# The Kinetics of Peptide Reactions with Class II Major Histocompatibility Complex Membrane Proteins

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Class I and class II major histocompatibility complex (MHC) proteins are polymorphic, heterodimeric membrane proteins that serve to bind and display peptides to cytotoxic T-cells and helper T-cells,[1,2] respectively. Class I and class II MHC proteins are particularly challenging from the point of view of chemical kinetics and structural chemistry. The biological function of these membrane proteins requires that (i) each type of MHC molecule be able to bind many different peptides and that (ii) the dissociation lifetimes of these complexes are long. In addition, there is a third requirement: (iii) portions of the bound peptide surface must interact with a third protein, the T-cell receptor.[3] The challenge to chemical understanding is made even greater by the fact that the MHC molecules are highly polymorphic in an animal population, such as the human population. In general, different humans have different MHC molecules on their cell surfaces, except for identical twins. Thus, the three requirements above must be met by a large number of different proteins, and this can be considered a fourth requirement (iv). There is evidence that these MHC proteins have additional structural and functional properties.

A breakthrough in the understanding of MHC–peptide complexes has come from X-ray crystallographic determination of the structure of class I–peptide complexes.[4,5] [The structure of the MHC class I molecule HLA-A2 was used originally to model the binding site of MHC class II molecules[6a] (Figure 1, upper). The structure of the MHC class II molecule HAL-DR1 has recently been determined[6b] (Figure 1, lower).] These structures have been of great value in seeing how some of the above requirements are met. For example, not unexpectedly, requirement i is met by a protein structure in which many stabilizing interactions are between the backbone of the peptide and the protein.[7-9] A small number of peptide amino acid side chain–protein interactions give some added peptide binding selectivity and stability. These interactions take place between the peptide and residues in the $\beta$-pleated sheet "floor" of the binding site, and inward-facing residues of the two $\alpha$-helices that form

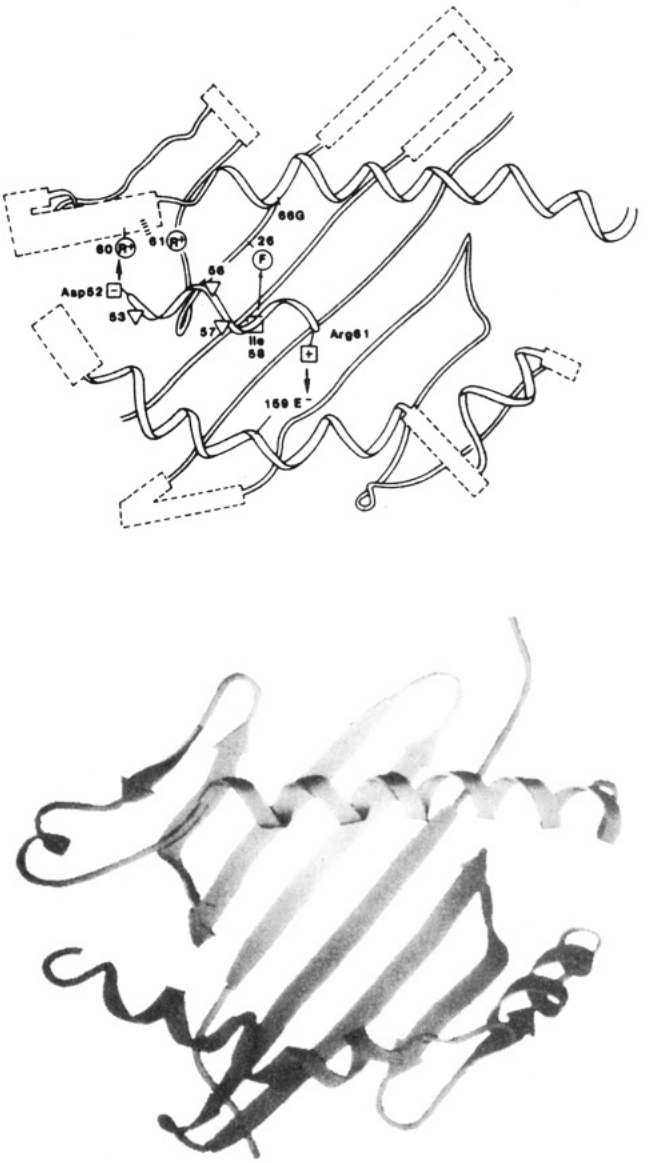


**Figure 1.** Structure of the binding site regions of MHC class II molecules. Upper: Model of the peptide binding region of the murine I-$A^k$ molecule extrapolated from a class I structure. The peptide sketched in the binding site is a fragment of hen egg lysozyme HEL (52–61). The structure is taken from Brown et al.[6a] Lower: Peptide binding region in the structure of the human MHC class II molecule, HLA-DR1, taken from Brown et al.[6b]

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the two sides of the binding site. Requirement iii is met by the peptide lying in a long binding site groove in the protein so that the T-cell receptor can interact, presumably via the variable J-diversity regions and D gene segments, with the MHC protein as well as the bound peptide. That is, the amino acid residues giving rise to the polymorphism of the MHC molecules lie within this binding site region, so as to contact the peptides. The fourth requirement (iv) above is also understandable in terms of the X-ray structures. On the other hand, at least at present the X-ray structures give almost no clue concerning the kinetics of peptide–MHC reactions.

From an immunological point of view it is clear that the lifetimes of MHC–peptide complexes on the surfaces of cells should be long. If a virus-infected cell is to be killed by a cytotoxic T-cell, the class I MHC complex with a viral peptide must persist on the surface of the infected cell until a cytotoxic T-cell specific for the class I MHC peptide complex comes into contact with the membrane of the infected cell. Similarly, in a case of an antigen-presenting cell (macrophage, dendritic cell, or B-cell) the class II MHC–peptide complex must persist on the surfaces of the cells until this complex is found by specific T-helper cells. As discussed in this Account, observed half-times for dissociation of peptides from MHC proteins are indeed long, on the order of days.

The combination of relatively nonspecific peptide binding and long half-lives virtually guarantees that a kinetic study of MHC peptide reactions will be rewarding from the point of view of physical chemistry, and possibly from the point of view of some immune functions. Of course, in a biological cell it is likely that additional proteins participate in the loading and unloading (or digestion) of MHC molecules with peptides.[10]

## Experimental Techniques

In this Account we deal almost exclusively with reactions involving peptides and class II MHC proteins, since that is the area of our experience. The reactions between class II MHC proteins and peptides have been studied using peptides labeled with either radioactive iodine or a fluorescent molecule, as well as by means of bioassays that are specific for the combination of peptide and class II MHC protein. Noncovalently associated heterodimeric ($\alpha\beta$) class II MHC molecules are membrane bound *in vivo*. *In vitro* kinetic experiments using affinity-purified class II MHC molecules require that the proteins either be in lipid bilayers,[11] or be solubilized by detergents,[12] or be modified so that the hydrophobic transmembrane domains of the $\alpha$ and $\beta$ chains are removed either genetically or enzymatically.[13,14] The molecular weights of the $\alpha$ and $\beta$ subunits are ~30K and ~28K, respectively.

Studies of the kinetics of peptide binding to class II MHC molecules are beset by an abundance of technical difficulties. For example, ≥90% of the binding sites of affinity-purified preparations of these proteins are saturated with tightly bound "self" (endogenous) peptides.[15] These peptides arise for example from proteins synthesized by the same cells as those producing the class II MHC molecules, or from proteins in the serum or cell supernatant in which the cells grow. Further, most preparations of class II MHC membrane proteins use detergent to solubilize the $\alpha\beta$ heterodimers, and the peptide-free class II MHC molecules have proved to be more or less unstable in detergent and split into separate $\alpha$ and $\beta$ chains, depending on the detergent and other experimental conditions.[16] In some cases this "instability" may be enhanced by attack on empty $\alpha\beta$ by trace concentrations of proteolytic enzymes.

Many assays of peptide binding to class II MHC proteins have used peptides radioiodinated at tyrosine residues. A problem arising from this iodination ($^{125}I$) is that the high-energy $\gamma$-emissions promote the fragmentation of both peptide and protein. Fragmentation affects the concentration and integrity of peptide and protein during a lengthy experiment. By contrast, the use of fluorescently labeled peptides, where the fluorescent tag is attached covalently to the N-terminus, eliminates the fragmentation problem. Bioassays have shown that many N-terminally labeled fluorescent peptides retain their specific antigenicity.

At the onset of a kinetic experiment the initial state of affinity-purified class II MHC molecules is often poorly defined: either all sites are occupied with self peptides or a small fraction of the binding sites are unoccupied. If all sites are initially occupied, then the binding of added, labeled peptide is due to either peptide replacement kinetics or peptide exchange kinetics, as discussed below.

## Association Kinetics

We preface the discussion of our kinetic experiments with simulations of two elementary kinetic models, both of which assume empty binding sites initially. These simulations will facilitate the discussion of the experimental kinetics. In each case, the effect of peptide on the initial rate of complex formation and the half-time of complex formation is examined. The half-time for peptide binding is the time required for the reaction to proceed halfway to equilibrium. This is a convenient mechanism-independent empirical parameter that is well-defined when the reaction proceeds to equilibrium.

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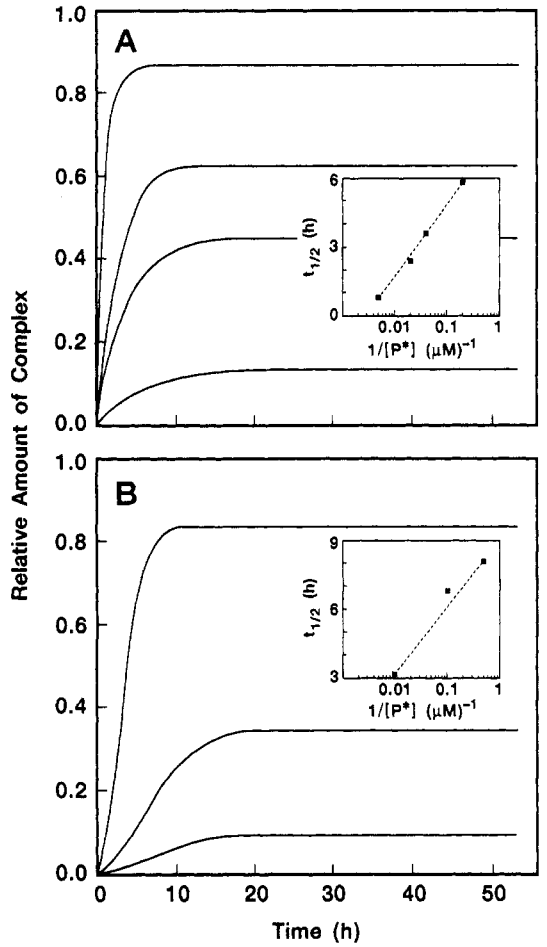


**Figure 2.** Simulation of a simple reversible reaction P* + $\alpha\beta \rightleftharpoons$ $\alpha\beta$–P* (A) and two consecutive reversible reactions P* + $\alpha\beta \rightleftharpoons$ $\{\alpha\beta\text{–P*}\}_i \rightleftharpoons \{\alpha\beta\text{–P*}\}_t$ (B). In panel A, the relative amount of fluorescent complex $[\alpha\beta\text{–P*}]/[\alpha\beta]_0$ is plotted versus time. In panel B, the relative amount of fluorescent complexes $(\{\alpha\beta\text{–P*}\}_i + \{\alpha\beta\text{–P*}\}_t)/[\alpha\beta]_0$ is plotted versus time. Conditions: (Panel A) $[\alpha\beta]_0 = 1$ μM; $[\alpha\beta\text{–P*}]_0 = 0$; [P*] = 5, 25, 50, and 200 μM; $k_{on} = 1\ M^{-1}\ s^{-1}$ and $k_{off} = 3 \times 10^{-5}\ s^{-1}$. For these reagent concentrations $t_{1/2} \approx \ln 2/(k_{on}[P^*] + k_{off})$. (Panel B) $[\alpha\beta]_0 = 1$ μM; $[\alpha\beta\text{–P*}]_i(t=0) = 0$; $\{\alpha\beta\text{–P*}\}_t(t=0) = 0$; [P*] = 2, 10, and 100 μM; $k_1 = 1\ M^{-1}\ s^{-1}$, $k_{-1} = 1.2 \times 10^{-3}\ s^{-1}$, $k_2 = 4 \times 10^{-4}\ s^{-1}$, and $k_{-2} = 6.3 \times 10^{-6}\ s^{-1}$. (Insets) Plot of $t_{1/2}$ vs 1/[P*].

All simulations involve numerical integration of the rate equations.

Consider a simple reversible reaction with a second-order step (forward reaction 1) and a first-order step (reverse reaction 1). The reactions depicted in eq 1

$$P^* + \alpha\beta \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \alpha\beta\text{–P*} \qquad (1)$$

were simulated with $k_{on} = 1\ M^{-1}\ s^{-1}$ and $k_{off} = 3 \times 10^{-5}\ s^{-1}$ (Figure 2A). Upon increasing the initial concentration of P*, while keeping the protein concentration fixed, (i) the equilibrium concentration of complex $[\alpha\beta\text{–P*}]_{eq}$ increases; (ii) the half-times for complex formation decrease; and (iii) the initial rates of complex formation increase. The plot of the calculated half-times ($t_{1/2}$), obtained from the simulated formation curves, versus 1/[P*] has a positive slope over a range of peptide concentrations (Figure 2A, inset). P* refers to a peptide that is tagged, either with radioactive iodine or with a fluorescent group.

The majority of previous investigators have assumed without justification that peptide binding to class II MHC molecules follows eq 1 and have reported second-order rate constants on the order of 1–200 $M^{-1}\ s^{-1}$. Reported dissociation constants are on the order of $10^{-6}$–$10^{-8}$ M.[12,17-20] The reported second-order rate constants were obtained either by (i) conducting kinetic binding experiments at high initial P* concentrations and then dividing the pseudo-first-order rate constant by the P* concentration or by (ii) conducting binding experiments at low P* concentrations and dividing the initial rate by the total protein and peptide concentrations. As discussed below, it is likely that many of these reported rate constants are incorrect.

There are a number of theoretical reaction mechanisms that can lead to $t_{1/2}$ variations that do not depend strongly on added peptide concentration, [P*], at least for certain ranges of assumed rate constants and peptide concentrations. An example is the two-step sequential reactions depicted in eq 2. At large peptide concentrations ([P*] ≫ [$\alpha\beta$]), the rate of complex formation $d\{\alpha\beta\text{–P*}\}_t/dt$ is governed by a two-step sequential reversible first-order reaction. The reactions in eq 2 are simulated with $k_1 = 1\ M^{-1}\ s^{-1}$, $k_{-1} = 1.2 \times 10^{-3}\ s^{-1}$, $k_2 = 4 \times 10^{-4}\ s^{-1}$, and $k_{-2} = 6.3 \times 10^{-6}\ s^{-1}$ (Figure 2B). As expected, both the equilibrium concentration of the two complexes ($\{\alpha\beta\text{–P*}\}_i$ and $\{\alpha\beta\text{–P*}\}_t$) and the initial rate of complex formation increase as the concentration of peptide is increased from 2 to 100 μM. A plot of the calculated formation half-times shows that $t_{1/2}$ increases at low peptide concentrations (Figure 2B, inset). The lag phase in the simulated formation curves at low peptide concentrations ([P*] < 10 μM) is less pronounced, or even absent, at large $k_1$.

$$P^* + \alpha\beta \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{\alpha\beta\text{–P*}\}_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \{\alpha\beta\text{–P*}\}_t \qquad (2)$$

Additional simulations of the two-step sequential kinetic model show that when the first step is very fast and the second step is slow, the observed formation half-times may not vary significantly over a given range of added peptide concentration. In those cases, the slope in the plot of $t_{1/2}$ vs 1/[P*] is slightly positive, nearly zero.

In this laboratory we observed that the half-times for peptide binding to affinity-purified mouse class II MHC proteins I-$A^d$ and I-$E^k$ in detergent are also weakly dependent on added peptide concentration, in contrast to the results expected for a simple bimolecular reaction (Figure 2A). The explanation for this result is peptide replacement:[21,22]

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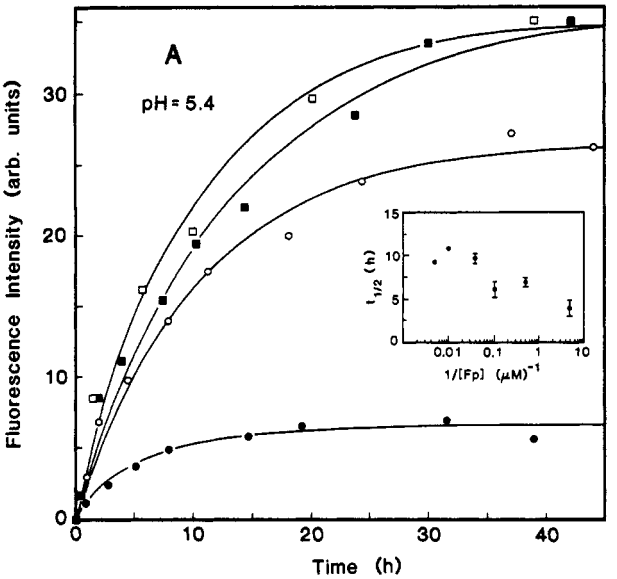


**Figure 3.** The kinetics of I-E$^k$-pCytc* complex formation in the detergent dodecyl β-D-maltoside. Panel A: Samples of purified I-E$^k$ were incubated at 37 °C with the indicated concentrations of pCytc*, and aliquots were periodically injected to determine the amount of fluorescent complex. [I-E$^k$] = 0.25 μM. [pCytc*] = (□) 200 μM; (■) 25 μM; (○) 2 μM; and (●) 0.2 μM. Data points were fit to an exponential function (solid lines). (Inset) Half-time for complex formation versus 1/[pCytc*]. Error bars represent the SEM of duplicates.

$$\alpha\beta\text{–P} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \alpha\beta + \text{P} \tag{3}$$

$$\alpha\beta + \text{P*} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \alpha\beta\text{–P*} \tag{4}$$

Here, αβ–P is the complex of the αβ heterodimer with self-peptide, and αβ–P* is the complex with the added, labeled (fluorescent) peptide. Strong evidence for this reaction pathway was obtained upon investigating peptide replacement between I-A$^d$ complexed with Texas Red-labeled OVA peptide (OVA represents amino acids 323–339 of chicken ovalbumin) and added fluorescein-labeled OVA peptide (OVA*).[21,23] Under the experimental conditions, the half-time for fluorescein peptide binding was found to be approximately constant and equal to the half-time for Texas Red peptide dissociation. Related work has shown that at pH 5 the rate of binding of a moth cytochrome *c* peptide to a sample containing a complex of sperm whale myoglobin peptide is equal to the rate of dissociation of sperm whale myoglobin peptide.[20] As discussed below, a detailed analysis of the formation half-times as a function of peptide concentration can be used as strong evidence for reactions 3 and 4 even when the peptides are endogenous self peptides.

Figure 3 shows formation curves for the reaction between a fluoresceinated peptide representing amino acids 89–104 of pigeon cytochrome *c* (pCytc*) and I-E$^k$ in the nonionic detergent dodecyl β-D-maltoside.[22] An intriguing aspect of the plot of the formation half-times versus reciprocal peptide concentrations is that $t_{1/2}$ *decreases* (the reaction gets faster) at *low* peptide concentrations (Figure 3, inset). Such a decrease in $t_{1/2}$ at low peptide concentrations is not expected either

(23) Texas Red is the trade name of a sulfonyl chloride derivative of sulforhodamine 101 (Molecular Probes, OR).

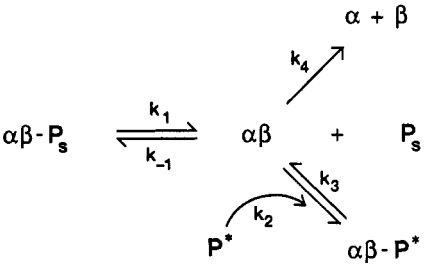


**Figure 4.** Proposed scheme for class II MHC–peptide complex formation.

for the simple reaction P* + αβ ⇌ αβ–P* or in the two-step sequential kinetic model (Figure 2), when the initial conditions are those in which the protein binding sites are empty.

The origin of the seemingly peculiar effect—a shorter $t_{1/2}$ at lower added peptide concentrations—is easily understood in terms of reactions 3 and 4 in the limiting case where the on-reaction 4 is very fast, the reaction goes to completion, and the concentration of added peptide is less than that of the protein. In this case the reaction is half over when enough time has elapsed that the loss of self-peptide equals half the concentration of added fluorescent peptide. This time is clearly shorter for lower concentrations of added peptide. In the presence of a large excess of added peptide, the half-time for peptide binding is equal to the half-time for peptide dissociation.[21,22] Under appropriate conditions a shorter $t_{1/2}$ could also occur at lower added peptide concentrations in the two-step sequential reaction 2, again in the presence of prebound class II MHC–peptide complexes. That is, the same effect could arise if the sample at the beginning of the reaction contained a small fraction of empty binding sites, and/or if there were a spectrum of endogenous peptide off-rates. See Appendix A for further discussion.

A significant additional kinetic finding was made in a study of the reaction between pCytc* and I-E$^k$ in the nonionic detergent *n*-octyl β-D-glucoside (OG).[16] Unlike the formation kinetics shown in Figure 3, where apparently stable asymptotes are reached at long times, there is a marked *reduction* in the αβ–pCytc* signal at long times when I-E$^k$ is incubated in OG. A reasonable explanation of the rapid loss of αβ–pCytc* signal at long times is that peptide-free αβ heterodimers cleave:

$$\alpha\beta \rightarrow \alpha + \beta \tag{5}$$

This splitting reaction can be inhibited by large added peptide concentrations, which reduce the concentration of the peptide-free heterodimer. An instability of empty class II MHC molecules in the presence of the detergent sodium dodecyl sulfate (SDS) has been noted.[14]

From the combined kinetic results obtained using detergent-solubilized class II MHC proteins we have proposed that a slow, first-order reaction generates a reactive intermediate which undergoes competing first- and second-order reactions, irreversible cleavage (αβ → α + β), and peptide rebinding (αβ + P* → αβ–P*), respectively (Figure 4). Detailed simulations of the reactions depicted in Figure 4 showed that depending on the ratio of the cleavage rate constant to the binding rate constant either apparently stable asymptotes are reached at long times or the asymptotes tend to zero at long times.[16,22]

**Table I. Dissociation Half-Times of Peptides from I-A$^d$ at pH = 5.3 and 40 °C**

| peptide[a] | | $t_{1/2}$ (h) |
|---|---|---|
| F-ISQAVHAAHAEINEAGKY-$NH_2$ | FOVA(323–340) | 107 |
| Ac-QAVHAAHAEINEAGKY-F | OVA(325–340)F | 99 |
| F-AERADLIAYLKQATAK | FpCytc(89–104) | 92 |
| F-GAAHA-$NH_2$ | FOVA(328–332) | 96 |

[a] The pCytc peptide was labeled with fluorescein isothiocyanate at the N-terminus.[22] OVA peptides were prepared as described.[26] F, Ac-, and -$NH_2$ denote fluorescein, N-terminal acetylation, and C-terminal amidation, respectively. OVA(325–340) is labeled with fluorescein at the ε-amino group of lysine ($K^{339}$). There is a 5–10% uncertainty in the half-times.

Assays for class II MHC proteins are frequently carried out using SDS–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions.[24] In such gels one can see silver-stained bands due to the $\alpha\beta$ heterodimer, along with bands due to the separate $\alpha$ and $\beta$ chains.[25] Microfluorescence scans of such gels prepared following a brief preincubation with fluorescent peptides generally show fluorescence associated with the $\alpha\beta$ heterodimer as well as the separate $\alpha$ and $\beta$ chains. In the presence of the denaturing detergent SDS, the $\alpha\beta$ heterodimer, as well as the peptide-loaded heterodimer $\alpha\beta$–P*, may split, according to eq 5 and

$$\alpha\beta\text{–P*} \rightarrow \alpha + \beta\text{–P*} \quad (6)$$

$$\alpha\beta\text{–P*} \rightarrow \alpha\text{–P*} + \beta \quad (7)$$

When $\alpha\beta$–P* complexes are incubated at low pH in either DM or OG, and peptide dissociation ($\alpha\beta$–P* → $\alpha\beta$ + P*) is monitored using HPSEC, $\alpha$–P* and $\beta$–P* complexes are not detected.[16]

## Dissociation Kinetics

As noted earlier, the long dissociation half-times for peptide release from MHC molecules are a highly plausible requirement from the point of view of biological function. One might anticipate that the rate of dissociation of bound peptide from class II MHC molecules is peptide structure dependent. For example, a truncated version of the parent OVA 17-mer, such as an OVA 5- or 6-mer, would be expected to dissociate more rapidly than the parent peptide. It was therefore surprising when it was found that complexes between I-A$^d$ and OVA* 5- and 6-mers are long-lived.[26] Recently, we have measured the rates of dissociation of bound peptides from four different I-A$^d$–peptide complexes.[27] The site of attachment of the fluorophore, the sequence, and the length of the peptide do not significantly affect the rate of peptide dissociation from I-A$^d$ in detergent solution (Table I): all four I-A$^d$–peptide complexes have dissociation half-times of 100 h at pH = 5.3 (40 °C). During the course of these peptide dissociation experiments, $\alpha\beta$ absorbance was monitored at 280 nm; apparently $\alpha\beta$ also splits, but more slowly ($t_{1/2}$ = 200 h).

The kinetic results shown in Table I are consistent with a slow, rate-limiting conformational change from a "closed" nondissociating heterodimeric state to an "open" dissociating heterodimeric state. This mechanism is discussed below in more detail.

## Kinetic Intermediates

There are two experimental reasons to suspect that kinetic intermediates play a significant role in the reactions of antigenic peptides with class II MHC molecules. The data in Table I are most easily rationalized if the dissociation reaction is a two-step process:

$$(\alpha\beta\text{–P*})_t \underset{k_{it}}{\overset{k_{ti}}{\rightleftharpoons}} (\alpha\beta\text{–P*})_i \quad (8)$$

$$(\alpha\beta\text{–P*})_i \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} (\alpha\beta) + \text{P*} \quad (9)$$

If reaction 8 is the slow rate-limiting step, then this may be a slow protein conformational change that is—to a good approximation—peptide structure independent. The putative peptide structure-dependent reaction 9 is not rate limiting, and thus the overall rate of peptide dissociation shows no significant dependence on peptide composition.

Early evidence for an intermediate was obtained using I-E$^k$ incorporated in lipid bilayers on glass microscope slides.[28] By incubating pCytc* with I-E$^k$ for various periods of time, and then measuring peptide off kinetics, it was observed that the peptide dissociation rate is biphasic; this was attributed to the presence of two complexes. Bound peptide dissociates from the intermediate complex $(\alpha\beta\text{–P*})_i$ with a half-time of the order of 20 min, whereas the longer half-time was 30 h, due to the loss of peptide from the terminal complex $(\alpha\beta\text{–P*})_t$ (see eq 2). As expected, the amplitudes of these short and long half-life decays depended on the time of prior incubation. Recent additional evidence for this kinetic intermediate has been obtained from using the human class II MHC protein DR1, genetically engineered so as to be water soluble, and a viral peptide.[29] Further evidence has also been obtained by the present authors for a similar kinetic intermediate in the case of detergent-solubilized mouse class II MHC protein I-A$^d$ and both the OVA and pCytc peptides, where the fast off-time is also of the order of 20 min.

The fact that class II MHC proteins can bind many different peptides and retain each peptide in the binding site for long periods of time suggests that a substantial protein conformation change *may* be involved in the MHC peptide reaction. In this case the intermediate in eqs 8 and 9 might involve a heterodimer with a structure $(\alpha\beta)_o$ that is substantially different than the heterodimer in the terminal complex $(\alpha\beta)_c$, where "o" and "c" refer to "open" and "closed". Let us consider a possible correspondence with the species in eqs 8 and 9:

$$\alpha\beta = (\alpha\beta)_o \quad (10)$$

$$(\alpha\beta\text{–P*})_i = (\alpha\beta\text{–P*})_o \quad (11)$$

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$$(\alpha\beta\text{–P*})_t = (\alpha\beta\text{–P*})_c \quad (12)$$

With these identifications, the expected kinetics of peptide dissociation from class II MHC molecules are not different from those described above.

We note that complex formation according to reverse reaction 9 followed by reverse reaction 8 is the same as the two-step sequential kinetic model given in eq 2. However, if a nonreactive species $(\alpha\beta)_c$ exists in equilibrium with $(\alpha\beta)_o$, and the equilibrium concentration of $(\alpha\beta)_c$ is much larger than that of $(\alpha\beta)_o$, then the complex formation kinetics can be distinctly different than those expected from the two-step sequential kinetic model:

$$(\alpha\beta)_c \rightleftharpoons (\alpha\beta)_o \quad (13)$$

Under these circumstances complex formation kinetics can mimic those described earlier for detergent-solubilized class II MHC molecules with prebound self-peptides, even when self-peptides are absent. [This would give the negative slope in the plot of $t_{1/2}$ vs $1/[P^*]$ as in Figure 3 (inset) if $(\alpha\beta)_c$ were the dominant species in the absence of added peptide.] It is possible that the "floppy" conformation of class II MHC molecules plays a role in some of these reactions. See Appendix B.

Speculations concerning peptide–class II MHC reactions have often understandably invoked "open" and "closed" pictures of the binding groove between the two helices of the $\alpha$ and $\beta$ chains. However, there is evidence that separate $\alpha$ and $\beta$ chains alone can bind peptides, with long half-lives against dissociation.[30] The reverse of these reactions, the formation of $\alpha$–P* and $\beta$–P* complexes from single chains and peptide, has been reported.[31] Further, recent experiments have also shown that single chain subunit–peptide complexes can be recognized by specific T-helper cells.[32] Does the $\alpha\beta$ heterodimer have any unique peptide binding property not shown by the separate $\alpha$ and $\beta$ chains? Many experiments have shown that the kinetics of peptide binding to and dissociation from class II MHC molecules are increased at lower pH values,[20,22,33] and immunologists have suggested that this serves the function of enhanced peptide loading (and unloading) in endosomes and lysosomes where the pH is low. The kinetics of peptide dissociation from the separate $\alpha$ and $\beta$ chains of I-A$^d$ are not increased by lower pH.[30] This then raises the possibility that the enhancement in the rate of class II MHC–peptide complex formation at low pH may involve interactions between the $\alpha$ and $\beta$ chains of the heterodimer, such as salt bridges between $\alpha$ and $\beta$.

## Complexes of MHC Class II Molecules with Two Peptides; Two-Peptide Exchange

When detergent-solubilized I-A$^d$ is incubated with two peptides, one labeled with fluorescein and one labeled with Texas Red, one can observe energy transfer between these fluorophores upon scanning an SDS gel with a fluorescence microscope.[34] Recently, evidence has been obtained that two-peptide complexes with class II MHC may also play a role in the reaction kinetics.

One kinetic manifestation of two-peptide binding is a "push-off" effect. Thus, the addition of one peptide $P_2$ to a solution containing the complex $\alpha\beta$–$P_1$ can enhance the dissociation of $P_1$. Examples involve I-E$^d$ and I-E$^k$ with the peptides dynorphin A(1–13) and HEL-(107–116):[35,36]

$$\alpha\beta\text{–}P_1 + P_2 \rightarrow \alpha\beta\text{–}P_1P_2 \quad (14)$$

$$\alpha\beta\text{–}P_1P_2 \rightarrow \alpha\beta\text{–}P_2 + P_1 \quad (15)$$

It has also been observed that one peptide X can catalyze the replacement of a self-peptide by an added peptide:

$$\alpha\beta\text{–}P_s + P^* \rightarrow \alpha\beta\text{–}P^* + P_s \quad (16)$$

Presumably this catalysis proceeds by reactions such as eqns 14 and 15: X pushes off $P_s$ and is in turn pushed off by P*. An example of reaction 16 is the displacement of self-peptide by HEL(107–116) in the presence of the catalyst X, where X is lysine$_n$ ($n$ = 14–16).[36]

These push-off reactions are not universal; we have attempted and failed to see OVA push off OVA* from I-A$^d$, or to see pCyt*c* push off pCyt*c** from I-E$^k$. Nonetheless, there are cases where a (biologically antigenic) peptide will push itself off. (HEL pushes HEL* off from I-E$^d$.) The two peptide class II MHC complexes may provide a clue as to one type of kinetic intermediate. That is, the pCyt*c** kinetic intermediate considered earlier in eq 2 may be one in which only part of the peptide is bound to the protein [in $(\alpha\beta\text{–P*})_i$] and all of the peptide is bound in $(\alpha\beta\text{–P*})_t$. In this event one might anticipate observing a push-off of pCyt*c** from $(\alpha\beta\text{–P*})_i$ but not from $(\alpha\beta\text{–P*})_t$.

## Conclusions

Many of the technical obstacles to studying the kinetics of class II MHC–peptide reactions are now being overcome, and one can look forward to the possibility of obtaining definitive chemical reaction kinetics. Then it will be possible to attempt to understand these chemical kinetics in terms of molecular composition and structure. For example, an interesting step in this direction has been taken recently by investigators who studied the effect of ionic strength on the binding of pCyt*c* to I-E$^k$ and related their results to charge complementarity between the peptide and protein.[37]

Related objectives of the present work include the goal of devising a simple technique to prepare MHC–peptide complexes of defined composition, since such complexes are preferred for physical–chemical studies and may also be of therapeutic value in terms of anergizing T-cells.[38] Accurate *in vitro* chemical kinetics for these reactions in solution provide a benchmark for comparative studies of the reactions in biological cells. Further, since there are strong correlations between specific peptides and class II MHC alleles and serious

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autoimmune diseases,[39] our studies offer the possibility, albeit remote, that abnormalities in the kinetics will reveal molecular lesions related to disease.

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## Appendix A: Simulated Peptide Replacement

The peptide replacement reactions (eqs 3 and 4) are deceptively simple. In practice, peptide replacement might be misidentified as two-peptide exchange (eqs 14 and 15) or even as two-step sequential kinetics (eq 2). This is because these reactions show saturation kinetics at high peptide concentration where the rates approach a maximum rate, $v_{max}$. In the two-step sequential kinetic model $v_{max}$ ($s^{-1}$) = $k_2$ (eq 2). In the peptide replacement kinetic model $v_{max}$ ($s^{-1}$) = $k_{off}$ (eqs 3 and 4). We believe that in some cases the peptide replacement model can be distinguished from the two-step sequential model, as discussed below.

An idealized case of peptide replacement, where each binding site is occupied with the same unlabeled peptide P at time zero, is simulated. Peptide dissociation (eq 3) precedes the binding of labeled peptide P* (eq 4). The following parameters are used: $k_{on}$ = 1–1000 $M^{-1}$ $s^{-1}$, $k_{off}$ = $3 \times 10^{-5}$ $s^{-1}$, [$\alpha\beta$–P]($t$=0) = 1 $\mu$M, [$\alpha\beta$]($t$=0) = 0, [$\alpha\beta$–P*]($t$=0) = 0, [P]($t$=0) = 0, and [P*]($t$=0) = 0.2–200 $\mu$M. Simulated peptide replacement with $k_{on}$ = 1 $M^{-1}$ $s^{-1}$ is shown in Figure 5. Simulations of reversible reaction 1 (where $k_{on}$ = 1 $M^{-1}$ $s^{-1}$ and $k_{off}$ = $3 \times 10^{-5}$ $s^{-1}$) and of the two-step sequential kinetic model (eq 2) are given in Figure 2.

We note that at long times and at low concentrations of P* there is competition for binding with dissociated peptide P.

The slope in the plot of $t_{1/2}$ vs 1/[P*] is either positive, zero, or even negative, depending on the magnitude of the second-order rate constant (Figure 5, inset). When the binding of added, labeled peptide is slow ($k_{on}$ < 10 $M^{-1}$ $s^{-1}$), the slope in the plot of $t_{1/2}$ vs 1/[P*] is positive; in contrast, when peptide binding is more rapid ($k_{on}$ > 100 $M^{-1}$ $s^{-1}$), the slope in the plot of $t_{1/2}$ vs 1/[P*] is negative. A positive, or nearly zero, slope in the plot of $t_{1/2}$ vs 1/[P*] is consistent with both the peptide replacement and the two-step sequential kinetic models. However, a decrease in $t_{1/2}$ at low peptide concentrations is not observed in the simulation of either the simple reversible binding reaction (eq 1) or the two-step sequential kinetic model (eq 2). Thus, the decrease in $t_{1/2}$ at low peptide concentrations seems to be a unique feature of the peptide replacement reaction (eqs 3 and 4). On the basis of these simulations, the observed ~3-fold decrease in $t_{1/2}$ at low pCytc* concentrations (Figure 3, inset) is consistent with a peptide replacement reaction in which $k_{on}$ ~ 1000 ± 250 $M^{-1}$ $s^{-1}$.

## Appendix B: Compact and Floppy Class II MHC Conformations

SDS gels of many class II MHC proteins show two distinct bands due to the $\alpha\beta$ heterodimer. These bands have been dubbed "compact" and "floppy", for the proteins of greater and lower mobility.[24] Although these two bands have sometimes been regarded as gel artifacts, this is almost certainly not the case. For one reason, the relative intensities of the two bands depend on prior treatment of the protein sample, in such a way as to suggest that floppy $\alpha\beta$ is on the pathway to the disassembly of the heterodimer: compact $\alpha\beta \rightarrow$ floppy $\alpha\beta \rightarrow \alpha + \beta$.[24] The compact form is stabilized at acidic pH in the presence of antigenic peptides that form stable complexes with the class II MHC proteins.[14,16,40] Further, it has been found that only the floppy form is produced by mutant cells that are deficient in the invariant chain.[41] Both floppy and compact bind fluorescent peptides, and floppy can bind two long peptides as well as two short peptides, whereas compact can only bind two short peptides.[34] For the present, we have avoided the temptation to identify floppy with one or another of the kinetic intermediates until there is solid evidence for this assignment. One way to probe the role of SDS on the formation of floppy is to use an alternative chromatographic technique such as capillary zone electrophoresis, which does not require SDS.

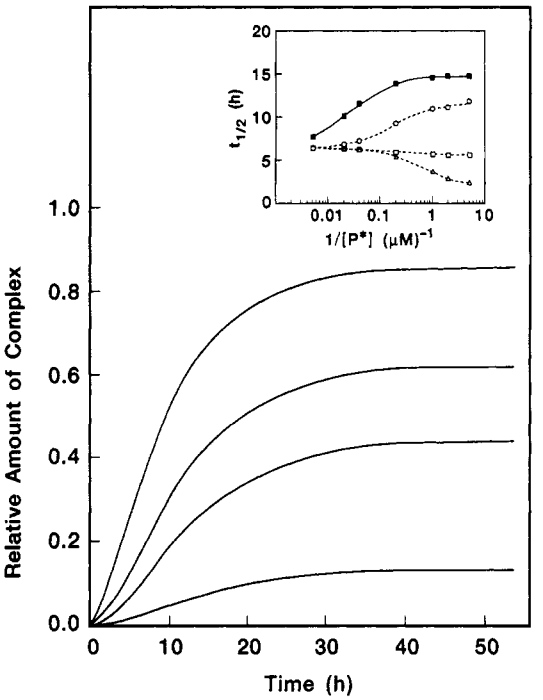


**Figure 5.** Simulated peptide replacement. Reactions 3 and 4 are simulated with the following parameters: [$\alpha\beta$–P]$_0$ = 1 $\mu$M; [$\alpha\beta$]$_0$ = [$\alpha\beta$–P*]$_0$ = [P]$_0$ = 0; [P*]$_0$ = 5, 25, 50, and 200 $\mu$M; $k_{on}$ = 1 $M^{-1}$ $s^{-1}$ and $k_{off}$ = $3 \times 10^{-5}$ $s^{-1}$. Replacement was also simulated using $k_{on}$ = 10, 100, and 1000 $M^{-1}$ $s^{-1}$, with $k_{off}$ = $3 \times 10^{-5}$ $s^{-1}$ (curves not shown). Note the sigmoidal shape in the binding curves, corresponding to the requirement of prior dissociation of $\alpha\beta$–P. This lag is not present in the simulated binding curves when the kinetic on rate constant $k_{on}$ is large. (Inset) Plot of $t_{1/2}$ vs 1/[P*]: (■) $k_{on}$ = 1 $M^{-1}$ $s^{-1}$; (○) $k_{on}$ = 10 $M^{-1}$ $s^{-1}$; (□) $k_{on}$ = 100 $M^{-1}$ $s^{-1}$; (△) $k_{on}$ = 1000 $M^{-1}$ $s^{-1}$. The lines through the points are to help guide the eye.

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