pH Affects both the Mechanism and the Specificity of Peptide Binding to a Class II Major Histocompatibility Complex Molecule†

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ABSTRACT: We have compared the contribution of electrostatic forces in the binding of antigenic peptides to the class II MHC molecule, IE^k, at weakly acidic (pH 5.4) and neutral (pH 7.5) pH values. The binding of specific moth cytochrome *c* (MCC) and hemoglobin (Hb) peptides to IE^k is very sensitive to ionic strength at pH 7.5 but not at pH 5.4, indicating that the mechanism of peptide binding is pH-dependent. Substitution of the C-terminal Lys in MCC for an Ala residue selectively destroyed peptide binding at neutral pH and increased the dissociation rate at least 30-fold, implicating this residue in the pH-dependent electrostatic interaction. The presence of a C-terminal Lys in many of the peptides that are restricted to IE^k suggests that this electrostatic interaction is widely used to bind peptides to this MHC molecule. We also probed the electrostatic environment of the peptide binding groove adjacent to the N-terminus of the bound peptide by rapid-diffusion fluorescence energy transfer using a terbium-labeled MCC peptide. In this region of the peptide binding groove, more negative charge is present at pH 7.5 than at pH 5.4. These findings indicate the importance of MHC carboxylates to the mechanism and specificity of peptide binding. The biological importance of having two distinct mechanisms of peptide binding at different pH may be that it acts to broaden the spectrum of antigenic peptides that can be presented to T-cells.

Class I and class II major histocompatibility complex (MHC)[1] molecules are noncovalently-linked glycoprotein heterodimers that bind peptide fragments of antigen and present them to T-cells. The recognition of peptides in association with MHC molecules is a pivotal event in the initiation of T-cell response and indirectly a requirement for most B-cell responses as well, since the production of antibody does not usually proceed without T-cell help [reviewed in Davis (1990)]. The limited number of allelic variants requires that each MHC molecule binds many different peptides. High-resolution crystal structures of class I MHC molecules indicate that antigenic peptides bind in a deep cleft between two antiparallel α helices that rest on a floor of an eight-stranded β sheet (Bjorkman et al., 1987; Madden et al., 1992; Garrett et al., 1989; Fremont et al., 1992; Matsumura et al., 1992). The recent determination of the DR1 crystal structure indicates that class II MHC molecules are very similar to class I molecules (Brown et al., 1993), as was previously proposed (Brown et al., 1988).

While class I MHC molecules associate with antigenic peptides in the endoplasmic reticulum (Townsend et al., 1989; Schumacher et al., 1990), class II molecules are thought to bind peptides in an acidic endosomal compartment (Guagliardi et al., 1990; Peters et al., 1991; Lotteau et al., 1990). In this regard, many class II MHC molecules show accelerated association rates for the binding of antigenic peptides at acidic pH (Reay et al., 1992; Jensen, 1991; Witt & McConnell, 1991; Mouritsen et al., 1992; Sette et al., 1992), which has led to the hypothesis that at low pH class II MHC molecules undergo a shift in the equilibrium from a "closed" to an "open" (peptide receptive) state (Jensen, 1990). SDS–PAGE analysis has shown evidence for pH- and peptide-dependent conformational transitions in class II MHC molecules (Dornmair et al., 1989; Wettstein et al., 1991; Sadegh-Nasseri & McConnell, 1991; Sadegh-Nasseri & Germain, 1991). Additionally, some spectroscopic approaches have been employed to study the structure of class II MHC molecules (Kropshofer et al., 1991; Lee et al., 1992; Driscoll et al., 1993; Tampe et al., 1991). Despite these studies, we have little understanding of the chemistry of peptide/MHC binding.

One possibility is that the enhanced peptide binding at acidic pH may occur by the titration of charge on the MHC molecule itself. Antigenic peptides could directly interact with titratable residues, or the titration of charge may only affect the MHC structure and the availability of contact sites for the peptide. The conservation of charged residues in antigenic peptides has suggested that electrostatic interactions may play a direct role in peptide/MHC binding (Collawn et al., 1989; Bogen & Lambris, 1989). Both moth and pigeon cytochrome *c* (MCC and PCC) peptide analogs with increased activities in T-cell activation assays have been prepared by adding positively charged amino acids N-terminal to residue 95 (Fox et al., 1987; Collawn et al., 1989). These IE^k-restricted superagonist activities may result from new or altered electrostatic interactions between IE^k and the N-terminus of the peptide. We also reasoned that the relative contribution of forces involved

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[1] Abbreviations: MHC, major histocompatibility complex; MCC, moth cytochrome *c*; Hb, hemoglobin; PCC, pigeon cytochrome *c*; OVA, ovalbumin; HEL, hen egg lysozyme; SWMb, sperm whale myoglobin; EqMb, equine myogobin; MoMb, mouse myoglobin; rep, λ repressor; HSVgD, herpes simplex virus glycoprotein; SNase, staphylococcal nuclease; LLO, listeriolysin; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; H/M, HEPES/MES: ELISA, enzyme-linked immunoadsorbant assay; 2-D NMR, two-dimensional nuclear magnetic resonance; Tb, terbium.

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in peptide binding may depend on pH. These hypotheses were tested by comparing the effect of ionic strength on the binding of antigenic peptides to IE^k at different pH. Additionally, we have probed the electrostatic environment of the MHC near the peptide N-terminus at different pHs by rapid-diffusion fluorescence energy transfer. Our results are consistent with a model in which buried, nonpolar regions of IE^k are exposed at acidic pH and show that the peptide binding groove has charged residues that titrate between pH 5.3 and 7.5. Proximal to the N-terminus of bound peptide are titrating charges that may explain the superagonist activities mentioned above. Another charged residue of IE^k appears necessary for efficient association and retention of peptides at neutral pH, but is not important for association at low pH, which is both faster and less sequence-dependent. Further, both the mechanism and specificity of peptide/IE^k binding change between these two pH values.

MATERIALS AND METHODS

HEPES/MES Buffer. A HEPES/MES (H/M) buffer system was employed for both energy-transfer and peptide association/dissociation studies. This biological buffer allowed a stable pH in the range of 5.3–7.5 with a negligible affinity for metal ions. The buffers were 20 mM each of HEPES and MES, plus the indicated amounts of NaCl. The pHs of the buffers were adjusted with concentrated HCl or NaOH. The Henderson–Hasselbalch equation was used to calculate the relative contribution of buffer species at each pH, and the ionic strengths were then determined by $i = 1/2\Sigma C_i X_i^2$, where i is the ionic strength and C and X are the molar concentration and charge of each species, respectively. The contributions of NaOH and HCl to the ionic strength were included.

Preparation of Soluble IE^k. Soluble IE^k was prepared as previously described (Wettstein et al., 1991), with minor modifications. Briefly, Chinese hamster ovary (CHO) cell transfectants, expressing GPI-linked IE^k, were grown to high density on hollow-fiber bioreactors (Cell Pharm I). Phosphatidylinositol-specific phospholipase C (PI-PLC) was prepared as described by Koke et al. (1991). Soluble IE^k was harvested by pumping PI-PLC (0.2 unit/mL) into the cell side of the bioreactor at a flow rate of 400 mL day^{-1} $pharm^{-1}$. The harvest was adjusted to 5 mM azide/5 mM EDTA, pH 8.0, filtered, and applied to an immunoaffinity column (MAb 14.4.4 coupled to cyanogen bromide activated Sepharose 4B). Purified IE^k was then concentrated into PBS by Centricon 30 ultrafiltration (Amicon). A molar extinction coefficient of 1.4×10^5 L/(mol·cm) at 280 nm, determined by amino acid analysis, was used to calculate concentrations of IE^k.

Synthetic Peptides. All peptides were prepared by the Stanford Univerisity PAN facility using standard FMOC chemistry. For N-terminal biotinylation, resin (post-piperidine deprotection) was washed with DMF and then ethyl acetate and dried under vacuum. The dry resin was then suspended in DMF containing a 10-fold molar excess of NHS-biotin (Sigma) and 1-hydroxybenzotriazole hydrate (Aldrich) and allowed to react with agitation for 4 h at room temperature. The resin was washed, dried as above, cleaved, and deprotected for 2 h in 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol, and 2% anisole. The success of the biotinylation was confirmed by reverse-phase chromatography using a Beckman Ultrasphere column (ODS, 5 μm, 4.6 × 25 cm) and a TFA/acetonitrile solvent system. Peptides used for direct binding were biotinylated moth cytochrome *c* (82–103), biotin-AGIKKKANERADLIAYLKQATK (MCC); biotin-AGIKKANERADLIAYLEQATA (MCC103A); and biotinylated mouse hemoglobin b^{dmin} (53–76), biotin-AIMGNPKVKAHGKKVITAFNEGLK. The E at position 99 of MCC103A is oriented up in MHC toward the T-cell receptor (Jorgensen et al., 1992) and has no effect on MHC affinity (Jorgensen et al., 1992; Reay et al., 1993). The peptide used for energy transfer was MCC(88–103), ANERADLIAYLKQATK.

Modification of MCC for Energy Transfer. The procedure for the synthesis of terbium-labeled MCC (Tb-MCC) was adopted from "Hnatowich et al. (1983) and modified by performing the reaction in the presence of $TbCl_3$. Briefly, diethylenetriaminepentaacetic acid anhydride (DTPA) was suspended at 1 mg/mL in diethyl ether, and an aliquot representing a 5-fold molar excess of reagent was removed into a separate tube and dried under a stream of N_2. Peptide, at a final concentration of 0.2 mM, was added to the dry DTPA in a 3:1 mix of H/M(7.5)/0.15 M NaCl and 10 mM $TbCl_3$ in water. The sample was vortexed immediately for 1 min and incubated for 10 min at room temperature. The mixture was then diluted 2.5-fold with water and purified by reverse-phase chromatography using an ammonium acetate (pH 6.0)/acetonitrile gradient. A single product with a unique retention time was obtained, dried by Speedvac, suspended in water, and assayed for Tb fluorescence. The product was found to be N-terminally labeled by mapping with endopeptidase-Lys C. The chelated terbium has a net charge of −1.

Preparation of IE^k/Tb-MCC. Complexes of IE^k containing fluorescent peptide were prepared as previously described (Matsui et al., 1991). Soluble IE^k (30 μg/mL) was incubated with Tb-MCC at 37 °C for 3 days in citrate/phosphate buffer (McIvaine, 1921), pH 5.1, containing 100 mM NaCl. The binding reaction was then neutralized, concentrated to 1 mL, and applied to a Sephacryl S300 HR column (30 × 1.5 cm) equilibrated with PBS (pH 7.4) and fractionated at a flow rate of 7 mL/h. Fractions corresponding to MHC heterodimer were then concentrated and exchanged into H/M (pH 7.5)/0.15 M NaCl buffer by Centricon 30 ultrafiltration for energy transfer.

Preparation of $(EDTACo)^{2-}$. $(EDTACo)^{2-}$ was prepared by mixing 4 mL of 1 M ethylenediaminetetraacetic acid (EDTA) with a 2–4-fold molar excess of cobalt(II) (Co^{2+}). Unbound Co^{2+} was removed from the mixture by cation-exchange chromatography using a Dowex AG50X8 column (2.5 × 26 cm) in water.

Fluorescence Energy Transfer. Two hundred thirty microliters of a 5–10 μM solution of terbium (Tb) as Tb-MCC or IE^k/Tb-MCC, at the indicated salt concentration and pH in H/M buffer, was placed in a quartz cuvette. Co^{2+}, $(EDTACo)^{2-}$, or NaCl were added as indicated, and the lifetime of Tb was measured as described below. The pH of the solution, which was checked before and after each experiment, did not change by more than 0.1 unit. After one experiment, the loss of Tb from the IE^k/Tb-MCC for the pH 5.4 sample was compared with that of the pH 7.4 sample. The MHC was separated from unbound Tb-MCC and Tb by a gel filtration column (Sephadex G100), and the ratio of the concentration of Tb to tryptophan was measured. The values of the pH 7.4 and pH 5.3 samples were within 10% of each other.

Tb was excited at 100 Hz with a 50-μs-wide pulse of 488-nm light, generated by placing a rotating slit in the path of an 80–700-mW beam of an argon ion laser. The fluorescent light was focused onto a cooled photomultiplier tube (PMT) at a right angle to the laser beam and separated from scattered light by a 520-nm long-pass dichroic and colored glass filter. Tb fluorescence was collected from 0.1 to 6.7 ms after the

multichannel analyzer was triggered by a fast photodiode illuminated by the pulsed laser beam. The PMT pulses were amplified with a Model SR445 amplifier (Stanford Research Systems) and accumulated into sequential 1.2-μs-wide bins on a Model SR430 multichannel scaler (Stanford Research Systems). The multichannel analyzer summed the PMT pulses following 50 000–100 000 illumination cycles of the laser. The data were fit by a Maquardt least-squares program to a single exponential: $y = a + be^{-(t/\tau)}$ where t is time, y is the fluorescence of Tb, a and b are constants, and τ is the lifetime of Tb. The efficiency of energy transfer (E) is the fractional decrease in donor fluorescence (Tb) due to energy transfer: $E = 1 - \tau_a/\tau_0$ where τ_a is the lifetime of Tb in the presence of an acceptor, either Co^{2+} or $(EDTACo)^{2-}$, and τ_0 is the lifetime of Tb in the absence of an acceptor. The distance of closest approach, assuming dipole–dipole transfer between Tb and Co^{2+}, was determined as previously described (Stryer et al., 1982; Thomas et al., 1978).

Peptide Binding Assay. Peptide binding to IE^k was measured using a capture ELISA described elsewhere (Reay et al., 1992). Association reactions were initiated by incubating IE^k at 35 μg/mL with a 30-fold excess of biotinylated peptide in H/M buffer containing the desired NaCl concentration and at either pH 5.4 or pH 7.5. Binding reactions were terminated by chilling and then adjusted to the same pH and ionic strength by diluting to 2 μg/mL in phosphate-buffered saline with 2% bovine serum albumin. Bound from free peptide was separated by capture of MHC onto the wells of an ELISA plate (Immulon 4) previously coated with an antisera directed against the GPI tail. After incubation with an avidin/alkaline phosphatase conjugate (Sigma), the binding was quantitated by the optical density at 405 nm by the catalysis of the chromogenic substrate *p*-nitrophenyl phosphate (Sigma 104 substrate). The binding of MCC and Hb can be completely inhibited by unbiotinylated versions of each but not by peptides restricted to other class II MHC molecules.

For measurements of dissociation rates, complexes were preformed as described above using 100 μg of IE^k and a 100-fold excess of peptide. After 3 days of binding, samples were chilled in an ice/water bath, and excess peptide was removed using a Centricon-30 at 4 °C. Dissociation was initiated by diluting the complex to 8 μg/mL in H/M buffer (at a particular pH and NaCl concentration) and incubating at 37 °C in the presence of a 50-fold excess of unbiotinylated MCC(88–103). The amount of bound peptide was then determined as described above except that samples were corrected for salt and pH before diluting to 2 μg/mL for capture. At zero time, the amount of MCC103A bound was about 40% of MCC, but a linear extrapolation of the natural-log-transformed data indicated that most of this difference could be accounted for by dissociation during the ELISA assay.

RESULTS

Effect of Ionic Strength and pH on Peptide/IE^k Binding. To determine the relative contribution of electrostatic forces in the binding of antigenic peptides to IE^k, the kinetics of association of two peptides were measured at different ionic strengths and pHs. At pH 5.4, the binding of both moth cytochrome *c* (MCC) peptide and hemoglobin (Hb) peptide is insensitive to ionic strength (Figure 1A,C) consistent with the lack of a net electrostatic contribution. However, at pH 7.5, the association of both peptides is very dependent on NaCl concentration (Figure 1B,D). The amount of MCC bound as steady state is approached (62 h) is 30–40% less at 0.15 M NaCl (physiological ionic strength) relative to H/M buffer without added salt. The binding of each peptide is almost completely inhibited at 0.75 M NaCl (Figure 1B,D). As the ionic strength is lowered at pH 7.5, the binding is significantly enhanced and approaches the equilibrium amount bound at pH 5.4 (compare Figure 1A with Figure 1B). To determine the effect of salt on the apparent association rate for MCC, early kinetic points from a separate experiment were fit by linear regression to obtain estimates of the initial velocities under these different conditions. When the inverse of this rate is plotted as a function of ionic strength, the slope of the line reflects the relative salt dependence (Figure 2). Clearly, at pH 7.5, the initial velocity of the reaction is much more dependent on ionic strength than at pH 5.4.

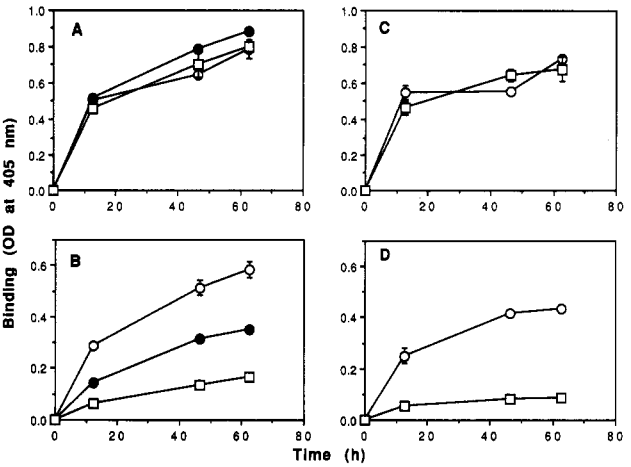


FIGURE 1: Effect of NaCl concentration and pH on the binding of MCC and Hb peptides to IE^k. Shown are the kinetics of binding of MCC (A and B) and Hb (C and D) peptides to IE^k in H/M buffer at either pH 5.4 (A and C) or pH 7.5 (B and D) with NaCl concentrations of 0 (open circles), 0.15 (solid circles), or 0.75 M (open squares). These data are from a single representative experiment that was repeated several times. The amounts bound (by OD) are directly comparable.

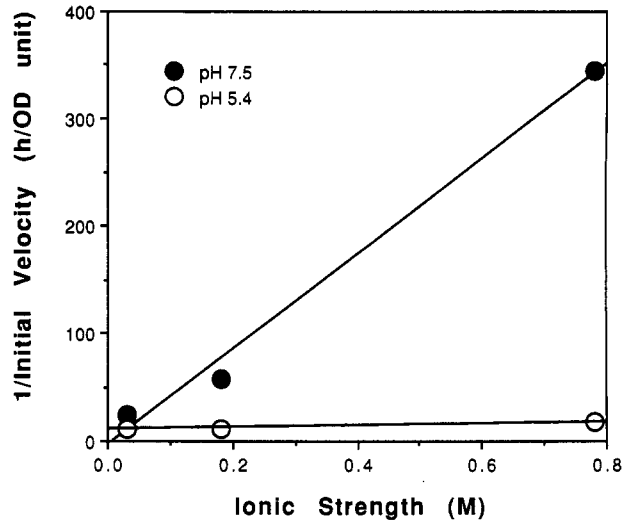


FIGURE 2: Effect of ionic strength on the initial velocity of MCC peptide binding to IE^k. Initial slopes were obtained by fitting early kinetic points from a separate experiment by linear regression.

The differential effect of ionic strength prompted us to examine the residues of MCC involved in binding. The major residues involved in the binding of MCC to IE^k at pH 5 have been mapped to Ile95, Gln100, and Lys103 (Reay et al., 1993). In addition, 2-D NMR analysis has indicated that Ala96 and Ala101 are also in contact with portions of the binding groove (Driscoll et al., 1993; see Table I). Of these residues, Lys103 is the most likely candidate for an electrostatic interaction with the MHC molecule. To test this, we measured the binding of a peptide variant, MCC103A, which has an Ala at position

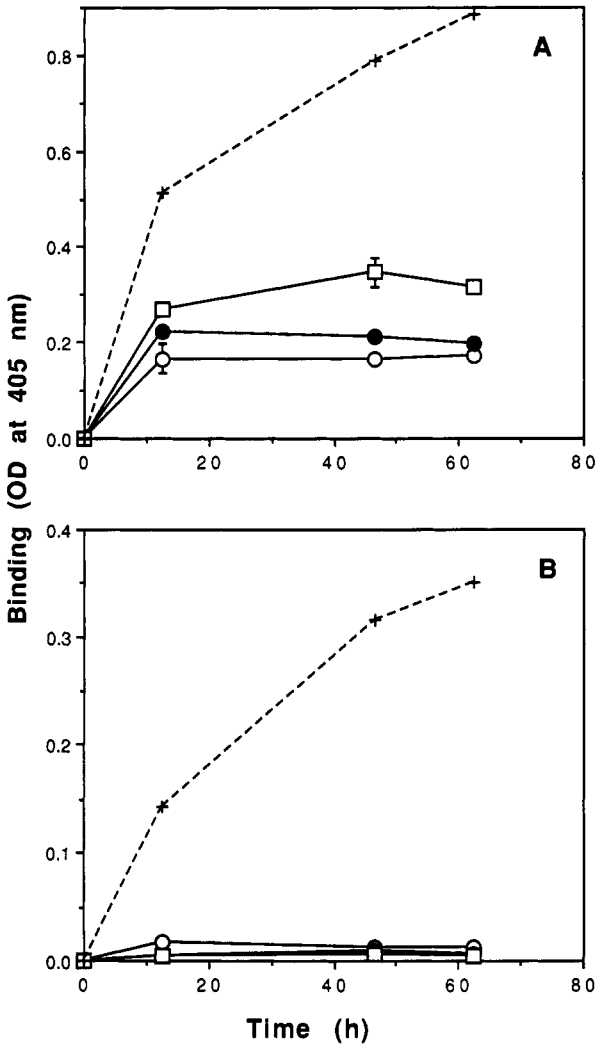


FIGURE 3: Effect of NaCl concentration and pH on the binding of MCC103A peptide to IE^k. Shown are the kinetics of binding of MCC103A to IE^k in H/M buffer at pH 5.4 (A) or pH 7.5 (B) with NaCl concentrations of 0 (open circles), 0.15 (solid circles), or 0.75 M (open squares). For comparison, the binding of the wild-type peptide at 0.15 M NaCl is shown at both pHs. These data are from a single representative experiment that was repeated several times. The amounts bound (by OD) are directly comparable.

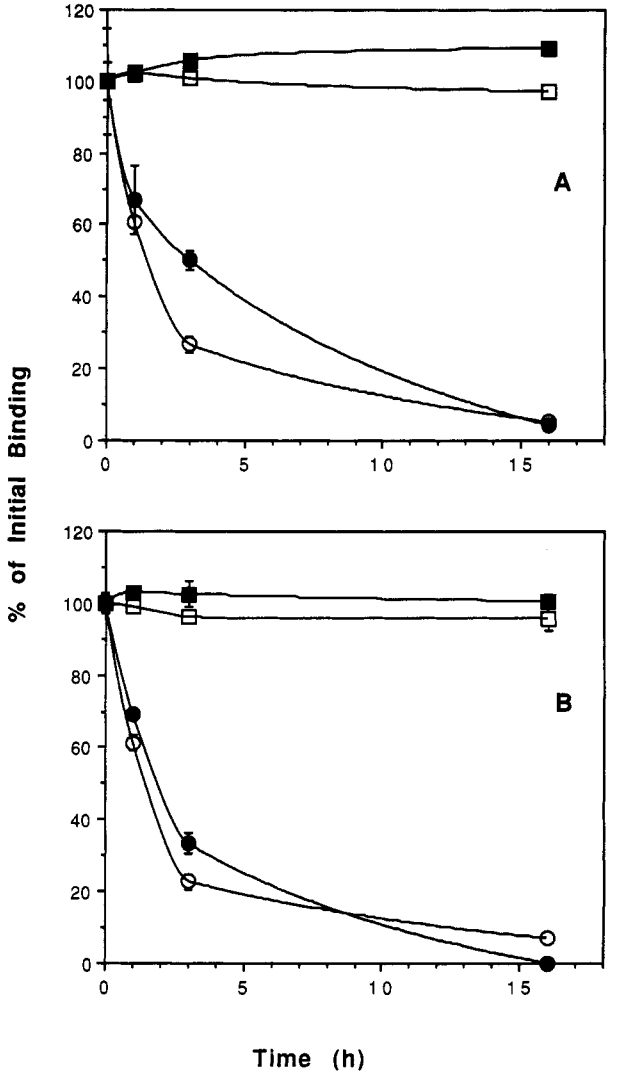


FIGURE 4: Dissociation kinetics of MCC and MCC103A. Shown are the kinetics of dissociation at pH 5.4 (A) or pH 7.5 (B) for MCC (squares) and MCC103A (circles) in H/M buffer with NaCl concentrations of 0 (open symbols) or 0.45 M (solid symbols).

103. Additionally, this peptide has a substitution at position 99 (Lys to Glu); however, this is a T-cell receptor contact residue and has no effect on MHC affinity (Jorgensen et al., 1992; Reay et al., 1993). Consistent with an electrostatic interaction involving Lys103, MCC103A is unable to bind the MHC molecule at pH 7.5 at any NaCl concentration (Figure 3B), whereas it is still able to bind at pH 5.4 (Figure 3A), although less efficiently than wild-type MCC.

Effect of Ionic Strength and pH on Peptide/IE^k Dissociation. To test whether the salt effect was due to a change in the association or dissociation rates, MCC/IE^k and MCC103A/IE^k complexes were preformed at pH 5.4, and dissociation was followed under different conditions. As shown in Figure 4, very little dissociation of MCC from IE^k occurs during the first 16 h, irrespective of pH or ionic strength. The inhibition of MCC binding by salt, discussed above (Figure 1), must therefore be an effect on the apparent association rate. In the case of the MCC103A/IE^k complex, the half-time ($t_{1/2}$) of dissociation is a few hours, indicating that the Ala103 substitution increases the rate of dissociation by at least 30-fold (Figure 4). This rapid dissociation rate is uncharacteristic of most antigens restricted to class II MHC molecules [reviewed by Rothbard and Gefter (1991)]. Therefore, it seems likely that the inability of MCC103A to bind IE^k at neutral pH is due to both a slower association (resembling the binding of MCC at pH 7.5 and high ionic strength) and a more rapid dissociation. The more rapid dissociation of MCC103A than MCC also explains the less effective loading of this analog at pH 5.4 (Figure 3). In fact, since the $t_{1/2}$ for dissociation of MCC103A is approximately equal to the time required for the separation of bound from free peptide (3 h), as much as half of the bound MCC103A may have dissociated during the assay. Taken together, these data indicate that an ionic interaction involving Lys103 is critical in accelerating the association rate at neutral pH and stabilizing the complex from dissociation.

To determine whether an electrostatic interaction may be of general importance to IE^k-restricted peptides, we compared the amino acid sequences contained within peptides known to bind to IE^k. When the sequences are aligned according to the IE^k binding motif of Reay et al. (1993), it was noted that 12 out of 15 peptides have a Lys at the C-terminus of the motif (Table I), supporting the stringent requirement for a positive charge in this location. The other two boxed positions are included to show the three amino acid motif used to align these peptide sequences (Reay et al., 1993). Interestingly, PCC, which has an Ala insertion that shifts Lys103 to position 104, has a reported $t_{1/2}$ for dissociation of 12 h at pH 7.5

Table I: Alignment of Antigenic Peptides That Bind IE^k

Antigenic Peptide	Amino Acid Sequence
MCC(95-103)	I A Y L K Q A T K
Hb(68-76)	I T A F N E G L K
PCC(95-104)	I A Y L K Q A T A K
OVA(255-263)	L E S I I N F E K
HEL(84-92)	L S S D I T A S V
HEL(88-96)	I T A S V N C A K
SWMb(69-77)	L T A L G A I L K
EqMb(69-77)	L T A L G A I L K
MoMb(69-77)	L T A L G A I L K
rep(18-26)	L K A I Y E K K K
HSVgD(9-17)	L K M A D P N R F
SNase(89-97)	L A Y I Y A D G K
SNase(125-133)	L R K S E A Q A K
SNase(104-112)	V R Q G L A K V A
LLO(218-226)	I A K F G T A F K

Table II: Lifetime of Terbium Bound to MCC

	pH	lifetime (ms)
Tb-MCC	7.4	1.74 (SD = 0.03, n = 10)
	5.3	1.71 (SD = 0.03, n = 11)
IE^k/Tb-MCC	7.4	1.81 (SD = 0.04, n = 18)[a]
	5.3	1.82 (SD = 0.05, n = 14)

[a] Since hydroxyl groups quench Tb fluorescence, the lifetime of Tb is inversely related to the number of coordinating water molecules. The 0.1-ms difference in the lifetime of Tb between IE^k/Tb-MCC and Tb-MCC indicates that the number of coordinating water molecules is essentially the same in the free and bound peptides (Horrocks et al., 1977).

(Witt & McConnell, 1991), a value intermediate between MCC (>100 h; Reay et al., 1992) and MCC103A (~3 h). The more rapid dissociation rate of PCC could be explained by a weakening of this salt bridge due to the shift in position of the terminal Lys.

Rapid-Diffusion Fluorescence Energy Transfer. The importance of charge–charge interactions in peptide/MHC binding was further assessed through fluorescence energy transfer. In these experiments, the MCC peptide (88–103) was N-terminally labeled with a chelating agent containing the fluorescent donor terbium (Tb) (Tb-MCC) and bound to soluble IE^k (IE^k/Tb-MCC). As shown in Table II, Tb has essentially the same excited-state lifetime on the unbound peptide or the peptide/MHC complex at either pH. The efficiency of energy transfer was determined by measuring the lifetime of IE^k/Tb-MCC following additions of the energy acceptor Co^{2+}. Because Co^{2+} diffuses rapidly, the efficiency of energy transfer depends on the time-averaged distribution of acceptor molecules around the donor molecule during the lifetime of the donor (~1 ms). This distribution is determined by the concentration of acceptor molecules, the depth that the donor fluorophore is buried in a macromolecule (such as IE^k), and the electrostatic environment surrounding the donor and acceptor fluorophores (Stryer et al., 1982). The rapid-diffusion limit applies here. The efficiency of energy transfer of Co^{2+} is expressed in terms of a shortening in the lifetime

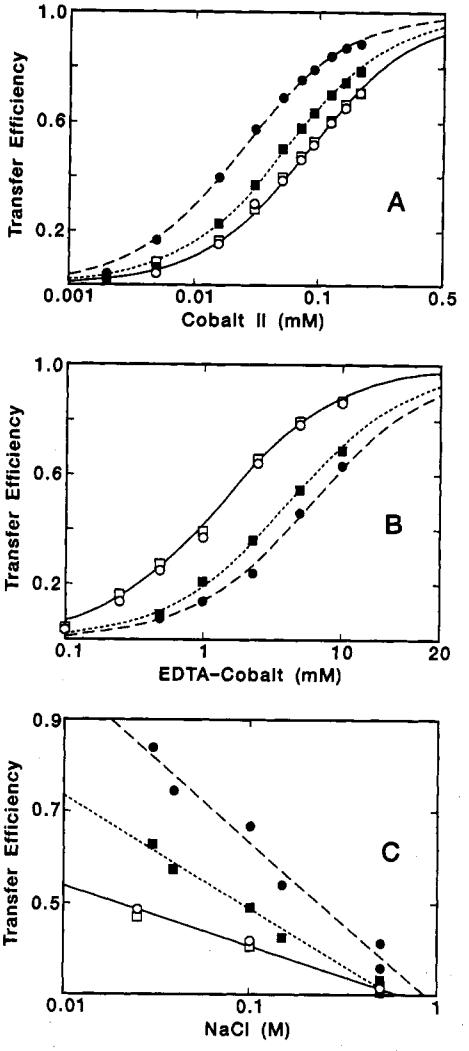


FIGURE 5: Efficiency of energy transfer from IE^k Tb-MCC and Tb-MCC to Co^{2+} (A and C) and $(EDTACo)^{2-}$ (B). (A and B) The efficiency of energy transfer was calculated from the measured lifetime of Tb-MCC at pH 5.3 (open squares) or pH 7.4 (open circles), and IE^k/Tb-MCC at pH 5.3 (solid squares) or pH 7.4 (solid circles). The lines represent a least-squares fit of the data assuming that the rate of energy transfer is proportional to ρ, where ρ is the density of acceptors. Tb-MCC was in H/M buffer, with 50 mM NaCl, and IE^k/Tb-MCC was in H/M buffer, with 100 mM NaCl. (C) The efficiency of energy transfer was calculated after addition of NaCl to Tb-MCC at pH 5.3 (open squares) or pH 7.4 (open circles), and IE^k/Tb-MCC at pH 5.3 (solid squares) or pH 7.4 (solid circles) in the presence of 50 μM Co^{2+}. The lines are empirically drawn. All curves were reproduced at least twice.

of Tb (see Materials and Methods). The high transfer efficiency at 50 μM Co^{2+} at pH 5.3 and 7.4 for bound and unbound peptide (Figure 5A) indicated that Tb to Co^{2+} transfer occurred by an exchange mechanism that required overlap of their electrons (Meares & Yeh, 1981). Hence the Tb atom on the amino terminus of the bound peptide is exposed to the aqueous solvent and not buried under protein at either pH. This is consistent with 2-D NMR studies which show that the N-terminus of MCC(88–103) extends out of the peptide binding groove and into the solvent (Driscoll et al., 1993).

The rate of energy transfer by the electron exchange mechanism to a charged acceptor is very sensitive to the electrostatic potential surrounding the donor Tb (Meares & Yeh, 1981; Stryer et al., 1982; Thomas et al., 1978; Wensel & Meares, 1983; Wensel et al., 1986; Meares & Rice, 1981). To monitor the charge environment of IE^k near the amino terminus of the peptide, the lifetime of Tb was compared

following additions of the energy acceptors, Co^{2+} or $(EDTACo)^{2-}$, to IE^k/Tb-MCC or Tb-MCC at pH 7.4 or 5.3. For Tb-MCC, the efficiency of energy transfer to Co^{2+} (Figure 5A) and $(EDTACo)^{2-}$ (Figure 5B) was independent of pH. Energy transfer from IE^k/Tb-MCC to Co^{2+} was higher than from Tb-MCC at both high and low pH; the difference was greater at high pH (Figure 5A). At a concentration of 16 μM Co^{2+}, energy transfer to the complex was twice as efficient at pH 7.4 as at pH 5.3. The reason for this difference is that Co^{2+} (the energy acceptor) is attracted to the negative charge of the MHC in proximity to the amino terminus of the bound peptide.

The reverse effect of pH on transfer efficiency is expected if a negatively charged acceptor is used in place of positively charged Co^{2+}. Indeed, repulsion of $(EDTACo)^{2-}$, due to more negative charge at pH 7.5 near the Tb, decreases the efficiency of energy transfer relative to pH 5.3 (Figure 5B). Hence, more negative charge is present on IE^k near the Tb on the bound peptide at pH 5.3 than on the unbound control peptide at either pH. Most importantly, and in line with the results described in the previous section for the C-terminus of MCC, IE^k is more negatively charged near the Tb on the bound peptide at pH 7.4 than at pH 5.3.

As in earlier experiments, we confirmed this apparent charge dependence by varying the ionic strength. We measured the lifetime of IE^k/Tb-MCC or Tb-MCC at pH 7.4 or 5.3 in the presence of a constant Co^{2+} concentration and varying concentrations of NaCl (Figure 5C). While energy transfer from bound or unbound Tb-MCC to Co^{2+} is decreased by NaCl at either pH, the steeper slope at pH 7.4 for the complex confirms the presence of more negative charge near the peptide binding groove at that pH.

DISCUSSION

One practical aspect of the data presented here is that care must be taken concerning the ionic strength of buffers used for measuring association reactions between peptides and MHC molecules. One common buffer used is McIlvaine's citrate/phosphate buffer (McIlvaine, 1921). Unfortunately, the ionic strength of this buffer changes from about 0.1 to 0.2 M in going from pH 4.8 to 7.2, which makes it unsuitable for comparisons of binding across this pH range. This indicates that the relative difference in antigen binding at acidic versus neutral pH can be buffer-dependent and this could be a factor in the variation in pH effects reported by different laboratories.

The binding of both Hb and MCC peptides to IE^k at pH 7.5 is very dependent on ionic strength, suggesting a large electrostatic contribution to binding at this pH. In contrast, the binding of both peptides at pH 5.4, although accelerated relative to binding at pH 7.5, is unaffected by large changes in ionic strength. Taken together, these data suggest that the mechanism of peptide binding is pH-dependent. The lack of an effect of ionic strength at acidic pH has been noted previously for the binding of a hen egg-white lysozyme peptide to IE^d (Jensen, 1991). Since binding at acidic pH lacks a significant electrostatic component, we conclude that the accelerated association rate may be due to an exposure of nonpolar pockets for van der Waals interactions and/or residues capable of forming hydrogen bonds. Alternatively, nonpolar binding sites could be produced by the protonation of ionized side chains in the absence of a conformational change. The binding of antigen to class I MHC molecules involves deep cavities in the peptide binding groove, referred to as "specificity pockets", that bind the termini of the peptide and some of the side chains (Madden et al., 1991; Garrett et al., 1989). It is possible that similar pockets in class II MHC molecules become more accessible at acidic pH. A number of experimental observations support this hypothesis. At weakly acidic pH, soluble IE^k becomes more susceptible to SDS dissociation and the binding of anilinonaphthalene-sulfonate, a probe for nonpolar pockets (Boniface et al., unpublished results). Low-pH treatment can produce a form of a class II MHC molecule, termed "floppy", that migrates more slowly on SDS–PAGE gels (Dornmair et al., 1989). Fluorescence spectroscopy studies (Kropshofer et al., 1991) have also suggested that a perturbation of Trp environments in HLA-DR occurs with peptide binding and these Trp residues are predicted to lie in deep pockets under the helices. Additionally, there is evidence for tight van der Waals contacts occurring between aromatic side chains of IE^k and Ile95, Ala96 and Ala101 of MCC (Driscoll et al., 1993). For class I MHC molecules, it also seems that a significant number of contacts occur through hydrogen bonds to main-chain "backbone" atoms of bound peptide (Madden et al., 1992; Matsumura et al., 1992; Fremont et al., 1992). Given that the formation of these bonds would be relatively insensitive to the ionic strength, they may be of similar importance for binding to class II MHC molecules. Although contributing significantly to the binding energy, interactions of this type would provide little specificity.

The lack of binding by MCC103A at pH 7.5 and the salt inhibition of MCC binding, together, strongly suggest that Lys103 is mediating an electrostatic interaction between peptide and IE^k at neutral pH. In addition, these data show that tolerance to some amino acid substitutions and, therefore, specificity change with pH. The association rates of MCC and MCC103A at pH 5.4 are similar, since the weaker binding of MCC103A can be explained by its rapid dissociation rate. The association rate of MCC103A at pH 7.5 must be much slower than at pH 5.4 since it is unable to bind IE^k at pH 7.5 and has similar dissociation rates at the two pH's. Hence, the modification of peptides can influence the association rate of one pH but not at another. It is unclear why the binding of MCC103A at pH 5.4 is stimulated by NaCl concentrations up to 0.75 M. This could be due to a salt stabilization of IE^k that prevents a competing $\alpha\beta$ dissociation, a reaction that has been shown to occur (Witt & McConnell, 1991; Tampe & McConnell, 1991; Boniface, unpublished observation), or to an effect on the structure of water that enhances the hydrophobic effect (McDevit & Long, 1952).

Interestingly, the rapid dissociation rate of the MCC103A peptide also suggests that the electrostatic interaction involving Lys103 is necessary for the stable association of IE^k and MCC. In support of this view are results showing that MCC analogs lacking residue 103 [e.g, MCC(88–102)] are 6000–40 000 fold weaker than MCC in stimulating relevant T-cells when presented by IE^k-expressing APC (Fox et al., 1987). A substitution of an Ala at position 103 in MCC, however, had little effect in this *in vitro* T-cell activation assay (Fox et al., 1987). The pH dependence of the salt effect on MCC and Hb binding suggests that the MHC carboxyl group contacting the C-terminal residue of the peptide titrates between pH 5.4 and 7.5. Alternatively, it is possible that the carboxyl is ionized at pH 5.4 but that its role in the charge–charge attraction of peptide is overshadowed by other forces or a conformational change. If this carboxyl group does titrate between pH 5.4 and 7.5, one puzzling question remains: At acidic pH, why does MCC not show a rapid dissociation, like MCC103A, due to the lack of a salt bridge? One possibility is that the long side chain of Lys makes a greater number of van der Waals contacts than does Ala. This seems unlikely given the large difference in dissociation rates. Alternatively, at pH 5.4, the

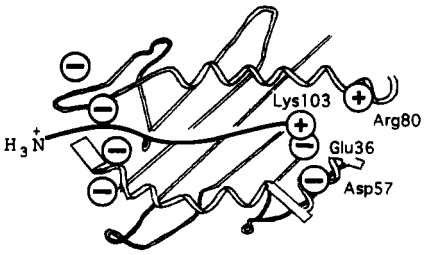


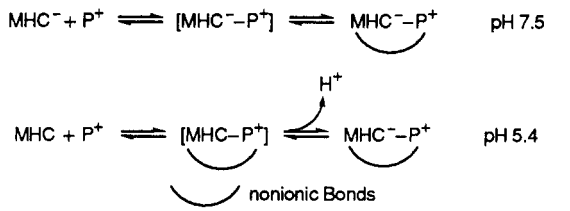
FIGURE 6: Shown is a model for possible electrostatic interactions occurring between MCC and IE^k. The ϵ-amino group of Lys103 of MCC is predicted to be 4–5 Å from the carboxylates of Glu36 and Asp57 of the β chain. Arg80 of the α chain is shown since it probably forms an interhelix salt bridge with Asp57 (Brown et al., 1988, 1993). The negative charges at the "left-hand" side of the binding groove are included to illustrate the approximate position of titrating charge-(s) determined by energy transfer.

carboxylate of interest in IE^k, although protonated in the empty molecule, may ionize upon peptide binding, due to the proximity of the positively charged side chain of Lys103. Although this is speculative, 2-D NMR data of MCC/IE^k complexes (Driscoll et al., 1993) indicate that the relative position of the peptide is the same at these two pH and thus Lys103 should be positioned next to the carboxylate at both pH values. This hypothesis may also help to explain why MCC and also other peptides have very different association rates but nearly the same dissociation at these two pHs (Reay et al., 1992; Sette et al., 1992; Jensen, 1991; Mouritsen et al., 1992). Whether electrostatic forces prove to be a general mechanism of forming long-lived class II–peptide complexes awaits further investigation.

To determine which negatively charged residues in IE^k might form a salt bridge with Lys103 of MCC, a model of IE^k based on the crystal structure of class I HLA-A2 was generated (M. Levitt, personal communication). MCC (residues 95–103) was docked in the peptide binding groove using available information on peptide structure, orientation, and position (Driscoll et al., 1993; Ronchese et al., 1987; Reay et al., 1993), with steric clashes eliminated by energy minimization. A cartoon summarizing the results of this modeling is shown in Figure 6. Two candidate carboxylates are Asp57 and Glu36 of the β chain (numbering based on IA; Klein, 1986), which are both located 4–6 Å from the ϵ-amino group of the Lys103. Asp57 probably forms a conserved interhelix salt bridge with Arg80 of the α chain (Brown et al., 1988, 1993) that may stabilize this end of the binding groove. The titration of this residue may provide an interesting mechanism of groove "opening" and raises the possibility that peptide may substitute for bonds in class II MHC molecules upon binding. Site-directed mutagenesis is currently under way to test whether either of these residues has a role in peptide binding or the pH-dependent conformational change.

The fluorescence energy-transfer data show that the peptide binding groove of IE^k adjacent to the N-terminus of the bound MCC also has electrostatic groups titrating between pH 7.5 and 5.4. The most likely mechanism for this would be the titration of uncompensated carboxyl groups of Asp and/or Glu residues or His residues that are salt-bridged to Asp or Glu residues. Since site-directed mutations of upper domain His residues did not reduce the enhancement of peptide binding at acidic pH (Wettstein and Davis, unpublished results), the former seems the more likely explanation. This observation is interesting for several reasons. First, the acidic residues detected by energy transfer are titrating in a pH range (pH 6–7) significantly above the normal range (pH 4–5). Second, they titrate in the same pH range in which peptide binding is sharply enhanced (Wettstein et al., 1991), and, therefore, they may be helping to drive the "opening" of the groove. Third, since they are present in the peptide binding groove, they could affect the electrostatic association of other peptides restricted to IE^k. Finally, since the exchange mechanism for energy transfer predominates at distances less than 11 Å (Stryer et al., 1982; Meares et al., 1981), we estimate the position of these acidic amino acids on IE^k to be at the extreme "left" side of the groove [as viewed in Brown et al. (1988); see Figure 6]. Therefore, these titratable charges may explain the superagonist activity of peptides with N-terminal positively charged residues as well as the weaker activity of PCC analogs with N-terminal negative charge (Fox et al., 1987; Collawn et al., 1989). The adverse effect of a repulsive electrostatic interaction between an antigenic peptide and an MHC molecule has recently been demonstrated (Boehncke et al., 1993). Knowledge of the location of unpaired charged residues in class II MHC molecules should allow the production of high-affinity peptide analogs either by adding attractive interactions that do not occur naturally or by deleting respulsive forces.

Scheme I



It has recently been proposed that peptide dissociation from IA^d is controlled by a rate-limiting conformational change in the MHC molecule and is independent of the amino acid sequence of the peptide (Witt et al., 1992). We show, on the contrary, that the dissociation kinetics of MCC from IE^k can be dramatically altered by amino acid substitution in the MCC peptide. We have also shown previously that a peptide (from sperm whale myoglobin) known to elicit a weak immune response, *in vivo*, has a 10-fold faster dissociation rate from IE^k than MCC peptide (Reay et al., 1992). Therefore, the results of Witt et al. are clearly not generalizable to all peptide/MHC combinations.

The mechanism shown in Scheme I summarizes a model that is consistent with out data and provides an explanation for the existence of a kinetic intermediate at neutral pH previously described (Sadegh-Nasseri & McConnell, 1989). We propose that peptide binding at neutral pH occurs by a sequential reaction in which the first step is the electrostatic interaction of peptide with a "closed" IE^k molecule containing the appropriate negative charge. This reaction, which could have fast-on, fast-off kinetics, leads to the formation of an electrostatic intermediate [MHC^-–P^+]. The effect of NaCl (Figure 2) would be to shield the two charged reactants and inhibit the overall reaction by diminishing the formation of [MHC^-–P^+]. A second reaction primarily involves the formation of nonionic bonds and occurs at a rate that is dependent on the transition to an "open" MHC conformation with exposed nonpolar pockets. At low pH, the "open" structure is stabilized. Because the MHC molecule lacks the appropriate negative charge, the peptide binds nonionically to form [MHC–P^+]. A second step involving a peptide-induced salt bridge may help to make the two complexes (formed at pH 5.4 or 7.5) indistinguishable by conformational probes (Driscoll et al., 1993). These reaction mechanisms represent the most prevalent pathway to form complex and do not include any competing reactions.

In conclusion, the mechanism of peptide binding to IE^k changes with pH because the relative contribution of the forces

involved is pH-dependent. Binding at pH 7.5 is absolutely dependent on Lys103 of MCC, while binding at pH 5.4 occurs when this Lys is replaced with an Ala residue. Additionally, it may be more difficult for IE^k to present an antigenic peptide that is more nonpolar in nature (of which MCC103A is an example) without exposure to an acidic environment. Hence, the specificity and degree of degeneracy of binding are also affected by pH. Knowledge of the forces required for peptide binding could allow the production of high-affinity peptide analogs that increase the effectiveness of peptide vaccine strategies or the treatment of autoimmune disorders. Since a class II molecule may encounter a range of pH in the endosomal pathway (Peters et al., 1991; Guagliardi et al., 1990) it is possible that different peptides will bind by different mechanisms. This trafficking of class II molecules through compartments of varying pH may allow the presentation of a broader spectrum of antigens and contribute to a more complete immune defense.

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