NMR spectra of both labels. This demonstrates that distant changes of amino acid residues can influence the conformation of the whole antigenic peptide inside the MHC binding cleft.

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Introduction

Class II major histocompatibility (MHC) molecules are $\alpha\beta$ heterodimeric transmembrane glycoproteins located on the surface of antigen presenting cells (APCs). Complexes between antigenic peptides and these proteins are normally

formed inside the cell during antigen processing, but they can also be formed on the cell surface by a direct peptide binding reaction. It is commonly assumed that a complex between a class II MHC protein and a given antigenic peptide adopts a single molecular structure, and that it is this structure that engages the T-cell receptor (TCR) of a CD4⁺ T-helper cell to generate an immune response (reviewed by Hedrick & Edelmann, 1993). However, there is extensive evidence from peptide dissociation kinetics for the existence of isomers of these peptide-protein complexes (Beeson *et al.*, 1996; Beeson & McConnell, 1994; Mason *et al.*, 1995; Mason & McConnell, 1994; Sadegh-Naseri *et al.*, 1994; Sadegh-Naseri & McConnell, 1989), and there is also evidence for distinct functional activities of such isomers with respect to T-cell triggering (Rabinowitz *et al.*, 1997).

Abbreviations used: APC, antigen presenting cell; A-¹⁹F, mono- β -fluoro-L-alanine; DMSO, dimethyl sulfoxide; ¹⁹FpF, *p*-fluoro-L-phenylalanine; Hb, hemoglobin; MHC, major histocompatibility complex; PBS, phosphate buffered saline; PCC, pigeon cytochrome *c* residues 89-104; TCR, T cell receptor. Single letter codes for the amino acids used: A, Ala; D, Asp; E, Glu; F, Phe; I, Ile; L, Leu; K, Lys; Q, Gln; R, Arg; T, Thr; Y, Tyr.

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Distinct T-cell responses in mice immunized with protein as compared with peptide (Viner *et al.*, 1996) may also involve conformational isomers. These biological and kinetic data, as well as recent work on the association (Rabinowitz *et al.*, 1998) and dissociation kinetics (Schmitt *et al.*, 1998) of water-soluble I-E^k, show that some class II MHC-peptide complexes are composed of conformational isomers. However, structural information so far is only available for long-lived class II MHC-peptide complexes (see, for example Brown *et al.*, 1993; Fremont *et al.*, 1996; Gosh *et al.*, 1995).

To gain insight into the structure of conformational isomers in solution, we have undertaken a ¹⁹F-NMR study in combination with side-chain-specific labeling of a class II MHC-peptide complex. In contrast to other NMR-active nuclei such as ¹³C or ¹⁵N, ¹⁹F has the advantages of 100% natural abundance, high sensitivity (83.3% sensitivity of ¹H), and absence of interfering background signals (Sykes & Hull, 1978). The van der Waals radius of ¹⁹F differs from ¹H by only 10% (Gerig, 1994). These advantages have been used previously to examine a variety of biological systems (see, for example Hardin & Horowitz, 1987; Hull & Sykes, 1975; Kimber *et al.*, 1978; Klimasauskas *et al.*, 1998; Metzler & Lu, 1989; Peerson *et al.*, 1990). In the case of a class II MHC-peptide complex, the absence of background ¹⁹F-signals and the high NMR sensitivity of the label are critical for detecting conformational isomers.

As a model system we chose a water-soluble version of the murine class II MHC molecule I-E^k (Wettstein *et al.*, 1991) complexed with an antigenic peptide derived from pigeon cytochrome *c* (PCC) residues 89-104 (see Materials and Methods). This class II MHC-peptide complex was the first one for which kinetic isomers were described (Sadegh-Nasseri & McConnell, 1989). Fluorine labels were introduced at position 97 (substitution of Y97 with *p*-fluoro-L-phenylalanine, ¹⁹FpF) and position 103 (substitution of A103 with mono-β-fluoro-L-alanine, A-¹⁹F; ¹⁹F-PCC 97,103). As has been shown previously, both of these positions are located within the MHC-binding epitope (Reay *et al.*, 1994). Based on these data, position 103 corresponds to a MHC contact residue, whereas position 97 is involved in TCR recognition.

Results

To confirm that the introduction of fluorine labels does not interfere with the dissociation kinetics of this class II MHC-peptide complex, the dissociation experiments shown in Figure 1 were performed using an N-terminally fluorescein-labeled version of each peptide (see Materials and Methods). For the wild-type complex (PCC/I-E^k), only a slow-dissociating complex was detected at pH 7.0 (Figure 1(a), filled circles). On the other hand, fast as well as slow-dissociating complexes were observed at pH 5.3 (Figure 1(b), filled circles).

The double-fluorinated complex ¹⁹F-PCC 97,103/I-E^k also shows a pH-dependent shift in the dissociation behavior (Figure 1, open squares). The observed rate constants of both MHC-peptide complexes are the same to within a factor of 1.5 (see Figure 1). The fraction of the fast-dissociating complexes was the same in both systems (14%). The shift in the dissociation kinetics from mono- to biphasic kinetics serves as an indicator for kinetic heterogeneity and is observed in the wild-type as well as the fluorinated system. This demonstrates that the introduction of fluorine labels does not change the structural basis for the kinetic heterogeneity.

The ¹⁹F-NMR spectra of the ¹⁹FpF97 label of ¹⁹F-PCC 97,103/I-E^k are shown in Figure 2. Upon binding, the signal undergoes a downfield shift of roughly 2.4 ppm (data not shown). As can be seen in Figure 2(a), the bound peptide displays two overlapping signals at pH 7.0 (labeled isomer 1 and isomer 2) at -38.09(±0.01) ppm (isomer 1) and -38.42(±0.01) ppm (isomer 2), indicating two different chemical environments of the fluorine label. Due to the overlapping of both signals, the ratio of both isomers could only be estimated on the basis of the signal heights (1.2:1). Because of the low complex concentration used (0.18 mM), the signal-to-noise is low. Nevertheless, spectra acquired at different positions of the carrier frequency or different digital resolution always showed two signals. Upon lowering the pH to 5.3, only one broad signal for ¹⁹FpF97 of the bound peptide (labeled isomer 1 + 2) at -38.13(±0.01) ppm with a linewidth of 294(±20) Hz is observed (Figure 2(b)). To measure the time-dependent dissociation kinetics, the complex was incubated for the indicated times at 25°C. As can be seen in Figure 2(b), a new signal (labeled free peptide) is formed over time. The chemical shift at -40.50(±0.01) ppm corresponds with the position of free peptide acquired under identical conditions in the absence of protein (spectra not shown).

Previous experimental data indicated no pH-dependent structural change of I-E^k at the peptide binding groove (Boniface *et al.*, 1996; Driscoll *et al.*, 1993; Runnels *et al.*, 1996). The observed pH-induced change of ¹⁹FpF97 bound to I-E^k as well as the conformational heterogeneity could be due to a chemical heterogeneity of the protein sample. To rule out this possibility, the complex remaining after essentially complete dissociation of the short-lived complex at pH 5.3 was isolated and the pH was raised to 7.0 (Figure 2(c)). Here and in other spectra (data not shown), two signals for ¹⁹FpF97 were observed again. The ratio of the signal heights and chemical shift of all spectra acquired were nearly identical with the spectra shown in Figure 2(a) (ratio of signal height ±10% and chemical shift ±0.01 ppm). Thus, the pH-dependent structural change of the protein-peptide complex is reversible. This also indicates that a change of pH and dissociation of antigenic peptide does

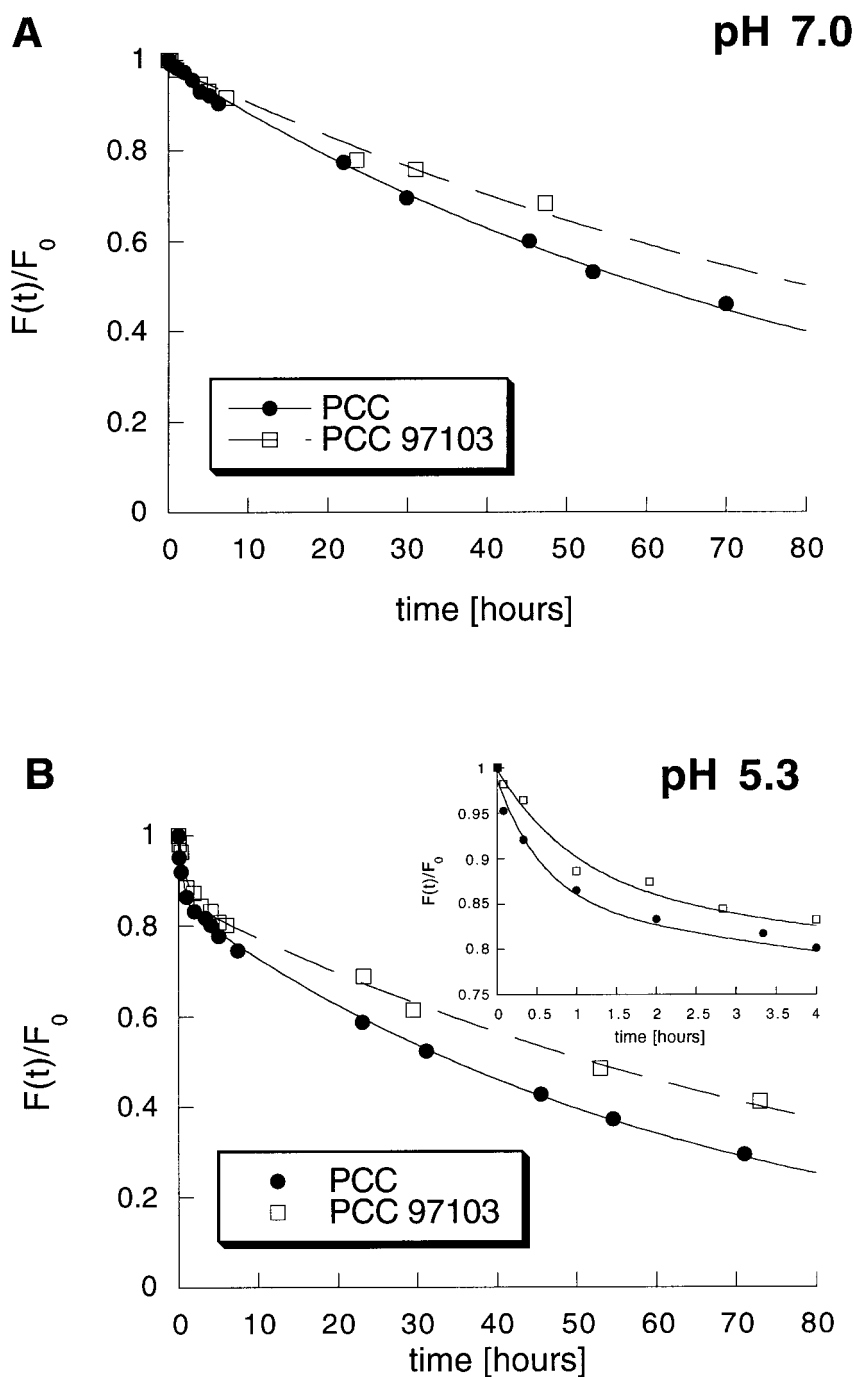


Figure 1. Comparison of the dissociation behavior of PCC/I-E^k (—●—) complex at pH 7.0 (a) and pH 5.3 (b) with the double-fluorinated mutant ¹⁹F-PCC 97,103/I-E^k complex (—□—) at pH 7.0 (a) and pH 5.3 (b) using N-terminal fluorescein-labeled versions of each peptide. Both complexes were prepared as described in Materials and Methods. While the dissociation at pH 7.0 (a) was strictly monophasic for both MHC-peptide complexes, biphasic behavior was found for both complex at pH 5.3 (b). The inset in (b) shows the first four hours of dissociation at pH 5.3, highlighting the observed biphasic decay. Half-times of dissociation ($t_{1/2}$) for each system were determined to be: PCC/I-E^k at pH 7.0 (a), $t_{1/2} = 51(\pm 1)$ hours and at pH 5.3 (b), $t_{1/2, \text{fast}} = 25(\pm 5)$ minutes (magnitude of fast-dissociating complex: $14.04(\pm 1.2)\%$ $t_{1/2, \text{slow}} = 46(\pm 1)$ hours). ¹⁹F-PCC 97,103/I-E^k at pH 7.0 (a), $t_{1/2} = 71(\pm 4)$ hours and at pH 5.3 (b), $t_{1/2, \text{fast}} = 42(\pm 10)$ minutes (magnitude of fast-dissociating complex: $14.07(\pm 1.3)\%$ and $t_{1/2, \text{slow}} = 66(\pm 2)$ hours).

not influence the ratio and occurrence of conformational isomers. Together, these data argue that the differences in the spectra of ¹⁹FpF97 at pH 7.0 (Figure 2(a) and (c)) and pH 5.3 (Figure 2(b)) reflect

a reversible pH-dependent structural change of the protein-peptide complex.

Figure 3 shows the ¹⁹F-NMR spectra of A-¹⁹F103 of the ¹⁹F-PCC 97,103/I-E^k complex at pH 7.0

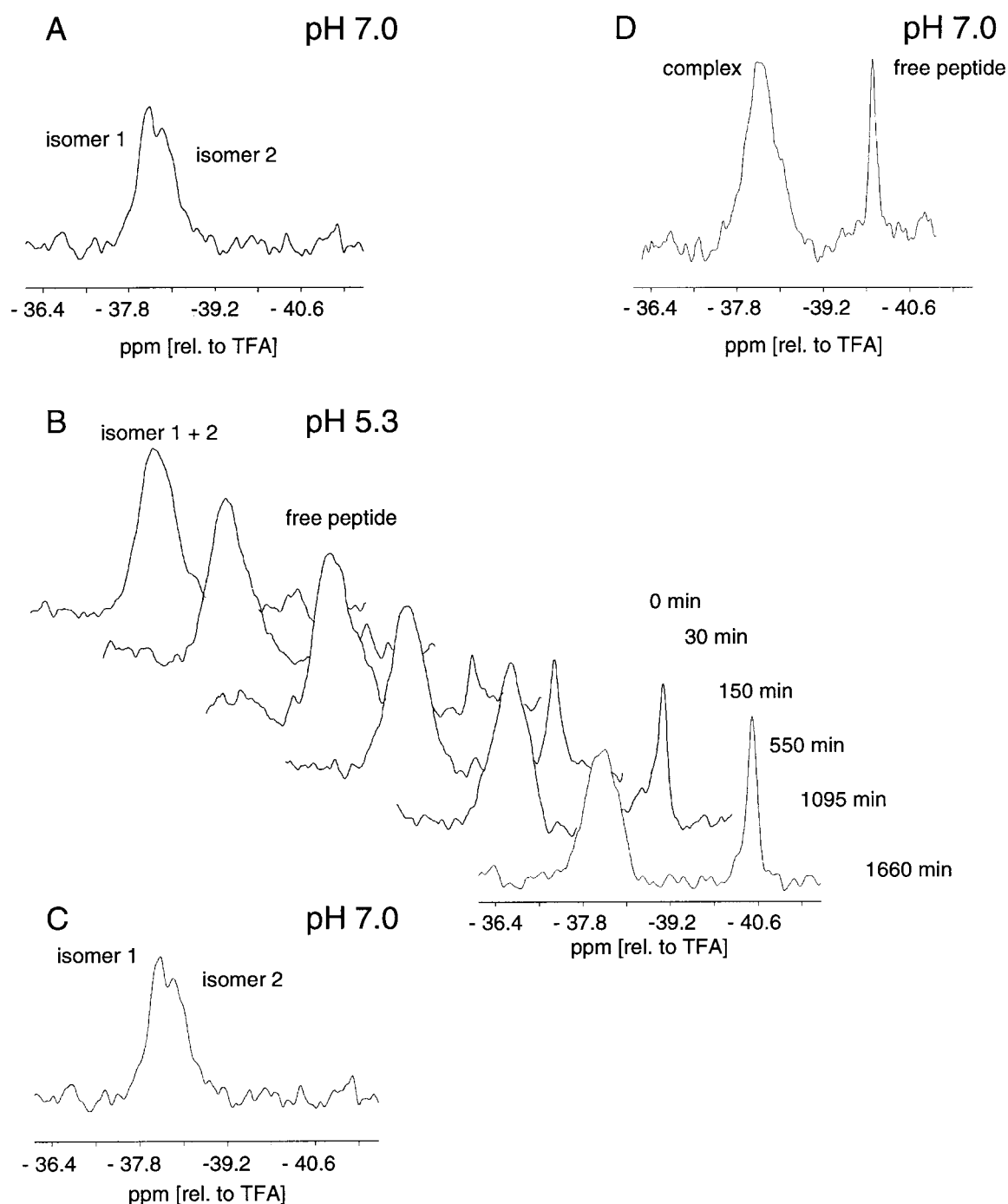
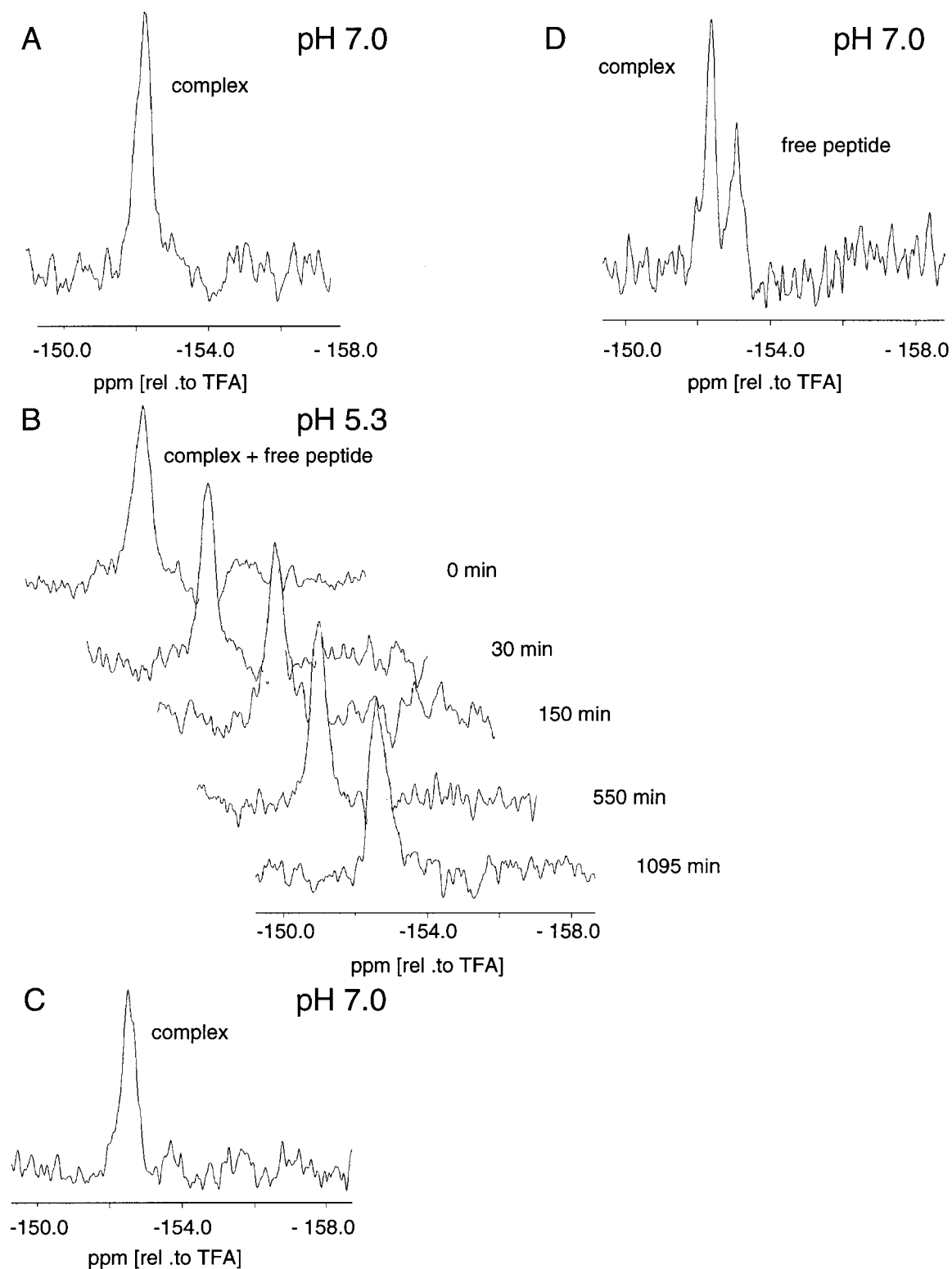


Figure 2. The ^{19}F -NMR spectra of $^{19}\text{FpF97}$ in the P3 position of ^{19}F -PCC 97,103/I-E^k (0.18 mM) in 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, 0.4 mM PCC (a) and (c), 100 mM citrate/phosphate (pH 5.3, 9:1), 150 mM NaCl, 0.4 mM PCC (b) and of 0.1 mM ^{19}F -PCC 97,103 Q100A/I-E^k in 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, 0.4 mM PCC (d) at 4 °C in 90% H_2O /10% $^2\text{H}_2\text{O}$. The pH was not corrected for $^2\text{H}_2\text{O}$. The complex was prepared and isolated as described in Materials and Methods. $^{19}\text{FpF97}$ bound to I-E^k: is labeled isomer 1, isomer 2, isomer 1 + 2, and complex, respectively, and ^{19}FpF in the uncomplexed form is labeled free peptide. (b) The MHC-peptide complex was incubated at 25 °C for the indicated times. To avoid any dissociation of peptide during data acquisition, all spectra were acquired at 4 °C. The dissociation half-time of the complex under these conditions is larger than 800 hours (data not shown). Rebinding of already dissociated ^{19}F -PCC 97,103 during the experiment was prevented by the addition of 0.4 mM PCC to the NMR buffer. All chemical shifts shown here were referred to TFA as internal standard.

(Figure 3(a) and (c)) and pH 5.3 (Figure 3(b)). In contrast to the spectra of $^{19}\text{FpF97}$, only one signal, at $-152.56(\pm 0.04)$ ppm (linewidth of $170(\pm 20)$ Hz),

was obtained at pH 7.0 (Figure 3(a) and (c)) indicating the presence of only one chemical environment of the fluorine label. Upon lowering the pH



to 5.3 and incubating the solution at 25°C for the indicated times, the spectra shown in Figure 3(b) were acquired. Because of a nearly identical chemical shift of free peptide ($-152.60(\pm 0.04)$ ppm, spectra not shown) and complex, both signals are not distinguishable.

To compare the dissociation kinetics of ^{19}F -PCC 97,103/I-E^k at pH 5.3 obtained by fluorescence (Figure 1(b), filled circles) and ^{19}F -NMR, we plotted the signal height of ^{19}F pF97 complexed with I-E^k versus time (Figure 4). Signal heights were taken from the spectra shown in Figure 2(b) and normalized to the signal height at time $t = 0$. As shown, the dissociation kinetics obtained by ^{19}F -NMR also followed a biphasic decay. The kinetic parameters obtained from the NMR data were: $t_{1/2, \text{fast}} = 16(\pm 9)$ minutes (magnitude of fast dissociating complex: $12(\pm 2.6)\%$) and $t_{1/2, \text{slow}} = 78(\pm 1.1)$ hours. These results are consistent with the dissociation experiments given in Figure 1(b) (filled circles) for fluorescein-labeled ^{19}F -PCC 97,103.

To relate our findings to available high-resolution structural data of the murine I-E^k molecule (Fremont *et al.*, 1996) we created a structural model of the PCC/I-E^k complex (Figure 5). For simplicity, the MHC molecule is shown as a surface model with the bound antigenic peptide in wire-frame representation. The fluorinated residues are highlighted as balls and sticks with fluorine in green, nitrogen in blue, and oxygen in red. In Figure 5, the ^{19}F -PCC 97,103/I-E^k is oriented so that the N terminus of the peptide is located on the bottom of the picture. In this model, which is consistent with mutational studies (Reay *et al.*, 1994), ^{19}F pF97 is predicted to lie at position P3 and A- ^{19}F 103 at position P9 of the MHC peptide binding groove. The P3 position is a shallow, solvent-exposed pocket, whereas the P9 pocket is a narrow hydrophobic channel (Fremont *et al.*, 1996). The model predicts that the methyl side-chain of A- ^{19}F 103 lies on top of the P9 channel and is solvent accessible; this might account for the lack of a change in chemical shift upon complex formation. On the other hand, the predicted orientation of ^{19}F pF located in the P3 pocket cannot explain the observed downfield shift upon complex formation. Thus, additional interactions between the peptide side-chain and MHC residues forming the P3 pocket, might alter the microenvironment of this fluorine label and create a more hydrophobic environment.

As noted by Fremont *et al.* (1996), one of the most unexpected structural features of the I-E^k complexes is the molecular architecture of the P6 pocket. Here, two acidic amino acid residues face each other and form the floor of the pocket. In the case of the PCC peptide, our model suggests that the amide side-chain of Q100 forms a hydrogen-bond network with these two amino acid residues. Despite the fact that this peptide residue is buried in the P6 pocket, it has been found that mutations at this position generate altered peptide ligands which are recognized differently by T-cells (Evavold & Allen, 1991; Evavold *et al.*, 1992).

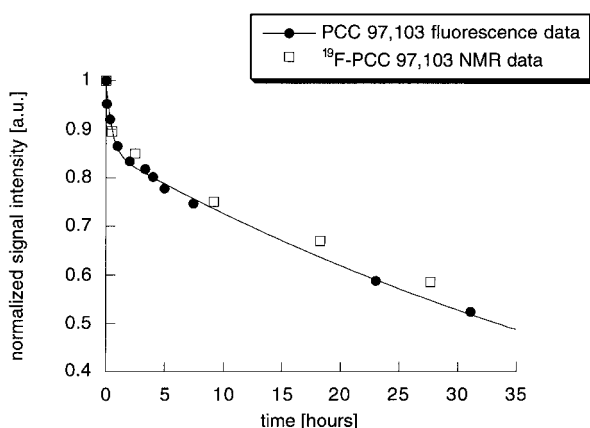


Figure 4. Comparison of the dissociation kinetics of ^{19}F -PCC 97,103/I-E^k complex using a fluorescein-labeled version of the peptide (fluorescence data, —●—) with the signal height of ^{19}F pF97 bound to I-E^k obtained by NMR (NMR data, —□—). The signal height was determined from the spectra shown in Figure 2(b) using the peak picking and optimizing routine of the software package Felix95 (MSI, San Diego, CA). Data points of the complex using fluorescein-labeled ^{19}F -PCC 97,103 were taken from Figure 1(b) and fitted to a biphasic decay function. In both cases the signal intensity was normalized to the intensity at time $t = 0$.

To examine the influence of substitutions of the P6 residue on peptide conformation, we prepared the ^{19}F -PCC 97,103 Q100A/I-E^k complex with the glutamine (Q) position 100 substituted with alanine and examined it by ^{19}F -NMR. Figures 2(d) and 3(d) show that this substitution alters the conformation of the P3 as well as the P9 position. Only one signal ($-38.39(\pm 0.01)$ ppm) was found for ^{19}F pF97 at the P3 position of ^{19}F -PCC 97,103 Q100A/I-E^k (Figure 2(d)). In the case of A- ^{19}F 103 (Figure 3(d)), two signals were detected. In comparison to the ^{19}F -PCC 97,103/I-E^k system, the A- ^{19}F 103 signal (labeled complex) is shifted by 0.15 ppm downfield. The second signal corresponds to free peptide. Due to a higher intrinsic off-rate of this complex, unbound peptide (labeled free peptide in Figures 2(d) and 3(d)) could be detected during data acquisition at 4°C. Nevertheless, the spectra in Figures 2(d) and 3(d) clearly demonstrate that a mutation in the P6 position of the antigenic peptide influences the chemical environment of the amino acid residues bound to the P3 as well as P9 pockets of I-E^k.

The presence of only one signal for ^{19}F pF97 of ^{19}F -PCC 97,103/I-E^k at pH 5.3 (Figure 2(b)) can be explained in two ways. Either a decrease in the exchange lifetime or a pH-dependence of the chemical shift of only one of the two conformations could result in a single signal for the two complexes at pH 5.3. These arguments hold also for the single signal of ^{19}F pF97 observed for ^{19}F -PCC 97,103 Q100A/I-E^k complex (Figure 2(d)). To examine the situation of ^{19}F pF97 in more detail, the change of chemical shift versus time was monitored

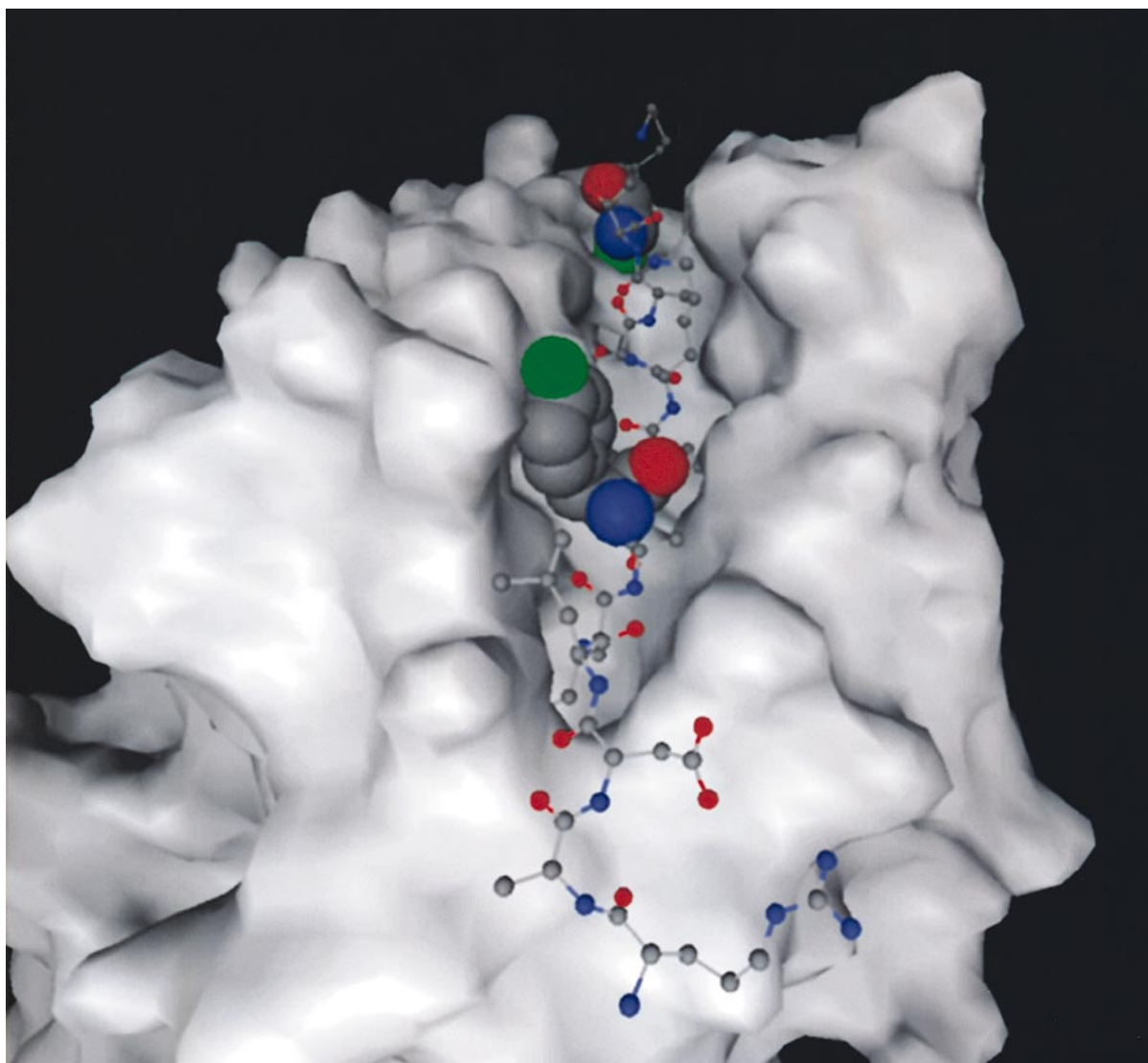


Figure 5. Structural model of the ^{19}F -PCC 97,103/I-E^k complex. The model was generated using the software package Look (Molecular Application Group, Palo Alto, CA) as described by Lee & McConnell (1995) and includes only residues 91-104 of ^{19}F -PCC 97,103. For simplification, the class II MHC molecule is shown as a surface model. The antigenic peptide (PCC residues 91-104) is shown in wire-frame representation with the amino acid residues in position 97 and 103 as balls and sticks (fluorine in green, nitrogen in blue, and oxygen in red), with the N terminus of the peptide at the bottom of the model. In the case of A- ^{19}F 103, only one proton of the methyl side-chain is substituted with fluorine, but the whole methyl group is shown in green. As shown, ^{19}F pF97 interacts with the α -chain of I-E^k and is located at the surface of the class II MHC molecule in the P3 position with the fluorine atom in a solvent-accessible position. The methyl group of A- ^{19}F 103 is located on top of the P9 binding pocket and is also solvent accessible.

(Figure 6). Whereas a time-dependent change for ^{19}F -PCC 97,103/I-E^k was observed (Figure 6, filled circles), the chemical shift for ^{19}F -PCC 97,103 Q100A/I-E^k was constant within experimental error (± 0.02 ppm; Figure 6, open squares). Therefore, only one chemical structure of ^{19}F pF97 bound to I-E^k is present in the ^{19}F -PCC 97,103 Q100A complex, whereas two isomers exist for the ^{19}F -PCC 97,103 complex at pH 5.3.

The monophasic dissociation at pH 7.0 (Figure 1(a)) is consistent with the two ^{19}F -signals of ^{19}F pF at pH 7.0 (Figure 2(a) and (c)) only if the two isomers have similar dissociation times. To distinguish the MHC-peptide complex kinetically,

we added DMSO (5-20 %) to the dissociation reaction of PCC/I-E^k using a fluorescein-labeled PCC peptide at pH 7.0 (Figure 7). DMSO is known to alter protein-ligand interactions (Huang *et al.*, 1995; Johansson *et al.*, 1997). Increasing amounts of DMSO added to the dissociation reaction resulted in the appearance of a fast-dissociating complex (Figure 7). The maximal magnitude of fast-dissociating complex (10 %) was observed at 10 % added DMSO. Higher concentrations of DMSO resulted in no further increase. This supports the idea that two isomeric complexes are indeed present at pH 7.0. In contrast, the dissociation of PCC Q100A/I-E^k using a fluorescein-labeled PCC

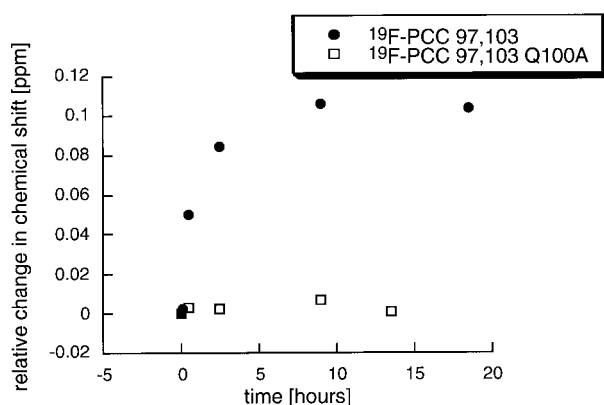


Figure 6. Relative change in chemical shift *versus* time for ^{19}F Pf97 bound to the P3 pocket of ^{19}F -PCC 97,103/I-E^k (—●—) and ^{19}F -PCC 97,103 Q100A/I-E^k (—□—) at pH 5.3. The chemical shifts for each data point were taken from Figures 3(b) and 4(b), respectively, and determined using the peak picking routine and optimizing of the software package Felix95 (MSI, San Diego, CA).

Q100A peptide in the presence of DMSO is strictly monophasic under all pH conditions (data not shown), indicating that only a single kinetic complex exists for this mutated peptide.

Discussion

The dissociation experiments shown in Figure 1 demonstrate that the introduction of fluorine labels in the binding epitope of PCC does not interfere with the kinetic heterogeneity of the complex. Like the wild-type complex, the ^{19}F -PCC 97,103/I-E^k complex follows monophasic kinetics at neutral pH but biphasic kinetics at acidic pH. Also, the magnitude of the fast-dissociating complex is the same for both systems. The kinetic experiments show that the ^{19}F -PCC 97,103/I-E^k is suited to provide

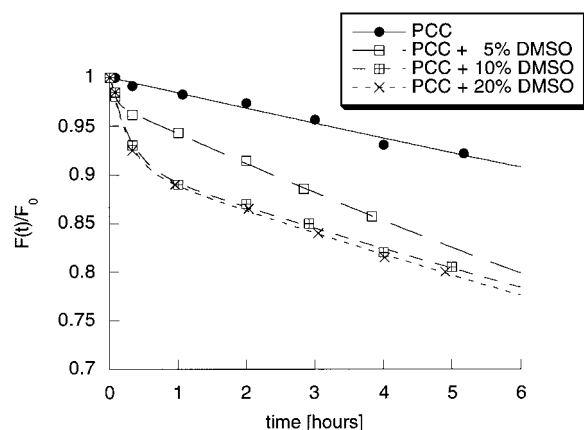


Figure 7. Influence of varying concentrations of DMSO (% v/v) on the dissociation kinetics of PCC/I-E^k at pH 7.0. Data points in the absence of DMSO are fitted to a monoexponential decay function, whereas data points obtained in the presence of DMSO are fit to a biphasic decay function.

structural information about conformational isomers of class II MHC-peptide complexes in solution without perturbing the system.

The ^{19}F -NMR spectra of the complex shown in Figures 2 and 3 reveal that conformational isomerism in the ^{19}F -PCC 97,103/I-E^k complex are located in the N-terminal part of the antigenic peptide. The observed downfield shift of ^{19}F Pf97 upon complex formation indicates a more hydrophobic environment around this label (Sykes & Hull, 1978). At pH 7.0, two signals were detected (Figure 2(a) and (c)). Because the ^{19}F Pf side-chain is symmetric with respect to rotation around the C α -C β bond, ring flipping can be ruled out as an explanation for the two signals. Therefore, ^{19}F Pf97 must exist in at least two different chemical environments, demonstrating structural diversity of the antigenic peptide in complex with I-E^k. The two distinct signals of ^{19}F Pf97 indicate that the lifetime of both isomers is much larger than seconds as discussed later. Thus, the lifetime of these conformational isomers is longer than the determined time of interaction between the TCR and class II MHC-peptide complexes (Matsui *et al.*, 1994). This implies that both isomers can be recognized as distinct structures inside the MHC peptide cleft. Lowering the pH to 5.3 resulted in only one detectable signal for ^{19}F Pf97 (Figure 2(b)). The chemical shift of ^{19}F -PCC 97,103 in the absence of protein showed no dependence on ionic strength or pH (data not shown). We conclude that the protein-peptide complex undergoes a pH-sensitive conformational change which influences the structure of the bound peptide. As shown in Figure 2(b), incubation of the complex at 25°C resulted in the appearance of a new signal which corresponds to unbound ^{19}F -PCC 97,103. A plot of the signal height of the complex signal *versus* time (Figure 4) showed a biphasic dissociation curve. The kinetic parameters agree to within experimental error with the parameter obtained for the fluorescein-labeled ^{19}F -PCC 97,103 (Figure 1(b), filled circles). Raising the pH to 7.0 (Figure 2(c)) resulted in the re-appearance of two isolated signals of ^{19}F Pf97. This demonstrates that the ratio of the two isomers is not affected by prolonged exposure of the complex to an acidic environment or dissociation of bound peptide. Consequently, the presence of conformational isomers of the ^{19}F -PCC 97,103/I-E^k complex is an intrinsic property of this protein-peptide complex rather than a protein heterogeneity or impurity.

The ^{19}F -NMR spectra for A- ^{19}F 103 are shown in Figure 3. Under all conditions examined only one signal was detectable, indicating only one conformation of this residue. No free peptide signal was detectable during the dissociation at pH 5.3 (Figure 3(b)), due to the nearly identical chemical shift of A- ^{19}F 103 in the bound and unbound form.

A molecular model of ^{19}F -PCC 97,103/I-E^k was created to compare our results with available high-resolution structural data. Based on the Hb (64-76)/I-E^k structure (Fremont *et al.*, 1996), ^{19}F Pf97 and A- ^{19}F 103 are aligned with the P3 and P9 pos-

ition of the MHC molecule, respectively. Our model predicts that the methyl side-chain of A- ^{19}F 103 (shown as a green ball in Figure 5) is located on top of the P9 channel. As a result, the fluorine label remains in a hydrophilic, solvent-accessible environment even after complex formation. This can account for the lack of change of chemical shift with complex formation. In position P3, ^{19}F pF97 underwent a downfield shift of 2.4 ppm upon complex formation (Figure 2(a)), but our model predicts that the fluorine label of ^{19}F pF97 (lower green ball in Figure 5) should be completely solvent accessible. Additional interactions not seen in our model, such as ring currents (Millett & Raftery, 1972), electrostatic, or van der Waals interactions (Gerig, 1994) with amino acid side-chains of the α -chain of I-E^k might account for the observed change in chemical shift.

As described by Fremont *et al.* (1996), the most striking feature of the Hb (64-76)/I-E^k crystal structure is the architecture of the P6 pocket. In addition, substitutions of E73 located at the P6 position with D73 resulted in a change of the T-cell response (Evavold & Allen, 1991; Evavold *et al.*, 1992). Both peptide residues are buried in the P6 pocket and are assumed not to interact with the TCR. To address this apparent inconsistency, we substituted Q100 of the ^{19}F -PCC 97,103 sequence which occupies the P6 position with A and examined the resulting complex by ^{19}F -NMR (Figures 2(d) and 3(d)). As shown in Figures 2(d) and 3(d), the substitution of the peptide residue located at the P6 position of I-E^k resulted in different chemical environments for both fluorine labels. This suggests a physical explanation for the observed changes in T-cell response of altered peptide ligands obtained by substitution of the peptide residue in the P6 position (Evavold & Allen, 1991; Evavold *et al.*, 1992). The same TCR may encounter the same MHC-peptide complex, but with subtle changes in the configuration of the peptide.

The analysis of the change of chemical shift *versus* time at pH 5.3 for ^{19}F pF97 of ^{19}F -PCC 97,103 (filled circles) and ^{19}F -PCC 97,103 Q100A (open squares) bound to I-E^k is shown in Figure 6. While a change over time was observed for ^{19}F -PCC 97,103, the chemical shift of ^{19}F -PCC 97,103 Q100A was constant over time within experimental error. The single ^{19}F -signal of ^{19}F pF97 is consistent with there being only one isomer for the ^{19}F -PCC 97,103 Q100A/I-E^k complex. On the other hand, the detected change for the ^{19}F -PCC 97,103/I-E^k complex demonstrates that at least two different isomers are present at pH 5.3. Furthermore, the time it takes for the chemical shift to reach a new plateau corresponds well with the lifetime of the fast-dissociating complex shown in Figure 1(b) (filled circles).

Based on the dissociation experiments shown in Figure 1, the ^{19}F -PCC 97,103/I-E^k complex is kinetically homogeneous at neutral pH but heterogeneous at acidic pH. On the other hand, the ^{19}F -NMR spectra of ^{19}F pF97 (Figure 2(a) and (c))

demonstrate that two conformational isomers are present at pH 7.0. To address this inconsistency, we performed the dissociation experiments shown in Figure 7 using fluorescein-labeled versions of the PCC peptide. The addition of DMSO to the dissociation reactions at pH 7.0 resulted in the appearance of a fast-dissociating complex. With increasing concentrations of DMSO, increasing amounts of fast-dissociating complex were detectable. At concentrations higher than 10% DMSO, no further increase of fast-dissociating complex was observed. Parallel with the presence of the kinetically distinguishable isomers, the overall stability of the fluorescein-labeled PCC/I-E^k decreased with increasing amounts of DMSO. This indicates that DMSO destabilizes both complexes, but to different extents. These experiments show that two isomeric complexes are indeed present at pH 7.0, consistent with the results of the ^{19}F -NMR spectra of ^{19}F pF97. Our failure to detect these isomers kinetically at pH 7.0 must be due to the near equivalence of the off-rates. The ability to resolve these isomers in the presence of DMSO shows that their rate of interconversion of these isomers must be low, on the order of hour⁻¹.

In summary, our data imply that kinetically distinguishable complexes possess structural counterparts that can be detected by ^{19}F -NMR. In all of the reported X-ray crystal structures of class II MHC-peptide complexes, a conserved pattern of interactions between protein and ligand (Brown *et al.*, 1993; Dessen *et al.*, 1997; Fremont *et al.*, 1996; Gosh *et al.*, 1995; Jardetzki *et al.*, 1994; Stern *et al.*, 1994) with a similar or even identical conformation of the complexed peptide (Jardetzky *et al.*, 1996) has been found. Here, we have demonstrated that an antigenic peptide can be bound in more than one structure within a single immunogenic complex. Most important, this conformational isomerism is localized at a TCR contact site in the N-terminal part of the peptide. This implies that more than one antigenic determinant can be presented to the TCR. Furthermore, a mutation of an amino acid bound to a minor MHC binding pocket changed the structure of the whole peptide, providing a structural clue for at least one type of altered peptide ligand (Evavold & Allen, 1991; Evavold *et al.*, 1992). The possibility that the ratio of these structures can vary due to different antigen processing pathways suggests a role of conformational isomers of class II MHC-peptide complexes accounting for differences between peptide *versus* protein immunization (Viner *et al.*, 1996).

Materials and Methods

Isolation of water-soluble I-E^k and complex preparation

Water-soluble I-E^k was purified and the class II MHC-peptide complexes were prepared and isolated as described (Boniface *et al.*, 1993). For complex preparation, 0.1 mg/ml water-soluble I-E^k was incubated

with a 30-fold molar excess of the corresponding peptide (PCC or ^{19}F -PCC 97,103) at 37°C for three days in 100 mM citrate/phosphate buffer (pH 5.3, 9:1; C/P), 150 mM NaCl, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ leupeptin. The association reaction was then concentrated by Centricon 30 ultrafiltration at 4°C. The complex was purified by size-exclusion chromatography on a Sephacryl S300 column (Pharmacia, Piscataway, NJ) equilibrated with PBS (pH 7.4) and fractionated at a flow rate of 0.5 ml per minute (dissociation experiments) or 2 ml per minute (NMR experiments) at 4°C. Fractions corresponding to the class II MHC-peptide heterodimer were pooled and stored at 4°C.

Peptide synthesis

Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer (Applied Biosystems, Forster City, CA) using standard Fmoc chemistry. Peptides were purified by reverse-phase HPLC chromatography using an optimized 0.1% TFA-acetonitrile/0.1% TFA gradient. Purity was generally larger than 98% and identity was confirmed by high-resolution mass spectroscopy. For the dissociation experiments shown in Figures 1 and 7, peptides were labeled N-terminally with NHS-5,6-carboxy-fluorescein (Molecular Probes, Eugene, OR) using standard protocols. Sequences of the peptides used in this study were as follows: PCC (89-104), AERA-DLIAYLKQATAK; ^{19}F -PCC 97,103 (89-104); AERA-DLIA(^{19}FpF)LKQAT(A- ^{19}F)K; and ^{19}F -PCC 97,103 Q100A (89-104); AERADLIA(^{19}FpF)LKAAT(A- ^{19}F)K, where ^{19}FpF denotes *p*-fluoro-L-phenylalanine and A- ^{19}F denotes mono- β -fluoro-L-alanine. ^{19}FpF was purchased in the Fmoc protected form from NovaBiochem (Nova-Biochem, San Diego, CA) and A- ^{19}F as the racemic mixture from Bachem (Torrance, CA). Enzymatic resolution of the racemate was performed by enzymatic hydrolysis of the acetylated amino acid by acylase I (Sigma, St. Louis, MO; Fodor *et al.*, 1949). The Fmoc group was introduced as described (Carping & Han, 1972).

Dissociation experiments

For the dissociation experiments, PCC/I-E^k, PCC Q100A/I-E^k, or ^{19}F -PCC 97,103/I-E^k complexes using fluorescein-labeled peptides were diluted into the appropriate buffer in the presence of a 20-fold molar excess of unlabeled PCC (89-104), and the dissociation started by incubating the sample at 25°C. Aliquots were taken at the indicated times and complex and peptide signal separated by size-exclusion HPLC. The fluorescence signal of the fluorescein-labeled peptide bound to the MHC molecule was normalized to the signal intensity at time $t = 0$ to compare each dissociation experiment quantitatively. Data points were fitted to either a monoexponential decay (pH 7.0) or a biexponential decay function (pH 5.3) using the software package KaleidaGraph (Synergy Software). Least-squares fits were performed using the Levin-Marquardt algorithm. The *R*-value (linear correlation coefficient) was larger than 0.996. Errors given in the text were taken from the standard error of each single value reported in the curve fit. For the dissociation experiments in the presence of DMSO (Figure 7), the indicated amounts of DMSO (% v/v) were added to the dissociation buffer.

NMR experiments

The ^{19}F -NMR spectra were collected on a GE omega 500 equipped with a $^1\text{H}/^{19}\text{F}$ dual probe at 470.5 MHz fluorine frequency with quadrature detection. Spectra were acquired at 4(±0.2)°C using a 90° pulse (37 μs) and averaging over 8000 transients with a delay time of three seconds (> five times t_1). Due to a possible negative nuclear Overhauser effect, no proton-decoupling was applied. Inverse gated decoupling was not employed in order to keep the acquisition time as short as possible. Digital resolution was 3.75 Hz for the spectra displaying ^{19}FpF and 7.5 Hz for A- ^{19}F . Prior to Fourier transformation, the first four data points were predicted using linear prediction (backward mode), zero-filled to 32,000 data points and an exponential broadening function of 30 Hz was applied using the software package Felix95 (MSI, San Diego, CA). Due to residual TFA remaining from the peptide purification, all spectra could be referred to TFA as an internal standard. For the experiments shown in Figures 2(c) and 3(c), remaining MHC-peptide complex was diluted into 10 mM phosphate (pH 7.0), 150 mM NaCl, 0.4 mM PCC 90% $\text{H}_2\text{O}/10\%$ $^2\text{H}_2\text{O}$ and washed four times with this buffer at 4°C by Centricon 30 ultrafiltration. Finally, the complex was concentrated back to the desired NMR concentration and used in the experiments shown in Figures 2(c) and 3(c). Errors in chemical shift and linewidth were determined in two ways. First, the signal of unbound peptide was used to obtain the standard deviation for chemical shift and linewidth. Second, a number of individual experiments were repeated. These were used to obtain the error of the signal of the complex. The largest resulting error is given throughout the text. Values for linewidth were determined using the software given in the software package Felix95. Individual signals were fit to Gaussian as well as Lorentzian lineshapes. Errors given by the fit procedure were generally smaller than 1%. Errors given in the text are based on the deviation of the linewidth of the free peptide signal or of the complex when the individual experiment was repeated. The largest resulting error is given and is not corrected for proton coupling.

Molecular modeling

The structure of the PCC/I-E^k complex including residues 91-104 of the antigenic peptide was modeled using the software package Look (Molecular Application Group, Palo Alto, CA) as described by Lee & McConnell (1995). Coordinates of the hemoglobin Hb (64-76)/I-E^k complex (Fremont *et al.*, 1996) were used as a starting point for the alignment procedure (Brookhaven Protein Data Bank entry 1IEA).

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