Long-lived Complexes between Peptide and Class II Major Histocompatibility Complex Are Formed at Low pH with No Requirement for pH Neutralization

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Summary

The binding of peptide antigens to class II histocompatibility glycoproteins can be markedly enhanced at pH values approximating those found in acidic endosomal compartments in antigen-presenting cells (APC). It has been proposed by others that low pH may increase the conformational flexibility of class II, facilitating both the association and dissociation of peptides. Neutralization of pH, as class II is expressed on the plasma membrane of APC, could then serve to trap peptide in a stable complex. If this were the only mechanism accounting for enhanced peptide binding at low pH, one would predict that there should be a concordance between the pH conditions required for enhanced binding and those associated with increased peptide dissociation. Furthermore, long-lived complexes of class II and peptide should not be observed at low pH without neutralization. In the present communization, I provide the data that support the generality of my previous conclusion that both affinity and maximal binding are regulated by pH in experiments using purified class II and biotin-labeled peptides. The pH profile for binding and dissociation using three different class II glycoproteins was analyzed, and the results demonstrated that enhanced binding is not coupled to enhanced dissociation. Peptide complexes were observed to be quite stable at pH 4.5 and above. This result was further substantiated in experiments where biotin-peptide/class II complexes were extensively dialyzed at low pH followed by analysis on Western blots probed with avidin. Finally, a low pH assay system was devised to analyze the formation of stable peptide/class II complexes without pH neutralization. Our results indicate that stable complexes can be formed at low pH without the requirement for a shift to neutral pH.

The antigen processing pathway includes various intracellular events required for the formation and cell surface expression of stable complexes composed of class II major histocompatibility glycoproteins and antigen-derived peptides (1, 2). Variation in the pH among different subcellular sites in APC may have a major effect in regulating these events by influencing antigen proteolysis, class II trafficking, and peptide loading. Previous studies from our laboratory have demonstrated that the binding of peptide Ag to class II is markedly facilitated at pH values approximating those present in acidic endosomal compartments in APC (3, 4). Initial experiments indicate that the functional association of peptide Ag with aldehyde-fixed B cells or isolated B cell membranes, as measured by T cell activation, was increased at pH 5 as compared with pH 7 (3). Subsequent experiments in our laboratory were focused on an analysis of the association of HEL(104-120) with purified I-E^d, and demonstrated that both the apparent affinity and the apparent concentration of peptide-binding sites are increased at pH 5 (4). The rate of association was increased at this pH, with no measurable effect on dissociation. It was proposed that the protonation state of a critical group(s) in class II regulates its capacity to form stable complexes with peptide.

An earlier study from this laboratory had demonstrated that peptide/class II complexes were remarkably stable and that acidification below pH 4 was required for rapid peptide dissociation (5). Membrane-associated complexes were observed to be stable during 2-h incubations at 37°C in pH 4 buffer. These results were confirmed and extended by Lee and Watts (6) in their analysis of the stability of complexes containing peptide and purified I-A^d. These complexes were observed to be stable for at least 8 h at 37°C in pH 5 buffer. Harding et al. (7) reported that the rate and maximal extent of binding of a labeled peptide to purified I-A^k was increased at pH 5.5 as compared with pH 7.2. Preformed peptide complexes were stable at pH 5.5, and dissociation was observed only at pH 4 or below (7). Thus, a variety of findings support the conclusion that pH values considerably below those required for enhanced peptide binding are necessary for enhanced peptide dissociation. By contrast, others have reported a concordance between the pH dependence of peptide association and dissociation such that increased dissociation was

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observed at the same hydrogen ion concentrations required for enhanced peptide binding (8, 9). This is an important issue because the latter results would imply that pH-regulated peptide dissociation may allow reuse of recycling class II for presentation of multiple antigens. Sadegh-Nasseri and Germain (9) have recently demonstrated rapid generation of long-lived peptide/class II complexes by initial incubation at low pH followed by a rapid stabilization event that occurs during neutralization. This may be visualized as a pH-dependent peptide trapping event. All of our previous assays for measuring peptide/class II complex formation required neutralization before analysis. Therefore, we could not exclude the potential importance of pH neutralization in stabilizing peptide/class II complexes. The current study was undertaken to address these issues.

Materials and Methods

Peptide Binding Assay. Class II was purified from detergent-solubilized A20 (10) or CH27 (11) B lymphoma membrane preparations using 10-2-16 (12) (I-A^k) or 14-4-4 (13) (I-E^k, I-E^d) immunoaffinity columns as previously described (4). Class II (50–100 nM) was incubated with biotin-peptide in microcentrifuge tubes at 37°C in 30 μl binding mixture, containing 0.2% NP-40, 50–100 mM citrate/phosphate, 1 mM PMSF, 2 mM EDTA, 5 mM *N*-ethyl maleimide, and 0.01% sodium azide (binding buffer). To measure bound peptide, samples were diluted to 200 μl in 200 mM Tris buffer, pH 7.5, containing 5% skim milk, 0.1% BSA, 0.1% Tween 20, and 0.5% NP-40, and incubated on microtiter assay plates coated with the same mAb used in class II purification. Assay plates were prepared by initial incubation with 50 μg/ml affinity-purified goat anti-mouse Ig followed by incubation with 50–200 μg/ml purified mAb. Plates were blocked with 50 mM Tris, pH 7.5 containing 5% skim milk, 0.1% BSA, and 0.1% Tween 20, and thoroughly washed before sample addition. Bound biotinylated peptide was detected by incubation with 5 μg/ml avidin-alkaline phosphatase for 60 min at 24°C followed by incubation with 1 mg/ml *p*-nitrophenyl phosphate in 4 mM $MgCl_2$, 0.5 M Tris, pH 10. Data represent mean absorbance at 405 nm. In some experiments, all steps in the immunoassay were performed at pH 5.5. Sample pH was adjusted with 1 M citrate/phosphate, and samples were diluted in 100 mM citrate/phosphate, pH 5.5, containing 5% skim milk, 0.1% BSA, and 0.1% Tween 20. A pH 5.5 solution containing 0.1% Tween 20, 0.15 M NaCl, and 100 mM citrate/phosphate was used in washing steps and during incubations with avidin-alkaline phosphatase.

Peptides. Peptides were synthesized in the Emory University Microchemical Facility as previously described (5). Sequences of peptides were HEL(104–120), GMNAWVAWRNRCKGTDVY; HEL(46–61), NTDGSTDYGILQINSR; pCc(91–104), RADLIAYLKQATAK; mCc(82–103), FAGLKKANERADLIAYLKQATK. HEL(104–120) and HEL(46–61) were biotinylated by reaction with excess biotin-amidocaproate *N*-hydroxysuccinimide ester in *N,N*-dimethyl formamide, followed by precipitation with acetone and HPLC purification (4). Biotin was attached through the free α amino in HEL(46–61) and through both α and ε amino groups in HEL(104–120). The modified peptides retained biological activity. Biotinylated derivatives of pCc(91–104) were inactive in functional and binding assays because of modification in critical COOH-terminal lysines. A biotin-labeled derivative of the longer related peptide, mCc(82–103), was therefore used in I-E^k binding experiments. The peptide, generously provided by Dr. Susan Pierce (Evanston, IL) was biotinylated by reaction with a 1.5-mol excess of biotin-ester. This derivative was active, presumably because of preferential reactivity with NH_2-terminal lysine amino groups.

Western Blot Assays. Purified class II (0.2 μg) was incubated with biotin-peptide for 18 h at 37°C in a 10-μl volume of 0.1% NP-40, 50 mM citrate/phosphate containing 2 mM EDTA, and 0.01% sodium azide. Samples were neutralized with 2 μl 1 M Tris, pH 7.5, followed by addition of 5 μl SDS sample buffer without 2-ME, incubation for 10–15 min without heating, and electrophoresis on linear 10% SDS-polyacrylamide gels. Gels were run at 190 V for ~50 min, and protein was transferred to nitrocellulose membranes for 1 h at 100 V in 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. Biotin-peptide/class II complexes were detected with avidin-alkaline phosphatase and chemiluminescence substrate (Western-Light Test Kit; Tropix, Bedford, MA).

Results and Discussion

We have previously reported that rate of peptide association, the affinity, and the apparent number of binding sites at saturation are increased when measuring binding of HEL(104–120) to I-E^d at pH 5, as compared with pH 7. The generality of these findings is further substantiated with experiments analyzing the binding of peptides to purified I-E^k and I-A^k. The rate of association of biotin-mCc(82–103) with I-E^k is substantially increased at low pH (Fig. 1 *a*) in agreement with the results of Wettstein et al. (14). Scatchard plots (15) of saturation binding data indicate that both the apparent K_d and maximum binding sites (B_{max}) are sensitive to proton concentrations in the range of pH 5–7. K_d values were calculated at 0.60 and 0.28 μM for pH 6.0 and 5.0, respectively (Fig. 1 *b*). The calculated B_{max} increased greater than threefold over this pH range. Too little binding is detected at pH 7 to allow calculations for this peptide/class II combination. Similarly, biotin-HEL(46–61) bound I-A^k better at pH 5.0 as compared with pH 7.0 (Fig. 1 *c*). Calculated K_d values decreased from 1.07 to 0.09 μM with a less impressive (~1.6-fold) increase in B_{max}. These results are similar to data previously published by Harding et al. (7). Note that the K_d value of 1.07 μM is in reasonable agreement with that originally reported for HEL(46–61)/I-A^k using a different assay (16). Thus, pH can have a variable relative effect on affinity and maximal binding depending on the peptide/class II combination studied, although both parameters are affected. Whether this is characteristic for individual class II glycoproteins remains to be determined.

Single time-point binding and dissociation experiments were performed to analyze the relationship between the pH dependence of association and dissociation for several class II glycoproteins (Fig. 2). Preformed biotin-peptide/class II complexes were incubated for 48 h at 37°C in the presence of 100 μM unlabeled peptide, neutralized, and remaining complexes measured by immunoassay. Substantial dissociation of peptide from I-E^k (Fig. 2 *a*), I-A^k (*b*), or I-E^d (*c*) required acidification below pH 4.5. By contrast, enhanced binding was observed at pH 4.5 and above (Fig. 2, *d–f*). There is a clear discordance between the pH conditions required for dissociation and those required for enhanced association. Indeed, very little binding was observed at hydrogen ion concentrations sufficient for significant peptide dissociation, consistent

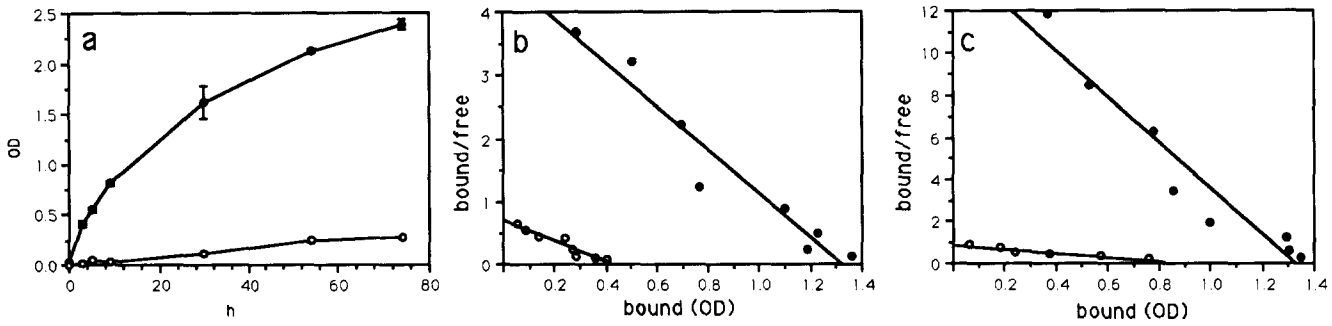

Figure 1. Effect of pH on binding of peptides to purified class II. (*a*) Increased rate of association of peptide with I-E^k at pH 5. Purified I-E^k was incubated with 5 μM biotin-mCc(82–103) for various time periods in triplicate tubes at pH 7.0 (*open symbols*) or pH 5.0 (*filled symbols*). (*b*) Scatchard plots of pH 6.0 (*open symbols*) and pH 5.0 (*filled symbols*) peptide/I-E^k binding data. Various concentrations of biotin-mCc(82–103) were incubated with I-E^k in triplicate for 68 h, and specific binding was determined by immunoassay as described in Materials and Methods. Nonspecific signal (<15% of maximum absorbance at each point) was measured by addition of 280 μM pCc(91–104) and subtracted from the data. (*c*) Scatchard plots of pH 7.0 (*open symbols*) and pH 5.0 (*filled symbols*) peptide/I-A^k binding data. Various concentrations of biotin-HEL(46–61) were incubated with purified I-A^k in triplicate for 68 h. Nonspecific signal (<10% of maximal absorbance) was determined in the presence of 250 μM unlabeled HEL(46–61).

with the possibility that dissociation may result from irreversible class II denaturation. It has been our experience that purified class II protein loses all peptide binding activity after exposure to pH 3.0–3.5 or below (data not shown).

The stability of peptide/class II complexes after exposure to low pH was further addressed using Western blot assays (Fig. 3) similar to those used by Sadegh-Nasseri and Germain (9). A significant fraction of class II heterodimers remain associated after exposure to SDS without heating when analyzed by SDS-polyacrylamide gels. Peptides remain associated with the dimers during electrophoresis (9, 17, 18). Purified I-E^k was incubated for 18 h with 3 μM biotin-mCc(82–103) at low pH, and unheated neutralized samples were analyzed on Western blots probed with avidin. Excess unlabeled pCc(91–104), but not HEL(46–61), blocked formation of biotin-peptide/I-E^k complexes corresponding to the known specificity of these peptides for binding I-E^k (Fig. 3 *a*). A reciprocal pattern of inhibition was observed with biotin-HEL(46–61)/I-A^k (Fig. 3 *b*). The pH profile for peptide binding measured in this assay corresponded reasonably

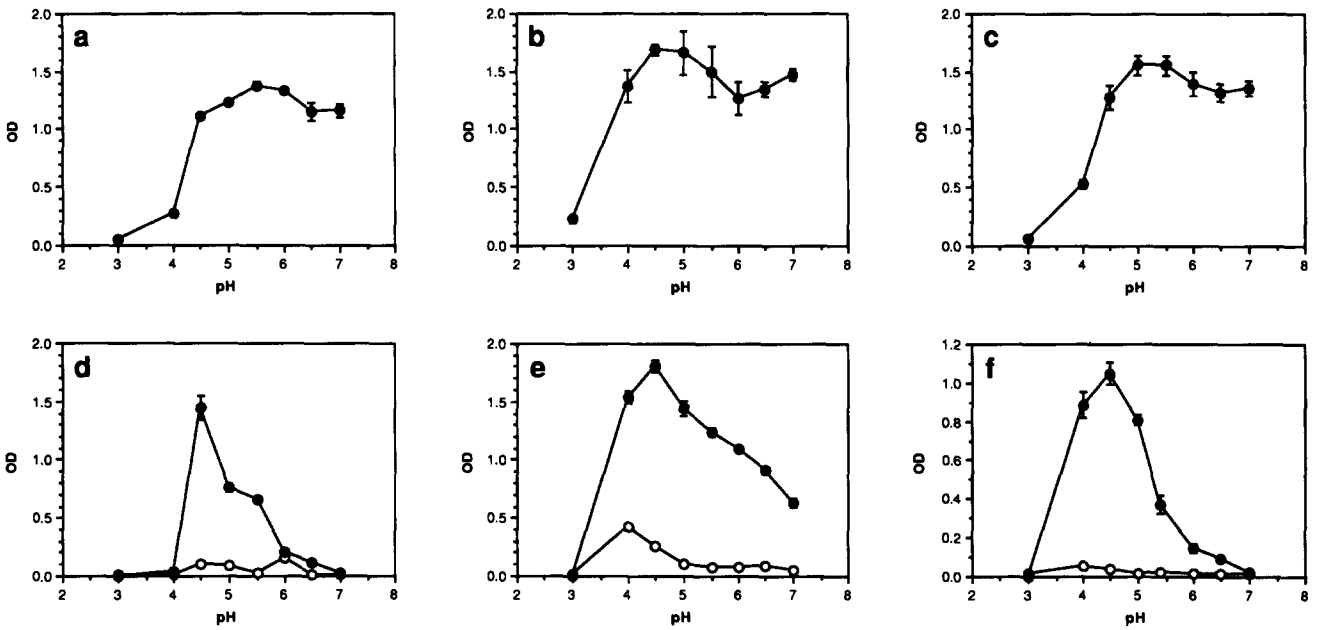

Figure 2. Discordance between pH conditions required for peptide dissociation and optimal peptide binding. (*a–c*) pH dependence of peptide dissociation. Triplicate tubes containing preformed biotin-peptide/class II complexes were incubated for 48 h at 37°C in binding buffer containing 100 μM unlabeled peptide and a total concentration of 100 nM class II at the indicated pH. Complexes were initially formed by incubation with 10 μM biotin-peptide at pH 5 followed by dialysis in PBS to remove free peptide. (*d–f*) pH dependence of peptide association. Purified class II was incubated with 2 μM biotin-peptide for 40 h at 37°C in triplicate tubes at the indicated pH. Specific binding (*filled symbols*) was determined by substraction of the absorbance measured in the presence of 200 μM unlabeled peptide (*open symbols*). Binding was measured by immunoassay as described in Materials and Methods. (*a* and *d*) Biotin-mCc(82–103)/I-E^k; (*b* and *e*) biotin-HEL(46–61)/I-A^k; (*c* and *f*) biotin-HEL(104–120)/I-E^d.

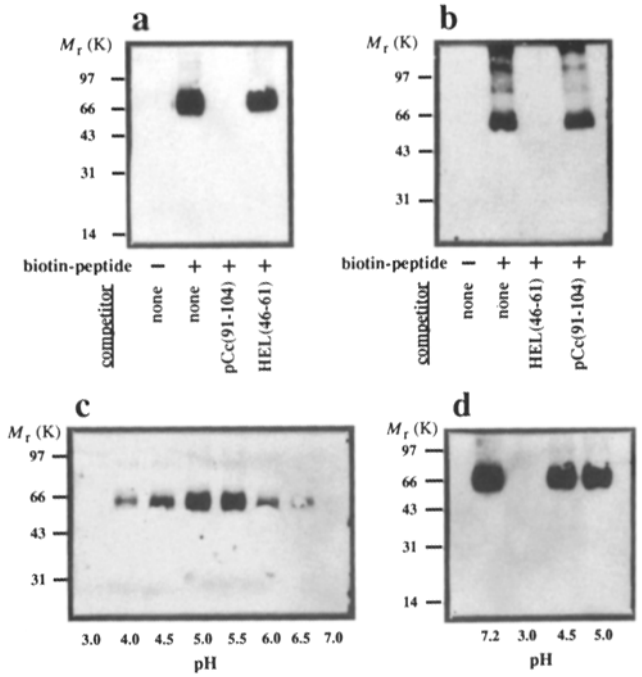

Figure 3. Western blot analysis of biotin-mCc(82–103)/I-E^k complexes. (*a*) Specificity of binding. Purified I-E^k (0.2 μg) was incubated with 3 μM biotin-mCc(82–103) for 18 h in a 10-μl volume of 0.1% NP-40, 50 mM citrate/phosphate, pH 4.5, in the presence or absence of 1 mM unlabeled peptide as indicated. Samples were neutralized with 2 μl 1 M Tris, pH 7.5, followed by addition of 5 μl sample buffer without 2-ME and electrophoresis on a linear 10% polyacrylamide gel. After transfer to nitrocellulose, complexes were detected with alkaline phosphatase–conjugated avidin and chemiluminescence. (*b*) Purified I-Ak was incubated with 3 μM biotin-HEL(46–61) and analyzed as described above. (*c*) pH dependence of complex formation. I-E^k was incubated with 0.5 μM biotin-mCc(83–103) for 18 h at the indicated pH followed by neutralization and Western blot analysis. (*d*) Stability of peptide complexes at low pH. Complexes were formed by incubation of I-E^k with 3 μM biotin-mCc(82–103) for 40 h at 37°C in pH 5.0 buffer. Aliquots were dialyzed against two changes of 0.15 M NaCl, 20 mM citrate/phosphate at the indicated pH for 48 h at 4°C, followed by neutralization and Western blot analysis.

well with that observed in functional assays in the binding immunoassay (Fig. 3 *c*). The extent of binding at pH 4.5 measured in this assay seems to be somewhat variable as compared with other assays. This could result from an increased sensitivity to SDS-induced denaturation after exposure to pH 4.5. Sadegh-Nasseri and Germain (9) have reported that biotin-peptide/I-E^k complexes are unstable after relatively brief exposure (1.5–3.5 h) to pH 4.5, dissociating into free α and β chains on SDS-PAGE, unless very high (100 μM) concentrations of peptide are present during sample neutralization. Slightly lower pH was required for peptide dissociation in our experiments (Fig. 2 *a*). This could reflect the use of different peptides or differences between particular preparations of I-E^k. To further examine the stability of complexes at low pH, I-E^k was incubated with 3 μM biotin-mCc(82–103) at pH 5.0, followed by 48 h of dialysis against buffers at pH 3.0, 4.5, 5.0, and 7.2 (Fig. 3 *d*). The complexes were quite stable at pH 4.5 and above. This result supports previous conclusions based on functional experiments (3, 5) and binding assays (Fig. 2) (4, 6, 7), indicating that complexes are stable at pH 5. We presume that very little biotin-peptide was present during much of the dialysis period and at the time of neutralization before electrophoresis in our experiments.

The results reported above support the conclusion that peptide/class II complexes are very stable in pH conditions that promote optimal peptide binding. The possibility that neutralization is required to stabilize the complexes by trapping them into a rigid conformation (9) could not formally be excluded because samples were neutralized in each experiment before measurement. Experiments were therefore carried out under conditions where class II was not neutralized after exposure to peptide at low pH. We found that the binding of mAb to class II and avidin conjugates to biotin were relatively unaffected in the range of pH 5.5–7.5. This allowed us to perform all steps in the immunoassay in parallel at either pH 5.5 or 7.2. Purified class II was incubated with biotin-peptide at pH 5.5 to allow complex formation. Samples were then dialyzed for 48 h at 4°C against buffers at pH 5.5 or 7.2. Aliquots were analyzed for stable peptide complexes using immunoassays performed at either pH. Neutralization of pH was clearly not required for the formation of long-lived complexes (Fig. 4). Indeed, shifting the pH during dialysis or at the time of immunoassay had no substantial effect.

Dornmair et al. (17) have described temperature-dependent effects on class II conformation that may relate to structural transitions important in peptide binding or dissociation. Low-pH dialysis was performed at 4°C in the experiments described in Figs. 3 and 4. We considered the possibility that low temperature may serve to stabilize peptide/class II complexes formed at low pH. To address this possibility, biotin-peptides were allowed to bind I-E^k or I-E^d at pH 5.5. Further binding was then inhibited by addition of a 750-fold excess of unlabeled peptide followed by incubation for 48 h at 37°C at pH 5.5 or 7.2 to allow dissociation of complexes that were not stable at 37°C. Remaining complexes were measured by immunoassay with incubations carried out at pH 5.5. The results indicate that stable complexes are readily formed at 37°C under conditions in which pH was never shifted above the initial value of 5.5 (Fig. 5).

Our initial observation that peptide/class II complex formation is facilitated at low pH has now been confirmed by a number of groups (7–9, 14, 19, 20). However, the mechanism underlying this effect remains controversial. We had previously reported that both the apparent affinity for HEL(104–120) and the maximal number of peptide binding sites on I-E^d are increased as hydrogen ion concentration is increased from pH 7 to 5 (4). Similar results are presented in the current study for two other class II glycoproteins, I-E^k and I-A^k (Fig. 1), supporting the generality of this finding and confirming the results of others (7, 14, 19). In preliminary experiments with ^{125}I-labeled I-E^k, 5–10% of labeled protein bound biotin-peptide under conditions approaching saturation at optimal pH. Therefore, the increased B_{max} observed at low pH cannot fully account for the surprisingly low fraction of class II–bearing peptide at saturation reported in many studies (8, 18), even if allowances are made for denatured protein. It is possible that only "empty" class II mole-

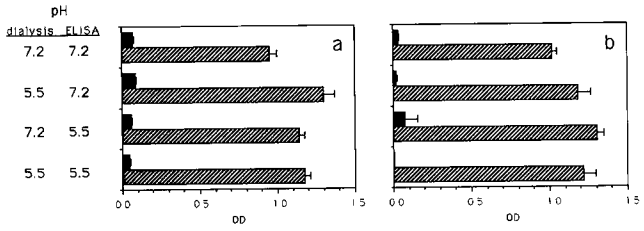

Figure 4. Generation of stable peptide complexes at low pH with no requirement for pH neutralization. (*a*) Purified I-A^k was incubated with 0.5 μM biotin-HEL(46–61) at pH 5.5 for 64 h at 37°C in the presence (*solid bars*) or absence (*hatched bars*) of 400 μM unlabeled peptide as a control for nonspecific binding. Unlabeled peptide (400 μM) was then added to all samples, and aliquots were dialyzed for 48 h at 4°C against three changes of buffer at pH 7.2 or 5.5., as indicated. Bound peptide was measured in quadruplicate by immunoassay with all steps carried out at pH 7.2 or 5.5. (*b*) I-E^d was incubated with 3 μM biotin-HEL(104–120) at pH 5.5 in the presence (*solid bars*) or absence (*hatched bars*) of 300 μM unlabeled peptide. The stability of peptide complexes was analyzed as described above.

cules can bind peptide and that the remarkable stability of most peptide complexes limits the generation of new sites by peptide dissociation during storage. It has been suggested that a substantial fraction of newly synthesized class II is expressed on the cell surface without bound peptide (21). However, mechanisms that may involve peptide exchange with replacement of a subset of the peptides already bound to class II cannot be excluded (22). Indeed, Stern and Wiley (23) have demonstrated that 100% of soluble recombinant DR1 molecules can bind peptide at neutral or acidic pH. A detailed analysis of the effect of pH on peptide binding in this system has yet to be done, and it is not clear that DR1 isolated from human cells binds peptide optimally at low pH (20). We have previously suggested that different class II proteins may differ with respect to the pH at which they generally prefer to bind

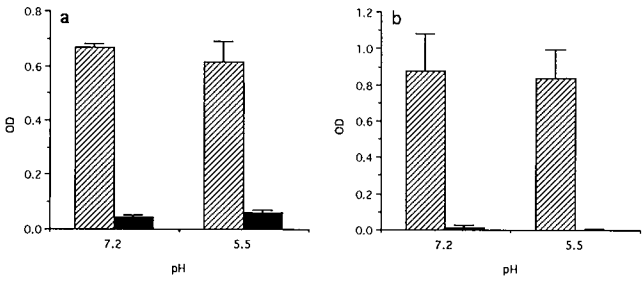

Figure 5. Stability of low pH complexes at 37°C. (*a*) Purified I-E^k was incubated with 2 μM biotin-mCc(82–103) in 30 mM citrate/phosphate buffer at pH 5.5 for 18 h at 37°C in the presence (*solid bars*) or absence (*hatched bars*) of 400 μM unlabeled peptide as a control for nonspecific binding. Samples were diluted threefold in 100 mM citrate/phosphate buffer, pH 5.5 or 7.2, containing protease inhibitors and a final concentration of 500 μM unlabeled peptide. Samples were incubated for a further 48 h at 37°C to allow dissociation of unstable biotin-peptide/class II complexes. Bound biotin-peptide was measured in quadruplicate by immunoassay with all steps carried out at pH 5.5. (*b*) I-E^d was incubated with 2 μM biotin-HEL(104–120) at pH 5.5 for 18 h in the presence (*solid bars*) or absence (*hatched bars*) of 400 μM unlabeled peptide. The stability of peptide complexes was analyzed as described above.

peptides (4). Reay et al. (19) have recently provided evidence that the maximum extent of peptide binding at equilibrium can be pH dependent in experiments using preparations of soluble I-E^k that are apparently free of bound peptides (19). This result and previous findings from our laboratory (3, 4) exclude a simple mechanism whereby available binding sites are increased after dissociation of previously bound peptides at low pH. We do note, however, that selected peptides may dissociate from class II more rapidly at pH 5 as compared with neutral pH (7, 8, 19). This could reflect electrostatic repulsion induced by alterations in the charge of peptide or class II (4).

We have previously proposed a model to account for the unusual characteristics of peptide/class II binding (4). We suggest that peptide may interact with class II in a rapidly reversible manner independent of pH in the physiological range. However, protonation of a critical group(s) in class II, in addition to an initial interaction with peptide, is required for a conformational transition that results in the stable complex. The protonation state of groups in class II may affect the conformational flexibility of the protein. Once formed, the complex is relatively insensitive to hydrogen ion concentrations in the range of pH 5–7, presumably because conformation is stabilized by interactions involving peptide or because of alterations in the pK or accessibility of ionizing groups in class II.

The results presented in the current report extend previous work by clearly demonstrating that there is no obligatory relationship between enhanced peptide binding observed at low pH and enhanced dissociation. Enhanced binding is observed at pH values well above those required for enhanced dissociation for the peptide/class II combinations used in our experiments (Fig. 2). Furthermore, the increased peptide binding observed in the range of pH 4.5–5.5 does not require a shift to higher pH to stabilize the complexes. This result would appear to contrast with that reported by Sidegh-Nasseri and Germain (9), who found that peptide/class II complexes were rapidly stabilized during brief exposure to neutral pH after an initial incubation at pH 4.5. We propose that their observations reflect a different pathway for peptide loading. This pathway requires exposure to pH values very near those required for irreversible denaturation of class II and requires high peptide concentrations and a pH shift for the recovery of stable complexes. The fraction of class II that binds peptide is apparently very high under these conditions, presumably because bound peptides are released during exposure to proton concentrations in the range that destabilize class II structure. A second pathway is observed at milder pH values above those required for destabilization of class II using lower concentrations of peptide. It is characterized by a selective loading of "empty " class II molecules and there is no requirement for a pH shift to form stable complexes. In both pathways interactions involving peptide are critical for stabilization of the complexes and effects induced by low pH on the structure of class II serve to enhance that rate of peptide association. We would expect that the latter pathway may be dominant under physiological conditions because pH

values required for destabilization of class II structure are less likely to be encountered. This leads to a further prediction that peptides are stably associated with newly synthesized class II molecules that have yet to reach the cell surface or encounter a neutral pH environment in APC.

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