A Structural Transition in Class II Major Histocompatibility Complex Proteins at Mildly Acidic pH

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Summary

Peptide binding by class II major histocompatibility complex proteins is generally enhanced at low pH in the range of hydrogen ion concentrations found in the endosomal compartments of antigen-presenting cells. We and others have proposed that class II molecules undergo a reversible conformational change at low pH that is associated with enhanced peptide loading. However, no one has previously provided direct evidence for a structural change in class II proteins in the mildly acidic pH conditions in which enhanced peptide binding is observed. In this study, susceptibility to denaturation induced by sodium dodecyl sulfate (SDS) detergent or heat was used to probe the conformation of class II at different hydrogen ion concentrations. Class II molecules became sensitive to denaturation at pH 5.5–6.5 depending on the allele and experimental conditions. The observed structural transition was fully reversible if acidic pH was neutralized before exposure to SDS or heat. Experiments with the environment-sensitive fluorescent probe ANS (8-anilino-1-naphthalene-sulfonic acid) provided further evidence for a reversible structural transition at mildly acidic pH associated with an increase in exposed hydrophobicity in class II molecules. IA^d conformation was found to change at a higher pH than IE^d, IE^k, or IA^k, which correlates with the different pH optimal for peptide binding by these molecules. We conclude that pH regulates peptide binding by influencing the structure of class II molecules.

$CD4^+$ T lymphocytes recognize antigen-derived peptides bound to class II major histocompatibility glycoproteins on the surface of APC. These peptide–class II complexes are generated in endosomal compartments in APC (1–4) where low pH is maintained by vacuolar H^+ ATPases (5). Agents that inhibit vacuolar acidification also inhibit antigen processing through several distinct mechanisms. Early studies demonstrated that chloroquine and ammonium chloride inhibit degradation of endocytosed protein antigens (6, 7). Antigen unfolding and degradation are necessary to generate peptides that can bind to class II molecules (8). Low pH is required for the optimal activity of acid proteases present in endosomes and lysosomes that degrade antigen, and acidification may also play a direct role in destabilizing the structure of native protein antigens (9). Chloroquine (10), monensin (11), and the highly specific H^+ ATPase inhibitor concanamycin B (12) also inhibit the release of invariant chain (Ii)[1] from newly synthesized class II molecules, causing accumulation of α/β–Ii complexes in endosomal compartments. The release of Ii requires the action of acid endopeptidases (13, 14), and Ii association prevents peptide binding by α/β dimers (11).

Hydrogen ion concentrations are also observed to directly regulate the binding of peptides to cell surface (15) and purified class II molecules (16–28). In general, peptide binding is markedly enhanced in the range of pH 4.5–6.5 compared with neutral pH. This range represents the hydrogen ion concentrations found in endosomal compartments (5). We and others have proposed that enhanced binding may be associated with a reversible conformational change induced by protonation of critical residues in class II molecules (16, 18–20, 27). Several studies have analyzed directly the effect of pH on the conformation of purified class II molecules, but no one has previously demonstrated a structural change at mildly acidic pH (pH $\geq$5.0). Dornmair et al. (29) reported that IE^k molecules assume a "floppy" conformation with higher apparent molecular size on SDS-PAGE during incubation at pH 4.0. However, Lee and Watts (30) observed no difference in the intrinsic tryptophan fluorescence of IA^d molecules at 3.0 and pH 7.6. In a later study, circular dichroism was used to analyze pH-dependent changes in the secondary structure of class II proteins (31). Changes in the structure of IA^d molecules were first observed at pH 4.9, with a progressive change in signal as pH was further reduced (31). Spectral changes in IE^d were observed at pH 3.0 but not at pH $\geq$4.0 (31).

[1] *Abbreviations used in this paper:* ANS, 8-anilino-l-naphthalene-sulfonic acid; CLIP, class II–associated invariant chain peptides; DP-PC, dipalmitoyl phosphatidylcholine; Ii, invariant chain; λ_{max}, emission peak maximum; SOG, 1-S-octyl beta-D-thioglucopyranoside.

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Sadegh-Nasseri and Germain (18) reported that after exposure to pH 4.5, IE^k molecules rapidly assume an unstable conformation and that rescue from irreversible denaturation requires exposure to high concentrations of antigenic peptide during pH neutralization. This structural change was not observed above pH 4.5 (18). Overall, previous studies have only demonstrated differences in class II structure at extremely acidic pH (<5.0).

Peptide-binding experiments have suggested that class II molecules may differ with respect to the pH at which they prefer to bind antigenic peptides (16, 19). IA^d molecules have significant peptide-binding activity at neutral pH, with optimal binding at pH 5.5–6.0. By contrast, very little binding to IE^d is observed at neutral pH. Incremental increases in peptide binding to IE^d are observed as hydrogen ion concentrations are increased in the range of pH 6.5–4.5. In all cases, peptide binding activity is significantly enhanced at mildly acidic pH in the range of pH 6.0–6.5. The class II structural changes described above were observed only at much lower pH values, raising questions about their relevance to the mechanism for pH regulation of peptide binding. In this study, we demonstrate that class II proteins undergo a reversible structural transition at mildly acidic pH. The pH dependence of this structure change corresponds to the observed pH dependence of peptide binding. Our results support the hypothesis that pH regulates peptide loading by controlling the conformation of class II molecules and that this effect does not depend on the nature of the specific peptides bound to these molecules.

Materials and Methods

Purification of Class II MHC Proteins. Class II proteins were purified from detergent-solubilized A20 (32) or CH27 (33) B lymphoma membrane preparations using 10-2-16 (IA^k), MKD6 (IA^d), or 14-4-4 (IE^k, IE^d) mAb immunoaffinity columns as previously described (16). Samples were stored in buffer containing 1% *N*-octylglucoside at 4°C and analyzed by SDS-PAGE and Coomassie blue staining. Protein concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

pH-induced SDS Denaturation Assay. Purified IE^d (0.2 μg/sample) or IE^k (0.35 μg/sample) were diluted into a 10-μl vol with 0.2% NP-40 and 20 mM citrate/phosphate buffer at the indicated pH and equilibrated for 15 min at 23°C. Samples were incubated for an additional 20 min with 4 μl SDS solution (4% SDS, 15% glycerol, and 10% bromophenol blue) before or after neutralization by addition of 3 μl 1 M Tris, pH 7.0. Alternatively, the pH of NP-40 or *N*-octylglucoside cell lysates was adjusted with 20 mM citrate/phosphate buffer, and SDS was added to a final concentration of 1–2%. After a 30-min incubation at 23°C, samples were neutralized with 1 M Tris, pH 7.0. Unheated samples (unless otherwise indicated) were resolved on linear 12% SDS-PAGE. Gels were run at 200 V for 50 min and protein was transferred to nitrocellulose membranes for 18 min at 12 V in 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol. IE was detected with anti-IEα rabbit serum, donkey anti–rabbit horseradish peroxidase, and chemiluminescent substrate (ECL kit; Amersham International, Little Chalfont, UK).

Peptide Rescue Assay. IE^k (0.4 μg/sample) with or without 150 μM pCc(91-104) was incubated in a 12-μl vol with 0.2% NP-40 and 20 mM citrate/phosphate buffer at various pH for 15 min at 23°C. The samples were further incubated with 4.8 μl 4% SDS solution for 20 min at 23°C before neutralization by addition of 3 μl 1 M Tris, pH 7.0. The unheated samples (unless otherwise indicated) were then loaded on a 12% SDS-PAGE, and IE was visualized by Western blotting as described above. pCc(91-104), RADLIAYLKQATAK, was synthesized as previously described (16).

Peptide Dissociation Assay. Purified IE^k (100 μl) was dialyzed against 50 mM acetate buffer, pH 7.0 or 5.5, containing 0.2% NP-40 (550 ml) for 4.25 h at 23°C using membranes with a cutoff size of 10 kD. The samples were then neutralized with 200 mM Tris, pH 7.0 and then SDS sample buffer was added to give a final concentration of 1.25% SDS. The unheated and heated samples were resolved using 12% SDS-PAGE (0.3 μg/lane), and IE was visualized by Western blotting as described above.

Heat Denaturation Assay. IE^k (0.37 μg/sample) was incubated in a 10-μl volume with 0.2% NP-40 and 20 mM citrate/phosphate buffer at the indicated pH for 30 min at 70°C. The samples were then neutralized with 3 μl 1 M Tris, pH 7.0, and equilibrated for 10 min at 23°C before addition of 5.2 μl of 4% SDS solution and incubation for 20 min at 23°C. The unheated samples (unless otherwise indicated) were loaded on a 12% SDS-polyacrylamide gel, and IE was visualized by Western blotting as described above.

8-Anilino-1-Naphthalene-Sulfonic Acid (ANS) Fluorescence Studies. Purified class II preparations were extensively dialyzed against 0.15 M NaCl containing 0.1% 1-S-octyl beta-D-thioglucopyranoside (SOG; Pfanstiehl Laboratories, Inc., Waukegan, IL) and adjusted to pH 7.2. Alternatively, IE^k (40 μg/ml) was reconstituted into dipalmitoyl phosphatidylcholine (DP-PC; Sigma Chemical Co., St. Louis, MO) liposomes (200 μg/ml). DP-PC was dissolved in chloroform and coated on the bottom of glass tubes by drying under a gentle stream of nitrogen. Solutions of 0.5% SOG with or without IE^k were added to the glass tubes and incubated 30 min at 23°C with rocking. The solutions were then extensively dialyzed against water. ANS fluorescence was measured on a spectrofluorimeter (model 8000; SLM Instruments, Inc., Urbana, IL). Excitation was set at 350 nm, and the emission spectrum was recorded between 420 and 600 nm. Samples contained 20 μM ANS, 0.3 or 0.6 μM protein, and 10–20 mM acetate buffer. After initial mixing, samples were kept in the dark for 2 min under constant mixing before recording emission spectra at 23°C. In pH titration experiments, samples contained 50 mM phosphate buffer and were maintained in the dark at 37°C with constant stirring. ANS fluorescence (excitation 350 nm) was recorded at the near emission maximum of 495 nm after successive addition of 1-μl aliquots of 1, 3, or 6 N HCl followed by pH measurement using a pH microelectrode. Values are reported as relative fluorescence, which is a measure of fluorescence intensity in arbitrary units (fluorescence intensity/reference intensity).

Results

Enhanced Susceptibility of Class II Molecules to SDS-induced Denaturation at Mildly Acidic pH. A proportion of mature MHC class II α/β heterodimers remain stably associated as dimers in 1–2% SDS without heating (18, 34, 35). Invariant chain dissociation and peptide binding have been found to be necessary for dimer stability (35, 36), and it is gener-

ally believed that empty α/β heterodimers are unstable and dissociate in SDS (35, 36). However, not all peptide-bearing class II molecules are stable in SDS at neutral pH (37, 38). We used SDS to probe the structure of purified IE–peptide complexes at low pH (Fig. 1). Purified IE^k and IE^d were incubated for 20 min in citrate/phosphate buffers at various pH in the presence of 1% SDS (Fig. 1, *a* and *b*). After neutralization, the samples were resolved by electrophoresis, and Western blots were probed with IEα antiserum. Fig. 1, *a* and *b*, shows that IE molecules become sensitive to SDS denaturation and dissociate from α/β dimers (*D*) to monomers (*M*) when exposed to SDS at low pH. A variable fraction of dimers dissociate into monomers at pH 6.5, and dissociation is nearly complete at pH 6.0. This effect is not a result of direct acid-induced denaturation because dimer stability is recovered when the pH is neutralized before exposure of IE to SDS (Fig. 1, *c* and *d*). This is consistent with previous results demonstrating that IE–peptide complexes are stable at pH 4.5 (19). Similar results are obtained in experiments in which IE is visualized by Coomassie staining (Fig. 1 *e*). Thus our results are not biased by potential differences in the affinity of the antisera for IE in different conformations.

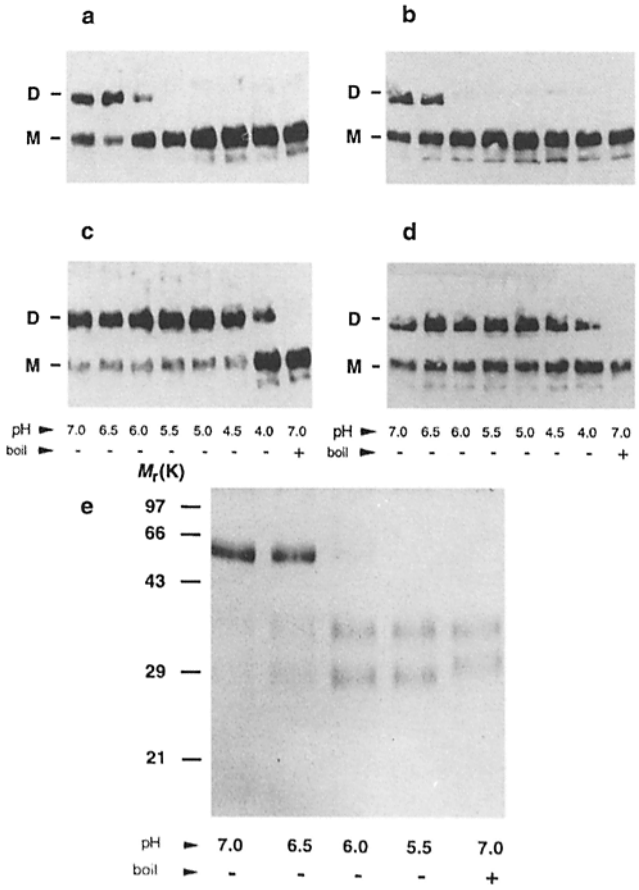

Figure 1. Acidic pH enhances denaturation of IE heterodimers by SDS. Purified IE^k (0.35 μg/sample; *a* and *c*) or IE^d (0.2 μg/sample; *b* and *d*) was equilibrated at 23°C for 15 min in 0.2% NP-40, 20 mM citrate/phosphate buffer of various pH. (*a* and *b*) SDS was added and samples were incubated at 23°C for 20 min before neutralization with 176 mM Tris, pH 7.0. (*c* and *d*) Samples were neutralized with Tris, pH 7.0, and incubated at 23°C for 10 min before addition of SDS and incubation for 20 min. After treatment, the unboiled samples (except lane *8*, which was boiled 5 min) were resolved by 12% SDS-PAGE, and IE was visualized by Western blotting as described in Materials and Methods. IE dimers (*D*; apparent molecular mass ~60–65 kD) and monomers (*M*; apparent molecular mass ~31–35 kD) are indicated. (*e*) Samples containing 6.5 μg of IE^k were equilibrated at 23°C for 15 min in 20 mM citrate/phosphate buffer of various pH. SDS was added followed by an additional incubation at 23°C for 20 min before neutralization with 143 mM Tris, pH 7.0. The unboiled samples (except lane *5*) were resolved by 12% SDS-PAGE and stained with 0.1% Coomassie blue.

The class II preparations used in this study were isolated from cell lysates by affinity chromatography and elution at pH 11.5. The relatively harsh conditions used for protein isolation, including exposure to high salt concentrations during column washing, could potentially select for a subset of molecules with altered physical properties. To address this possibility, freshly prepared detergent lysates of CH27 ($H\text{-}2^k$) and A20 ($H\text{-}2^d$) B cells were incubated with SDS in citrate/phosphate buffers at various pH. After neutralization, unheated samples were resolved by SDS-PAGE, and Western blots were probed with IEα-specific antisera (Fig. 2). Increased sensitivity of IE molecules in cell lysates to SDS-induced denaturation was observed at low pH. The pH required to induce complete dissociation into monomers was shifted 0.5–1 U compared with purified class II molecules. This difference results from titration of the SDS by components of the cell lysates, which can be overcome by increasing the SDS concentration (data not shown). We conclude that mildly acidic pH induces a change in the structure of IE–peptide complexes that increases sensitivity to SDS-induced denaturation. This change in structure is rapidly reversed after pH neutralization.

Peptides Remain Associated with Class II Molecules at Mildly Acidic pH. Sadegh-Nasseri and Germain (18) have reported that IE^k molecules adopt an unstable conformation at pH 4.5 that leads to irreversible denaturation unless high concentrations of specific peptide are present during pH neutralization. An experiment was performed to determine whether high concentrations of peptide can also rescue

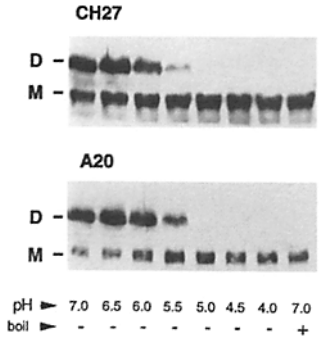

Figure 2. Enhanced denaturation of cell-associated IE molecules by SDS at low pH. (*a*) CH27 cells (5×10^5 cell equivalents/lane) were solubilized in 0.5% NP-40 in the presence of protease inhibitors, and insoluble material was removed by centrifugation. Lysates were adjusted to various pH with 20 mM citrate/phosphate buffers, and SDS was added to a final concentration of 2%. After incubation for 30 min at 23°C, the pH was neutralized with Tris, and samples were resolved by SDS-PAGE. IE was visualized by Western blotting as described in Materials and Methods. (*b*) A20 cells were solubilized in 1% *N*-octylglucoside, and cell lysates were incubated with 1.1% SDS at various pH for 30 min at 23°C. The fraction of IE present as dimers (*D*) or monomers (*M*) was determined as described above.

class II molecules from SDS-induced denaturation at milder pH values. Purified IE^k was incubated for 15 min at either pH 7, 5.5, or 4.5 in the presence or absence of 150 μM of pCc(91-104) (Fig. 3 *a*). SDS was added, and the samples were further incubated for 20 min, followed by neutralization and Western blot analysis. Lanes *1* and *4* show that at neutral pH with no heating, equivalent amounts of stable dimers are detected in the absence or presence of pCc(91-104). Although this peptide binds with high affinity to IE^k, it does not rescue the dimers from SDS-induced denaturation at low pH. Short-lived low-affinity interactions that occur in the presence of high concentrations of specific peptide have been reported to stabilize empty class II molecules at 37°C (39). Our results indicate that these interactions do not protect IE^k from denaturation by SDS at low pH.

Because empty class II molecules are unstable in SDS (35, 36), we considered the possibility that the increase in sensitivity to SDS observed at low pH results from rapid dissociation of previously bound peptides. To directly address this possibility, purified IE^k was dialyzed at pH 5.5 versus 7.0 to remove free peptides, followed by pH neutralization and SDS-PAGE analysis. The SDS-stable fraction is essentially unchanged in the acid-treated samples compared with IE^k molecules dialyzed at pH 7.0 (Fig. 3 *b*). We conclude that the majority of peptides remain associated with class II molecules at mildly acidic pH and that enhanced sensitivity to SDS at low pH is not a consequence of peptide dissociation.

Increased Sensitivity to Heat Denaturation at Low pH. Independent evidence for a structural change in IE at mildly acidic pH was obtained by measuring sensitivity to heat-induced denaturation. Purified IE^k was incubated for 30 min at 70°C in citrate/phosphate buffers over a range of hydrogen ion concentrations (Fig. 4). The samples were

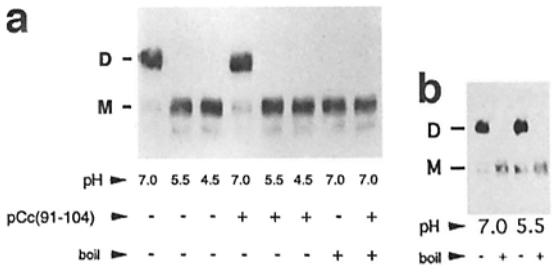

Figure 3. Peptides remain associated with class II molecules at mildly acidic pH. (*a*) Peptide does not rescue IE^k from denaturation by SDS at low pH. Purified IE^k (0.4 μg/sample) was incubated at 23°C for 15 min in 0.2% NP-40, 20 mM citrate/phosphate buffer at various pH in either the absence or presence of 150 mM pCc(91-104). SDS was added and samples were incubated at 23°C for 20 min before neutralization with 150 mM Tris, pH 7.0. The fraction of IE present as dimers (*D*) or monomers (*M*) was determined by SDS-PAGE and Western blotting. (*b*) Class II molecules remain stable in SDS after dialysis at mildly acidic pH. Purified IE^k was dialyzed against 50 mM acetate buffer, pH 7.0 (lanes *1* and *2*) or 5.5 (lanes *3* and *4*) in 0.2% NP-40 for 4.25 h at 23°C. Samples were neutralized with 200 mM Tris buffer, pH 7.0, and then SDS was added before heating (95°C for 5 min) half the samples (lanes *2* and *4*). The fraction of IE present as dimers (*D*) or monomers (*M*) was determined by SDS-PAGE (0.3 μg/lane) and Western blotting.

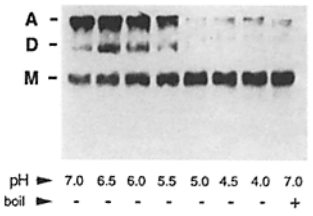

Figure 4. Decreased thermostability of IE^k at low pH. Purified IE^k (0.37 μg/sample) was incubated at 70°C for 30 min in 0.2% NP-40 with 20 mM citrate/phosphate buffer at various pH values. The samples were then neutralized with 230 mM Tris, pH 7.0, and equilibrated for 10 min at 23°C before the addition of SDS and further incubation for 20 min at 23°C. The fraction of IE present as aggregates (*A*), dimers (*D*), or monomers (*M*) was determined by SDS-PAGE and Western blotting.

neutralized, cooled to 23°C, and analyzed by SDS-PAGE and Western blotting. A major fraction of molecules is observed to aggregate (*A*) during incubation at 70°C, pH 7.0. However, the fraction of molecules present as monomers (*M*) is similar to that observed after incubation at 23°C, pH 7.0 (see Fig. 1 *c*). We assume that the aggregated molecules are composed of α/β heterodimers. At low pH, the aggregated molecules and unaggregated dimers (*D*) dissociate in parallel into monomers. This effect is largely complete at pH 5.0–5.5 (compare with Fig. 1 *c*). The transition to monomers is observed at higher pH during incubation at higher temperatures (data not shown). We conclude that the thermostability of IE molecules is reduced at mildly acidic pH.

ANS Fluorescence Studies Demonstrate a Reversible Structural Transition in Class II Molecules at Mildly Acidic pH. ANS is an environment-sensitive fluorescent probe that is used to monitor structural changes in proteins (40–44). The emission characteristics of ANS vary with the polarity of its environment. In a hydrophobic environment, the fluorescent quantum yield (intensity) increases, and the emission peak maximum (λ_{max}) shifts to shorter wavelengths. Protein structural changes that expose hydrophobic regions provide sites for ANS binding, with resulting changes in the fluorescence emission spectra of ANS solutions. To evaluate the effect of pH on the exposed hydrophobicity of class II molecules, solutions of IE^k, IE^d, IA^k, and IA^d were brought to either pH 7 or 5 and ANS was added (Fig. 5). After mixing for 2 min in the dark, samples were excited at 350 nm and scanned for emission wavelength spectra. For each class II protein, there was a marked increase in fluorescence intensity and a shift in λ_{max} at pH 5 compared with pH 7. The altered emission spectra occurred rapidly after acidification and was stable over time. ANS in identical conditions but in the absence of protein (Fig. 5, *a–d*) or in the presence of a control protein, hen egg lysozyme (Fig. 5 *e*) had essentially the same emission spectra at pH 7 and 5. The altered emission spectra were rapidly reversed after pH neutralization with Tris (Fig. 6). Control experiments were done to rule out bleaching during sample rescanning (data not shown).

To address the potential influence of detergent on class II structure or ANS fluorescence, IE^k was reconstituted in DP-PC vesicles. This allowed ANS spectra to be recorded in the absence of detergent. Fig. 7 shows that a similar effect is seen with the IE^k in DP-PC vesicles as was seen with

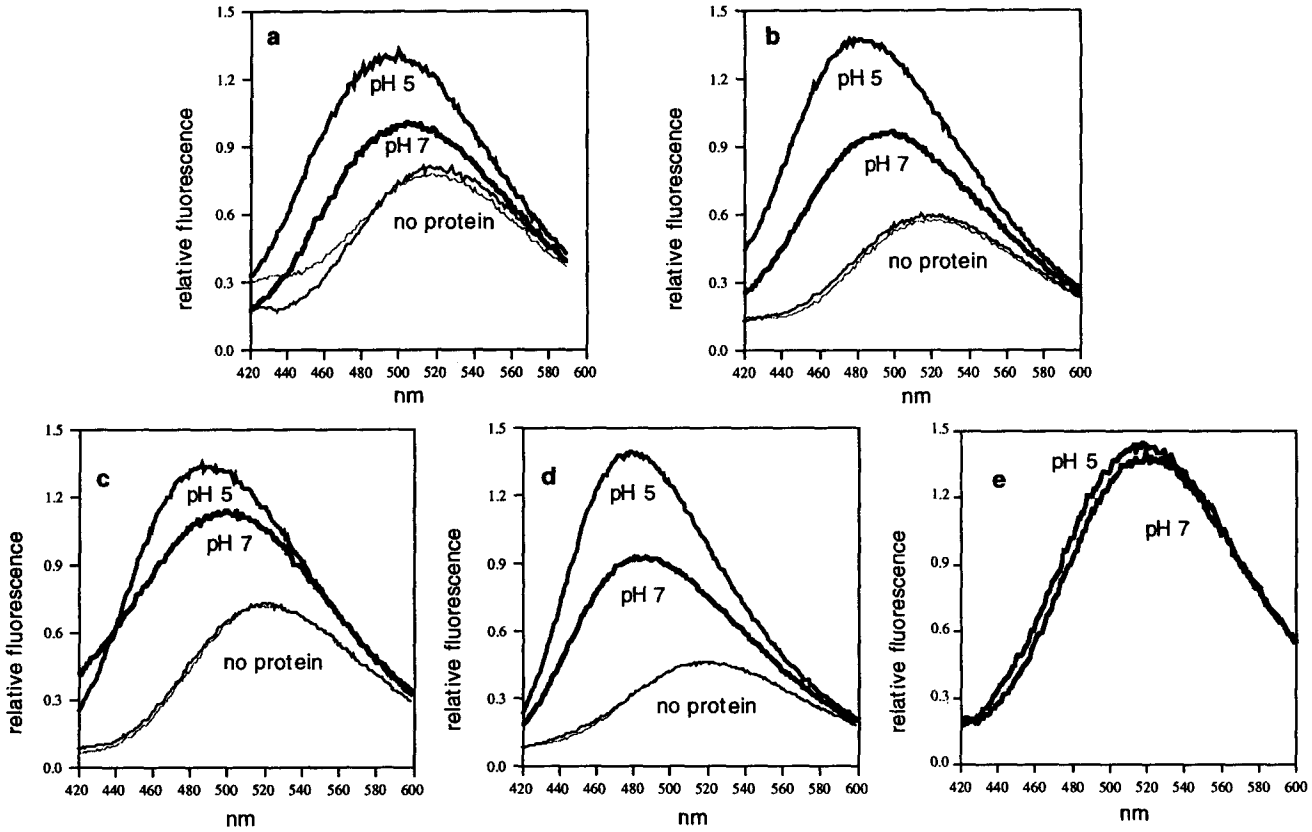

Figure 5. A change in the structure of class II proteins at pH 5 measured with the fluorescent probe ANS. (*a–d*) Purified class II molecules IE^k (*a*), IA^k (*b*), IE^d (*c*), and IA^d (*d*) were dialyzed against 0.1% SOG and 0.15 M NaCl. Samples (0.3 μM) were adjusted to pH 7 or 5 with 10–20 mM acetate buffer, and ANS (20 μM) was added. After mixing for 2 min in the dark, fluorescence spectra (420–600 nm) were measured by excitation at 350 nm. Controls were performed with the same reaction conditions but in the absence of protein. (*e*) A control protein, hen egg lysozyme (3.2 μM), was adjusted to pH 7 or 5 with 20 mM acetate buffer containing 0.1% SOG and 0.15 M NaCl. Fluorescence spectra were measured after addition of 20 μM ANS as described above.

IE^k solubilized in detergent. There is a substantial increase in ANS fluorescence intensity when pH is lowered from 7 to 5. This effect is fully reversible by pH neutralization. It is not clear why the shift in λ_{max} was smaller than that observed with detergent-solubilized molecules. However, the fluorescence intensity of ANS is more sensitive to polarity changes than λ_{max} (40). Together, the ANS fluorescence experiments provide strong evidence for a reversible structural transition in class II molecules exposed to mildly acidic pH that is characterized by an increase in surface hydrophobicity.

Structural Change in IA^d Occurs at Higher pH than Other Class II Proteins. In an effort to determine the structural transition points for different class II molecules, pH titration studies were done by gradually lowering the pH with HCl and measuring the ANS fluorescence intensity at 495 nm (Fig. 8). In each case there was a transition characterized by a major change in the slope of the titration curve, followed by a continuous increase in intensity as pH was lowered to 4.5. This structural transition in IA^d occurred in the range of pH 7.0–7.5. By contrast, the transition was observed at pH 6.0–6.5 for IA^k, IE^k, and IE^d. This result is consistent with the observation that purified IA^d and IA^d in cell extracts is mostly unstable in 1% SDS at pH 7.2 in contrast to the other three class II proteins (data not shown). We conclude that a structural change occurs in IA^d molecules at a higher pH than IA^k, IE^k, and IE^d, and that this change is characterized by an increase in exposed hydrophobicity and increased sensitivity to SDS denaturation.

Discussion

This study shows that murine MHC class II glycoproteins undergo a structural transition in the same pH range that regulates peptide binding. This conclusion is supported by experiments showing that, at pH 5–6, class II molecules are more sensitive to SDS-induced denaturation, have decreased thermostability, and increased surface hydrophobicity compared with pH 7.

The stability of class II heterodimers in SDS at room temperature has been widely used as a measure of peptide loading. Empty class II α/β heterodimers (36) and α/β–Ii complexes (35) are unstable in SDS. Peptide loading is required to generate stable complexes. However, not all peptides stabilize α/β complexes, and thus a fraction of mature peptide-bearing α/β complexes dissociates into monomers

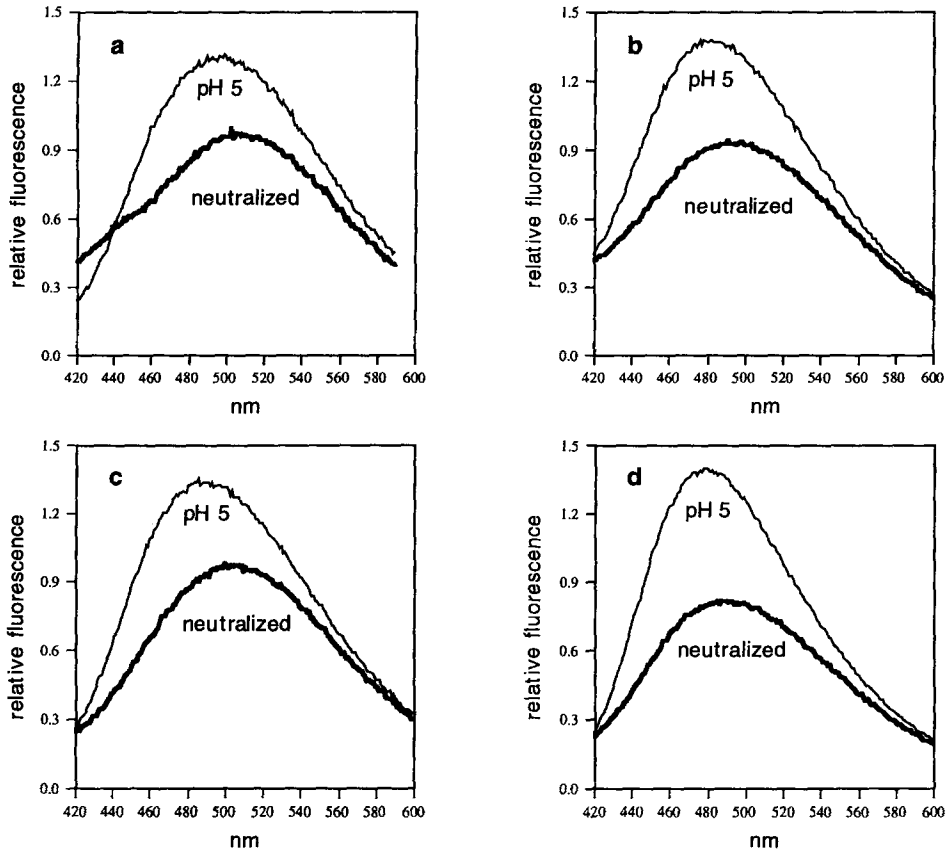

Figure 6. The pH-dependent change in class II structure is fully reversible. Purified class II molecules IE^k (*a*), IA^k (*b*), IE^d (*c*), and IA^d (*d*) were dialyzed against 0.1% SOG and 0.15 M NaCl. Samples (0.3 μM) were adjusted to pH 5 with 10–20 mM acetate buffer, and ANS (20 μM) was added. After mixing for 2 min in the dark, fluorescence spectra were measured by excitation at 350 nm. The pH was then neutralized by addition of 50 mM Tris, pH 7.5, and fluorescence was again measured.

when exposed to SDS in sample buffer at pH 6.8 (45). Results presented in this study demonstrate that all peptide complexes dissociate when exposed to SDS at mildly acidic pH in the range of pH 5–6 (Figs. 1 and 2). This effect is not due to the dissociation of previously bound peptides at low pH. Most peptides remain stably associated with class II at pH >4.5 (15, 16, 19, 20, 27, 46, 47), although some exceptions have been described (20, 27, 46, 47). Data presented in Fig. 1 suggest that class II complexes remain associated at low pH in the absence of SDS. The fraction of stable IE^d or IE^k heterodimers is not decreased by incubation at pH 4.5 and only slightly decreased at pH 4.0. Therefore, the vast majority of peptide complexes formed

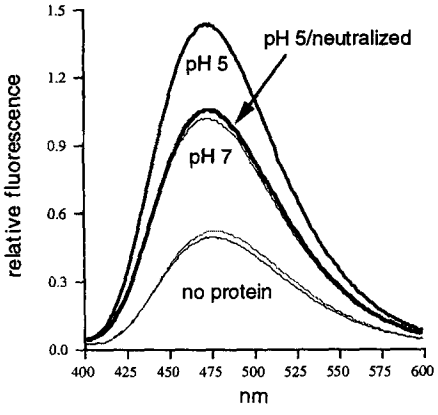

Figure 7. A pH-dependent change in class II structure in the absence of detergent. IE^k in DP-PC vesicles was prepared as described in Materials and Methods. IE^k (0.6 μM) in DP-PC vesicles was mixed with 20 mM acetate buffer, pH 5 or 7, and 20 μM ANS for 2 min in the dark at 23°C. The ANS fluorescent spectra were measured during excitation at 350 nm. The pH 5 sample was neutralized with 50 mM Tris, pH 7.5, and the ANS fluorescent spectra again measured. Control spectra were recorded with the identical conditions but using DP-PC vesicles containing no IE^k.

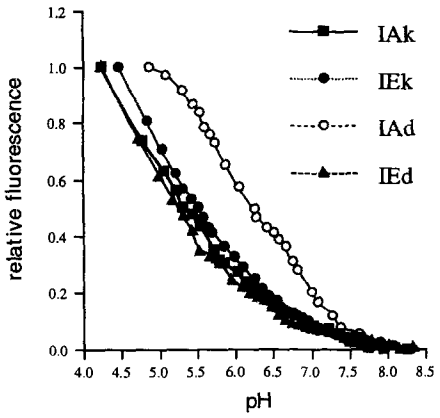

Figure 8. IA^d changes structure at a milder pH than other class II proteins. Samples containing purified class II molecules (0.3 μM) and 20 μM ANS in 0.1% SOG, 0.15 M NaCl, 50 mM phosphate buffer were mixed in a volume of 1.5 ml at 37°C. ANS fluorescence was measured at 495 nm after the pH was gradually lowered by addition of HCl. Values represent relative fluorescence after subtraction of fluorescence measured at pH 8.

under physiological conditions are stable in all of the hydrogen ion concentrations likely to be encountered in the cell, where a minimal pH of ~4.5 is found in lysosomes (5). The low pH–induced structural change that is associated with enhanced sensitivity to SDS is rapidly reversible. It is unlikely that this reversibility is a result of rebinding of dissociated peptides during pH neutralization. The fraction of IE^k molecules that remains stable in SDS is largely unchanged after dialysis at pH 5.5 to remove any dissociated peptides and prevent rebinding (Fig. 3 *b*). Furthermore, high concentrations of specific peptide do not rescue IE^k from SDS-induced denaturation at pH 4.5 or 5.5 (Fig. 3 *a*). We conclude that the low pH–induced structural change characterized by enhanced sensitivity of class II complexes to denaturation by SDS is not associated with peptide release.

Independent evidence for a structural change at low pH came from analysis of the thermostability of class II molecules (Fig. 4). IE^k molecules dissociated into monomers during incubation at 70°C at pH 5 but not 7. The degree of acidification required for dissociation was dependent on temperature such that dissociation was observed at higher pH during incubations at higher temperature. Evidence for conformational transitions in class II molecules at neutral pH has been obtained by measuring the fluorescence of intrinsic tryptophan residues as a function of temperature, but the effect of varying pH was not examined (29). Our results were complicated by the formation of SDS-stable aggregates during incubation at 70°C at neutral pH. We assume that these aggregates are composed of molecules in which the α/β dimers remain associated in a nativelike conformation. However, it is possible that the aggregates contain dissociated α and β monomers. In either case, a structural transition is evident at mildly acidic pH in which the molecules completely dissociate into free monomers.

Experiments with ANS provide a third line of evidence for a pH-regulated structural change in class II molecules. ANS is an environment-sensitive fluorescent probe that has been extensively used to monitor conformation changes in proteins (40–44). The ANS fluorescence intensity was markedly increased with an associated blue shift in λ_{max} in the presence of detergent-solubilized IA^d, IA^k, IE^d, and IE^k molecules at pH 5 compared with 7 (Figs. 5–8). These spectral changes suggest that there is an increase in exposed, hydrophobic ANS-binding sites in class II molecules at low pH. The low pH structure is rapidly reversed after pH neutralization. These effects were not observed with ANS solutions in the absence of protein or in the presence of a control protein, hen egg lysozyme. The potential effect of detergent on class II structure or ANS binding was excluded in experiments with IE^k reconstituted into lipid vesicles. This is important because our other experimental data were obtained in the presence of detergent and because proteoliposomes simulate better the natural environment of class II molecules in lipid bilayers.

The structural change measured by altered interaction with ANS appears to be a common property of murine class II proteins, including IA as well as IE molecules. Titration experiments with IE^d, IE^k, and IA^k demonstrated a transition in ANS fluorescence at pH 6.0–6.5, with a continuous increase in fluorescence as the pH was further lowered to 4.5 (Fig. 8). This is strikingly reminiscent of the effect of pH on peptide binding to these molecules, which is generally optimal at pH 4.5 (15, 16, 19–21, 24, 26). By contrast, a transition in ANS fluorescence was observed at pH 7.0–7.5 in solutions containing IA^d. IA^d binds peptides quite well at neutral pH, and binding is generally optimal at pH 5.5 (15, 16, 22, 24). Thus the pH dependence of the structural transitions measured with ANS corresponds well with the pH dependence of peptide loading. The conclusion that IA^d undergoes a conformational change at higher pH is further supported by measuring sensitivity to denaturation by SDS. Unlike IE^d, IE^k, and IA^k, the major fraction of IA^d molecules is unstable in SDS at neutral pH (data not shown). In addition, IA^d molecules have been reported to undergo a heat-induced neutral pH conformational change at lower temperature than IE^k (29). It is interesting to speculate that IA^d may be adapted to load with peptides in a range of intracellular compartments with higher pH compared with the other molecules that we studied. Nevertheless, our results strongly support the conclusion that class II molecules undergo a structural change at mildly acidic pH, and that this is associated with enhanced peptide binding.

Many proteins pass through a conformation intermediate, termed the molten globule state, during acid denaturation (48–50). This structure is a compact conformation with nativelike secondary structure but disordered side chains (tertiary structure). Analysis of far UV circular dichroism spectra indicates that secondary structure is largely preserved in class II molecules above pH 4 (31, 51). However, we do not believe that there is substantial loss of side-chain interactions in class II complexes at pH $\geq$4.5 because (*a*) quaternary structure is preserved, (*b*) the acid-induced structural change is rapidly reversible, (*c*) most bound peptides remain associated with no increase in the rate of dissociation, and (*d*) specific peptide loading with formation of stable complexes occurs at low pH with no requirement for pH neutralization (19). Not only is peptide binding enhanced at low pH, but the specificity of binding is conserved. Thus it is likely that many, if not all, of the interactions between amino acid side chains in the peptide-binding pockets of class II and anchor side chains in peptide (52) are preserved in the low pH conformation. It is possible that some of the conserved hydrogen bonds involving peptide main-chain atoms are disrupted in the acid conformation. These bonds are thought to contribute significantly to the stability of peptide–class II complexes but not to the specificity of peptide binding (52). This possibility would allow peptides to interact specifically with class II molecules at low pH and form long-lived complexes. Peptides with stronger anchor–pocket interactions would remain stably associated at low pH, whereas a smaller subset with weaker interactions may have increased dissociation rates at low pH. Although our knowledge of the specific structural

changes induced at low pH is only speculative, we suggest that this conformational transition is limited to localized changes in the relationship between selected sites in the molecule with little disruption of general tertiary structure. It is possible that the basic structure is unchanged and that there is an increase in the flexibility of local regions of the molecule.

A continuous increase in ANS fluorescence intensity was observed in titration experiments as the pH was reduced to 4.5 (Fig. 8). We do not believe that this is a result of heterogeneity in the molecules with respect to the degree of acidification required to induce a conformational change. Rather, it is likely that all molecules undergo a structural transition at mildly acidic pH and that structure is further destabilized as the hydrogen ion concentration is increased. This conclusion is supported by the observation that all molecules become sensitive to denaturation by SDS or heat at mildly acidic pH. Furthermore, Boniface et al. (51) demonstrate pH-dependent structural changes in IE^k molecules that are uniformly loaded with a single high affinity peptide.

Although all purified class II molecules undergo a conformational change, generally <10% bind peptide under optimal conditions. This fraction could be made up of empty molecules, or it could represent a subset bearing peptides that are easily removed at low pH by a peptide exchange mechanism. The latter possibility is favored by the observation that empty class II molecules are unstable at 37°C (39; and Sherman, M. A., and P. E. Jensen, unpublished observations). Extended incubation at pH 5 has little effect on the capacity of class II molecules to subsequently bind peptide at either pH 5 or 7 (15, 16, 19). Thus, it is unlikely that low pH simply induces the dissociation of a subset of peptides that have low affinity. Reay et al. (20) demonstrated that a recombinant chimeric form of IE^k binds stoichiometric quantities of peptide with markedly increased rate at low pH. It is possible that these molecules are empty. Alternatively, they may be uniformly associated with peptides that are able to be replaced in exchange reactions at low pH. We favor the hypothesis that these molecules, along with a subset of mature cell surface class II molecules, mimic newly synthesized class II molecules after proteolytic release of Ii. These class II molecules are associated with fragments from exon 3 of Ii (class II–associated invariant chain peptides [CLIP]) and have not bound antigenic peptides (53). The weight of evidence suggests that CLIP occupy the peptide-binding groove in a manner similar to antigenic peptides (54, 55). We propose that CLIP and a subset of other peptides sharing presently undefined physical characteristics are selectively able to be replaced in peptide-exchange reactions involving the low pH conformation of class II molecules. The fact that most antigenic peptides do not share this property explains why the rate of dissociation of previously bound labeled peptides is generally not enhanced in the presence of excess unlabeled peptide. These peptides are not easily replaced through an exchange mechanism. By contrast, some peptides are available for exchange, and the dissociation of these peptides is enhanced in the presence of other peptides (56, 57). We suggest that these peptides fall into a common group with CLIP and predict that the dissociation of CLIP can be enhanced in the presence of other peptides. Thus, we view the acid-induced conformation of class II molecules as described here as a conformation required for peptide-exchange reactions that lead to the release of CLIP and loading with antigenic peptides under physiological conditions.

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