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Conformational changes in mouse MHC class II proteins at acidic pH

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Abstract

MHC class II molecules bind peptide antigens and present them to T cells. Recent evidence suggests that peptide–MHC class II interaction is regulated by pH. Both peptide binding to and dissociation from MHC class II molecules is enhanced at acidic pH. In this report we use circular dichroism to investigate the possibility that the modulation of peptide association with MHC class II molecules at acidic pH is associated with changes in MHC class II structure. Our results show that a change in the structure of the MHC class II protein A^d occurs between pH 4 and 5, close to the pH optimum for peptide binding. More drastic changes in A^d structure occur at lower pH levels, where peptide dissociation is enhanced. The structural changes at pH 4–5 are fully reversible upon neutralization, while changes at pH 3 are not. The pH stability of purified E^d molecules is somewhat different, with changes observed only at pH <4. This may reflect a differential ocupancy of the purified molecules with endogenous peptides.

Introduction

MHC class II molecules bind peptide antigens of 10–20 amino acids in length (1–4) and present them to T cells of the CD4 subset. MHC class II molecules (human HLA DP, DQ, DR; mouse H-2A,E) are heterodimeric, highly polymorphic, transmembrane glycoproteins consisting of non-covalently linked 34 kDa α and 29 kDa β chains. Each chain consists of a short cytoplasmic tail, a transmembrane region, and two extracellular domains, each of ~95 amino acids length. The membrane proximal α_2 and β_2 domains are immunoglobulin-like and relatively conserved in sequence between MHC class II allotypes, while the N-terminal α_1 and β_1 domains are non-immunoglobulin-like and contain most of the protein's polymorphic amino acids. Based on the crystal structure of the MHC class I molecule (5,6), a model for the N-terminal domains of the MHC class II protein has been proposed (7). The N-terminal α_1 and β_1 domains are thought to form an eight-stranded β-pleated sheet on top of which sit two α helical regions. Together they form a 10×25 Å groove lined by many of the polymorphic amino acids in the protein. Comparison of the structures of three different HLA class I molecules (5,8,9) has shown that the α-carbon backbones of MHC proteins are remarkably conserved, but that the polymorphism between different allelic products gives rise to different specificity pockets, or subsites, within the peptide binding groove. These subsites are thought to influence peptide binding specificity. By analogy with the MHC class I molecule, antigenic peptide is thought to bind to MHC class II proteins in the groove formed by the N-terminal domains. Furthermore, the T cell receptor is believed to be in contact with upward pointing residues of the helices.

Although a large fraction of purified MHC class II molecules bind conformationally sensitive antibodies, only 10–20% of affinity purified A^d molecules bind the peptide ovalbumin (OVA)323–339 (10). This is thought to reflect the unusual stability of MHC class II–peptide complexes, with the result that a large fraction of MHC molecules co-purify with peptides that occupy their antigen binding site (11–13). Indeed, peptides of 13–17 amino acids are released from purified MHC class II molecules under strongly acidic conditions (13). In a lipid bilayer, the half-life of an MHC II peptide complex may be of the order of days (10,14,15), and even in detergents the complex is remarkably long-lived (16,17). Dissociation of peptides from MHC II molecules is enhanced at acidic pH (15,16,18). For the A^d/OVA323–339 complex in a lipid bilayer, dissociation is enhanced at pH levels <5 (15,18). On the other hand, co-purifying peptides may be even more tightly associated, in as much as it has been difficult to increase the availability of binding sites in purified MHC II

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molecules by acid pH treatment (12,15). Dissociation of peptide – MHC II complexes can also be accelerated in the presence of soluble competitor peptide (19), perhaps through an intermediate in which both peptides are bound to the same MHC class II molecule (20).

Peptide – MHC class II association is also remarkably slow at neutral pH (10,16) and is thought to proceed through a kinetic intermediate (21). Recent evidence suggests that the rate of peptide – MHC class II interaction is optimal at mildly acidic pH (pH 4.5 – 6) in most (18,22 – 26) but not all (18,27) cases. This observation is consistent with experiments suggesting that the site of MHC II – peptide interaction in an antigen presenting cell (APC) is a low pH compartment in the endosome – lysosome pathway (28,29).

In view of the profound effect of pH on the interaction between peptide and MHC class II proteins, it is tempting to speculate that pH-dependent conformational changes regulate the accessibility of the MHC II peptide binding site. In this study we have used circular dichroism (CD) to investigate the effects of acidic pH on the structure of the MHC class II molecule and find that exposure to mildly acidic pH causes a change in the CD spectrum consistent with an opening up of the protein.

Methods

Cell lines and reagents

The T cell hybrid DO-11.10 (30) was kindly provided by P. Marrack and J. Kappler (National Jewish Hospital, Denver, CO). The B lymphoma A20 (H-2^d), the hybridomas MKD6 and 14-4-4S, and the IL-2 indicator line CTLL were obtained from the American Type Culture Collection, Rockville, MD. All cell lines were grown in RPMI 1640 supplemented as previously described (10). Fluorescein-5-isothiocyanate (FITC) was purchased from Molecular Probes Inc., Eugene, OR.

Peptides

The peptide representing OVA323 – 339 (ISQAVHAAHAEINEA-GR) was synthesized, labeled at its N-terminus with FITC, and purified to homogeneity by reverse phase HPLC as previously described (31). The peptide contained a single fluorescein, and the concentration of fluorescein-labeled peptide was determined using the extinction coefficient of fluorescein at 488 nm (75 000/M.cm). Previous work has demonstrated that labeling this peptide at its N-terminus with fluorophores does not alter the activity of the peptide in T cell activation assays (31).

MHC class II purification and reconstitution

MHC class II molecules were purified as previously described (10). Briefly, A20 was grown in spinner cultures, lysed in NP-40, and the glycoproteins isolated by lentil lectin chromatgraphy. MHC molecules were subsequently purified from the pooled glycoprotein fractions from several litres of cells by antibody affinity chromatography using MKD6 – Sepharose for A^d and 14-4-4S – Sepharose for E^d. MHC molecules were eluted from the MKD6 or 14-4-4S columns using 1% octylglucoside in PBS, pH 11.5.

Peptide binding to the lymphoma A20

The A^d/E^d expressing APC A20 was fixed with glutaraldehyde (32). Fixed cells were resuspended at 1×10^6/ml in 10 mM phosphate, 10 mM citrate, 10 mM acetate, and 138 mM NaCl, and incubated with 0.1 μm OVA323 – 339. At various time points, cells were neutralized and unbound peptide washed away with pH 7.3 PBS. Then 1×10^5 APCs were cultured with 10^5 DO-11.10 in 200 μl of 10% FCS/RPMI. The following day, culture supernatants were assayed for IL-2 production by measuring thymidine incorporation by the IL-2 dependent indicator line CTLL.

Peptide binding to purified MHC class II molecules

Affinity purified A^d (20 or 25 μg/ml) was reconstituted with 200 μg/ml phosphatidylcholine by detergent dialysis (10). The lipid reconstituted protein was incubated with 40 or 100 μM FITC – OVA323 – 339 in 10 mM phosphate, 10 mM citrate, 10 mM acetate, and 137 mM NaCl at various pH levels for 24 h. Following the incubation, the sample was neutralized by the addition of saturated Tris, and the unbound peptide removed by dialysis against pH 7.5 PBS. Samples were then converted to planar membranes by incubation on glass slides followed by washing, and fluorescence was analyzed as described previously (10). Specific binding of FITC – OVA323 – 339 to the A^d samples was determined as the difference in fluorescence between A^d-containing membranes incubated with FITC – OVA323 – 339 and A^d-containing membranes incubated with FITC – OVA323 – 339 in the presence of a 10-fold molar excess of unlabeled OVA323 – 339

Circular dichroism

CD measurements were performed in a Jasco 700 CD spectropolarimeter using quartz cells with a path length of 0.1 cm. The spectra are reported as the average of 15 scans at 50 nm/min with a 0.1 nm step resolution. Data are reported as the mean residue ellipticity [θ], in units of deg cm^2/dmol. The conversion of raw data to mean residue ellipticity requires an accurate protein concentration determination. The extinction coefficients for A^d and E^d at 280 nm were calculated based on tyrosine and tryptophan content as 1.020 and 1.028 g^{-1} cm^{-1} respectively. Mean residue weights for A^d and E^d were calculated to be 112.7 and 117.1 g respectively. The concentrations based on absorbance were similar to those obtained by the Lowry protein determination. Protein concentrations used in the experiments varied from 75 to 120 μg/ml and were corrected for dilution during the titration.

Samples used for CD analysis were either in octylglucoside or were reconstituted into phosphatidylcholine vesicles. In each case, samples were buffered with 5 mM citrate – 5 mM acetate – 5 mM phosphate in 5 mM NaCl. Samples were acidified by addition of 20% acetic acid or by 0.2 M HCl. In samples acidified by acetic acid, the protein sample is ~0.6 M in acetate ions at pH 3, from the initial 0.005 M acetate concentration. In samples acidified with HCl, [Cl$^-$] increases from 5 mM to ~40 mM by pH 3. Samples were neutralized with 0.2 M NaOH. Changes in CD spectra at low pH are unlikely to be due to buffer effects, because two different acidification protocols, using either acetic acid or HCl, gave similar results.

Fraction α-helix and β-sheet were estimated using a program, CONTIN, developed by Provencher and Glockner (33). This program compares the far UV CD spectrum of an unknown protein with the spectra of 16 proteins of known structure. The spectral contributions are divided into contributions from α-helix,

β-sheet, or other (which includes turns and random coil). Data were entered in 1 nm intervals between 190 and 240 nm. For the samples at acidic pH, spectral measurements were not taken below 195 nm due to increased lipid scatter. The absence of the low wavelength data weakens the accuracy of the secondary structure predictions. Furthermore, the concentration determination significantly affects the secondary structure prediction. Therefore, while it is reasonable to interpret conformational changes within a single experiment, the absolute values of $[\theta]$ varied between experiments, making the absolute estimates of secondary structure less reliable.

Results

Low pH increases the rate of MHC – peptide interaction

Several groups have shown that the binding of antigenic peptide to mouse MHC class II molecules is optimal at mildly acidic pH levels (18,22 – 27). Figure 1 confirms that this is the case for our system. Aldehyde-fixed APCs, A20 (expressing A^d, E^d), were pre-incubated for various times with OVA323 – 339 at various pH levels, neutralized by washing, and allowed to present antigen to the A^d/OVA323 – 339-restricted T hybridoma DO-11.10. The formation of functional MHC – peptide complexes on the APC is slow at pH 7, but is dramatically faster at pH 5. The rate of peptide binding at pHs 4 and 6 is intermediate between the pH 5 and neutral pH rates. However, at long periods of peptide incubation (24 h), comparable levels of DO-11.10 stimulation occurred at all pH levels between 3 and 7. This suggests that the amount of peptide bound after prolonged incubation at each pH was sufficient to saturate the T cell response. In order to test more directly whether low pH increases the level of peptide bound to MHC II molecules under saturating conditions, we measured the binding of fluorescently labeled OVA323 – 339 to affinity-purified A^d.

Figure 2 shows the binding of OVA323 – 339 to purified A^d

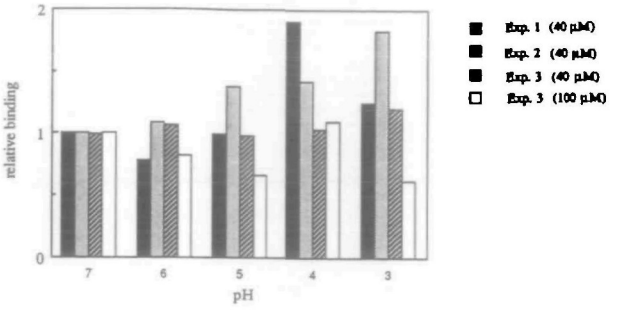

Fig. 2. Binding of OVA323 – 339 to purified A^d as a function of pH. Fluorescence measurements on planar membranes prepared from A^d-containing phospholipid vesicles that had been incubated with 40 μM or 100 μM FITC-OVA323 – 339 at pH values 3 – 7. The results of three different experiments are shown. Binding has been normalized to the pH 7 level. Specific fluorescence was calculated by subtracting the signal obtained from samples which had been pre-incubated with a 10-fold molar excess of unlabelled peptide over labelled peptide. Details are provided in Methods.

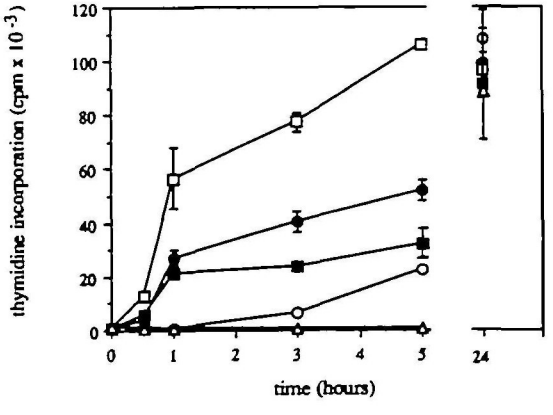

Fig. 1. Low pH increases the rate of peptide – MHC II complex formation. A20 (A^d/E^d expressing) antigen presenting cells were fixed with glutaraldehyde and resuspended to 1×10^6 cells/ml in a citrate – phosphate – acetate – saline buffer at pH 7 (○), 6 (●), 5 (□), 4 (■), and 3 (△). Cells were then incubated with 0.1 μM OVA323 – 339, and at various times aliquots were removed. Cells were washed with PBS (pH 7.3) both to neutralize the samples and to remove unbound peptide. The ability of the APCs to stimulate IL-2 production by the A^d/OVA323 – 339-specific T hybridoma was then measured. IL-2 levels in the supernatant were measured by determining thymidine incorporation by CTLL cells.

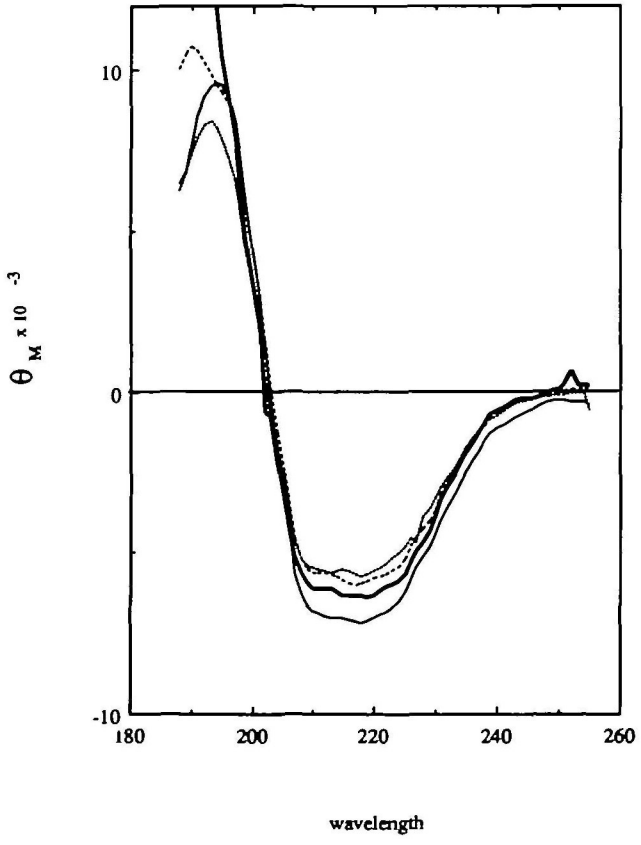

Fig. 3. CD spectra of the MHC class II molecules A^d and E^d. CD spectra of affinity purified A^d (heavy line) and E^d (light line) solubilized in octyglucoside (dashed lines) or reconstituted into phosphatidylcholine vesicles (solid lines).

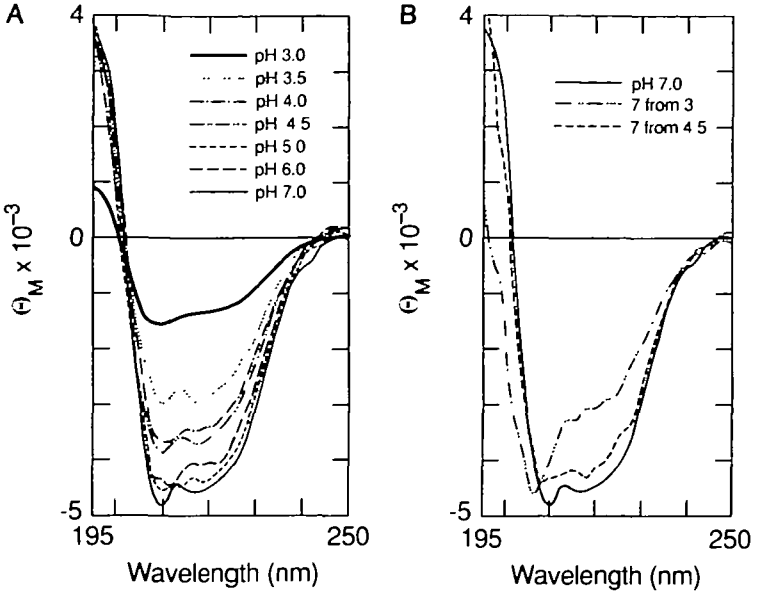

Fig. 4. Far UV CD spectra of A^d as a function of pH. A sample of lipid-reconstituted, purified A^d molecules were progressively acidified from pH 7 to 3 by addition of 20% acetic acid (A). The pH was checked at each point using a microelectrode. At pHs 4 5 and 3 the samples were neutralized and spectra were re-recorded after an interval of 20–25 min (B). Longer incubations did not change the refolding

as a function of pH. We used conditions of excess peptide and an incubation time known to be saturating for A^d binding at neutral pH (10). Figure 2 shows that when peptide concentration is saturating, the levels of OVA323–339 binding to A^d were comparable between pH 3 and 7. Apparent increases in peptide binding at pH 5, 4, and 3 at 40 μM peptide translate to an increase in occupancy of the A^d molecule from ~10 to a maximum of ~18%. These data suggest that low pH does not consistently increase the number of available peptide binding sites on a purified population of A^d molecules, but primarily allows more rapid binding of peptide to the MHC molecule.

CD spectra of A^d and E^d in detergent and lipid

Figure 3 shows representative far UV CD spectra of A^d and E^d in octylglucoside or after reconstitution into phosphatidylcholine vesicles. The spectra of both molecules are similar in detergent and lipid, suggesting that there are no large differences in secondary structure in detergent versus lipid. Furthermore, the A^d and E^d molecules have similar CD spectra. The CD spectra at neutral pH are similar to those obtained from papain solubilized human class I and II molecules (34).

Conformational changes in the A^d molecule as a function of pH

Figure 4(A) shows representative CD spectra of the A^d molecule at pH levels from 7 to 3. We used A^d reconstituted in phosphatidylcholine for these experiments, but other experiments using A^d in octylglucoside yielded similar results (data not shown). Although the lipid obscures CD signals below 195 nm, analysis of lipid reconstituted material may be more physiologically relevant than studies in detergent, in that the reconstituted samples more closely mimic the conditions in the APC. As shown in Fig. 4(A), a change in the A^d CD spectrum is detectable at pH 4.5. In four independent experiments, this change is first

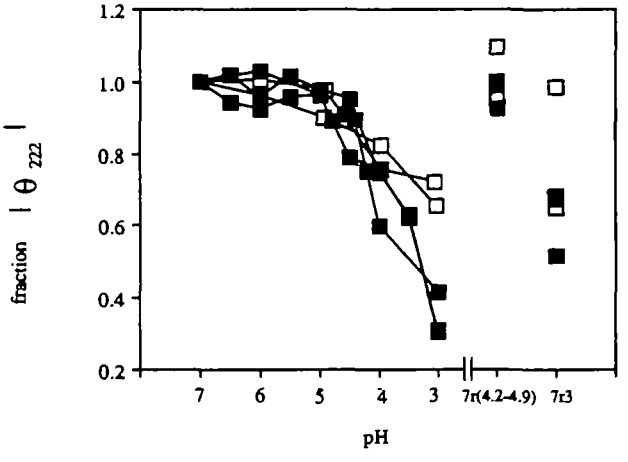

Fig. 5. θ_{222} as a function of pH for A^d. Five A^d samples from three purifications were acidified with acetic acid as in Fig. 4 (closed symbols) or using HCl (open symbols). The magnitude of the CD signal at 222 nm at each pH was measured and expressed as a fraction of the θ_{222} signals at pH 7. θ_{222} signals were also obtained for A^d samples neutralized with NaOH after acidification to between pH 4.2 and 4.9 (7r4.2–4.9) or pH 3 (7r3).

observable at a pH of between 4.2 and 4.9. As the pH is lowered to 3, there is progressively more loss in the ellipticity. Figure 4(B) shows that the change in secondary structure at pH 4.5 is fully reversible, in as much as the CD spectrum returns to its pH 7 shape if the pH is returned to neutrality. In other experiments (data not shown), the spectral change in A^d was fully reversible between pH 4.2 and 4.9. After exposure to more extreme acidic conditions (e.g. pH 3, Fig. 4B) there is a larger loss of ellipticity in the far UV, and upon neutralization the spectrum has a distinctly

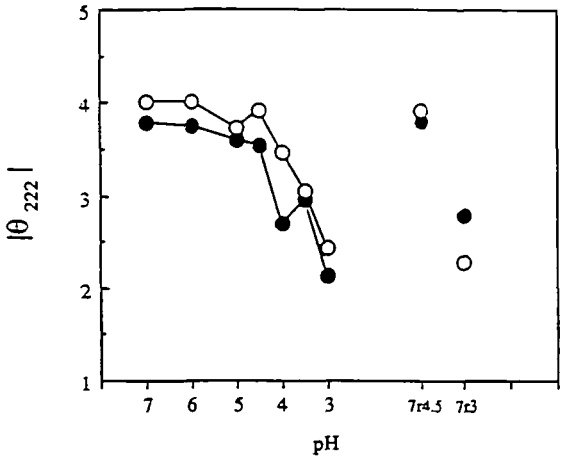

Fig. 6. Effect of peptide pre-incubation on CD spectral changes at low pH. A^d reconstituted in lipid was incubated with 40 μM OVA323–339 (closed circles) or 40 μM HEL46–61 (open circles) for 24 h. A^d binds OVA323–339 well but has a substantially lower affinity for HEL46–61 (47) Unbound peptide was dialyzed away and the sample progressively acidified, and then neutralized, as described in Figs 4 and 5.

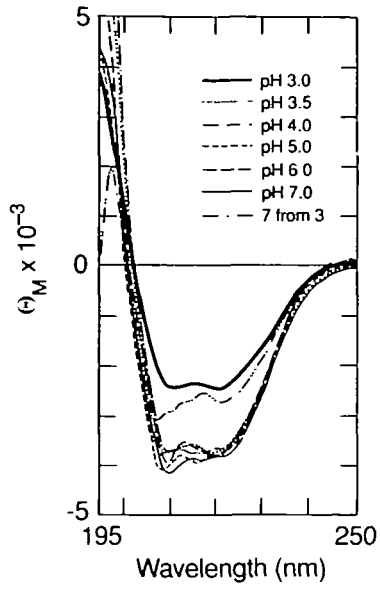

Fig. 7. Far UV CD spectra of E^d as a function of pH. A sample of lipid-reconstituted, purified E^d was progressively acidified by addition of 20% acetic acid as described in Fig. 4. At pH 5 and pH 3 the spectra was neutralized. Spectra have been corrected for dilution.

different shape, more indicative of a higher content of random coil.

Figure 5 shows A^d ellipticity at 222 nm (θ_{222}) as a function of pH for five pH titrations on three independently purified MHC preparations. The ellipticity at 222 nm is used to summarize the data from several different experiments because 222 nm represents the spectral minimum for α-helical polypeptides. Consistently, the pH titrations show that the A^d structure is stable until pH ~5. A progressively decreasing change in signal that is fully reversible occurs between pH 4 and 5. By pH 3 this structural change becomes irreversible. Although the magnitude of the θ_{222} signal of the pH 3 sample after neutralization was variable, in each case neutralization resulted in an altered spectral shape (not shown) as was seen in Fig. 4(B), indicating a lack of reversibility of the spectral change.

Figure 6 shows that pre-incubation of A^d with the OVA323–339 peptide does not change the pH where CD spectral changes are first observable. Pre-incubation with peptide altered neither the magnitude of the spectral changes nor the initial shape of the CD spectrum.

Effect of pH on the far UV CD of E^d

Figure 7 shows spectra from a representative pH titration of the E^d molecule. Figure 8 shows θ_{222} as a function of pH for four experiments on three independently purified preprations. Figures 7 and 8 both show that the E^d protein appears to be more acid stable than A^d, in that no change was observable until a pH of <4 was reached. As was the case for the A^d molecule, the change in CD spectrum at pH 3 was not reversible.

Effect of pH on the secondary structure of MHC class II molecules

An estimate of the secondary structure of proteins can be obtained by comparing the far UV CD spectra of proteins of known structure with that of an unknown protein. Table 1 shows the secondary structure predictions for the A^d and E^d molecules

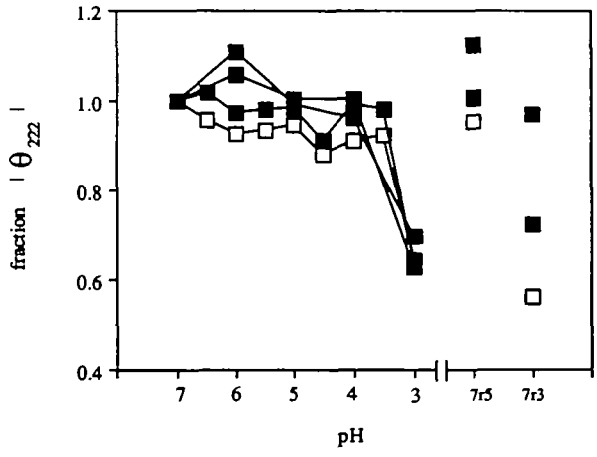

Fig. 8. θ_{222} as a function of pH for E^d. Four E^d samples from three purifications were acidified as in Fig. 4. The magnitude of the CD signal at 222 nm for each pH was measured and expressed as a fraction of the pH 7 θ_{222} signals. θ_{222} signals were also obtained for E^d samples neutralized after being acidified to pH 5 (7r5) and pH 3 (7r3). Samples were acidified with HCl (open symbols) or acetic acid (closed symbols).

as a function of pH, calculated using the program CONTIN (33). The estimates at neutral pH are consistent with the expected secondary structure of the MHC class II molecule based upon its proposed similarity to MHC class I proteins. Table 1 shows that at mildly acidic pH (pH ~4.5) there was a loss of α-helical content in the A^d molecule. As shown in Fig. 4, this loss of ellipticity was fully reversible upon return to neutral pH. At low pH (pH 3), on the other hand, there is an almost complete loss

Table 1. Secondary structure predictions for A^d and E^d as a function of pH

MHC	pH	% secondary structure		
		α-Helix	β-Sheet	Other
A^d	7.0	10 ± 1	41 ± 2	49 ± 3
	4.2 – 4.9[a]	4 ± 3	40 ± 3	56 ± 1
	3.0	1 ± 1	54 ± 8	46 ± 7
E^d	7.0	9 ± 1	45 ± 4	47 ± 6
	4.5	8 ± 1	46 ± 1	47 ± 2
	3.0	1 ± 1	53 ± 6	47 ± 6

Molar ellipticity values for MHC class II molecules reconstituted in lipid were analyzed by the CONTIN secondary structure determination program of Provencher and Glockner (33). Secondary structural estimates are expressed as a fraction of total secondary structure. Determinations are the average of four experiments for A^d and two experiments for E^d.

[a]For A^d, the secondary structure determination at pH 4.2 – 4.9 was calculated for each experiment at the pH level which first showed a significant decrease in ellipticity as a function of pH.

of predicted α-helical content for A^d. E^d seems to be more acid stable in that detectable changes in predicted α-helical content occur only below pH 4. However, like A^d, E^d is predicted to lose most of its helical content at pH 3.

Discussion

The data presented in this report show that: (i) the increase in A^d – OVA323 – 339 complex formation at low pH is primarily a kinetic effect, and increases the number of available peptide binding sites in the MHC class II protein only irreproducibly and to a limited extent; (ii) acidic pH treatment results in a reversible change in A^d structure at mildly acidic pH (4.2 – 4.9) and an irreversible change in A^d structure at more acidic pH (pH 3); and (iii) purified E^d molecules are more stable to acid pH treatment than are purified A^d molecules.

Several groups have reported (18,23,24) that the number of available binding sites increases upon incubation of peptides with MHC class II proteins at acidic pH. For example, Sette *et al.* (18) found a 2-fold increase in available A^d binding sites upon incubation of OVA318 – 339 at acidic pH. In our study we did not reproducibly find large changes in the occupancy of the MHC class II protein after incubation of peptides at acidic pH. Differences between our study and the others might reflect the increased ability to exchange labeled peptide for endogenous peptide in the presence of detergent, the longer incubation times used in the Sette *et al.* (18) study (48 h versus 24 h), or their use of a longer peptide (OVA318 – 339 versus OVA323 – 339).

Our previous work on the pH-lability of A^d – OVA323 – 339 complexes showed that these complexes dissociate only at pH levels <5 (15). Mild acid treatment did not appear to release endogenous co-purifying peptides in that prior acid treatment did not increase the number of available binding sites (15). Exposure to more extreme acidic conditions (pH 3), such as those used to elute co-purifying peptides from MHC class II proteins (13), also failed to lead to an increase in the number of available binding sites, presumably because such treatment produced irreversible denaturation of the MHC protein (15). Using CD spectroscopy, the present data confirm that exposure to pH levels which lead to removal of endogenous peptides from MHC class II proteins causes an irreversible change in conformation, possibly denaturation of the binding site. On the other hand, exposure to pH levels equivalent to those found in the endosome – lysosome pathway leads to a reversible change in MHC class II conformation.

It is tempting to speculate that the reversible change in A^d conformation at mildly acidic pH (4.2 – 4.9) is consistent with a reversible opening up of the binding site to facilitate entry of peptides. This conformational change is predicted to involve a loss of α-helical structure, but might also involve a change in the relationship between the α1 and β1 domains which combine to form the MHC class II binding site. Helical regions of MHC class II proteins are predicted to occur in both the transmembrane domains and in the N-terminal domains. Since transmembrane domains of proteins are highly resistant to denaturation (35,36), it is most likely that the conformational change we observe first is in the N-terminal, peptide-binding domains. Whether the changes we are observing involve empty molecules or molecules already occupied with endogenous peptides cannot be absolutely determined from these experiments. However, the magnitude of the change implies that a large fraction of the molecules are responding to the change in pH.

Sette *et al.* (18) showed that OVA323 – 339 binds to A^d with a broad pH optimum, between pH 4.5 and 6.5. We initially observe conformational changes in A^d only at the more acidic end of this optimum. It is likely that we do not detect conformational changes until a significant proportion of the A^d molecules have undergone the change. Because only 10 – 20% of the A^d molecules in our preparation bind peptide, and because the level of binding does not dramatically increase under saturating conditions at low pH, it is conceivable that the subpopulation which binds peptide undergoes the structural change at milder pH, but that this is not detectable by CD.

Several models can be proposed to explain the effect of pH on the interaction of peptides with MHC class II proteins (Fig. 9). In model A, MHC class II proteins are refractory to peptide binding at neutral pH, and a pH-dependent conformational change leads to an opening up of the structure. A second conformational change upon peptide binding is proposed to give rise to a long-lived, relatively acid-stable complex. This complex may be more stable to acid than molecules without bound peptide, thus allowing the complex to survive until it is neutralized upon transport to the cell surface. Model B is a variation of model A in which protons promote exchange of peptides in the MHC class II binding groove via an intermediate that binds two peptides (20). Alternatively, low pH might promote release of one peptide, followed by rebinding of another (not shown) (37).

Model C is similar to a model proposed by Jensen (23) and largely based on the observations of Sadegh-Nasseri and McConnell (21). In this model, peptide initially interacts weakly with the MHC class II protein, such that it is in rapid equilibrium between a free and bound form. This short-lived complex only slowly converts to a long-lived complex at neutral pH, but upon exposure to low pH conditions rapidly converts to the long-lived complex.

Our data are more consistent with model A than with model C, in as much as the spectral changes observed at pH 4.2 – 4.9 are consistent with an opening up of the structure at this pH.

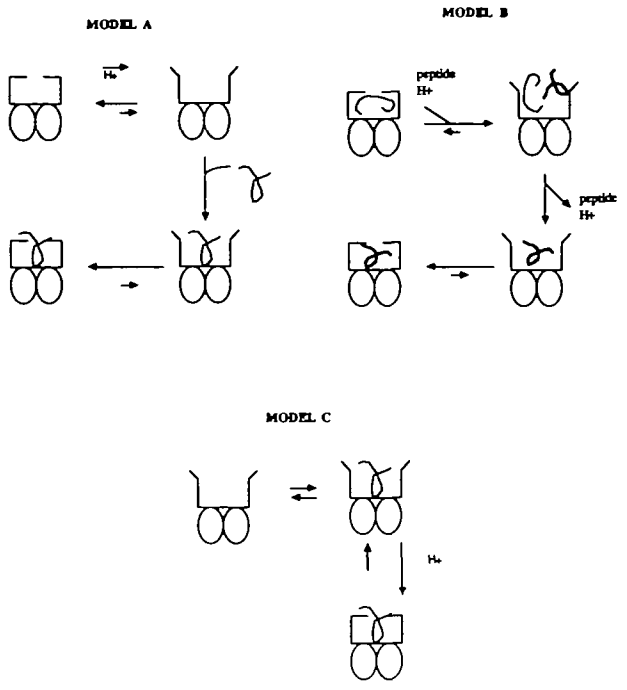

Fig. 9. Models for the effect of pH and peptide on the accessibility of the MHC class II peptide binding groove. In model A, at neutral pH the peptide binding groove is thought to be relatively inaccessible, resulting in slow on-rate for peptide binding. At acidic pH, the groove is thought to become more accessible. To explain the long-lived state of the MHC class II – peptide complex, we propose that a further conformational change in the MHC class II protein occurs upon peptide binding, promoting re-entry into a (perhaps different) closed state. In model B, simultaneous addition of peptide at low pH allows exchange with already bound peptide via an intermediate in which two peptides are bound to the MHC class II protein, presumably via different subsites. In model C, empty MHC class II proteins are thought to have relatively accessible binding sites, but only slowly convert to a long-lived stable complex upon peptide binding. Acidic pH conditions are thought to promote entry into the long-lived state.

However, we cannot absolutely determine from these measurements which part of the molecule is changing conformation at low pH, or whether we are measuring the behavior of all the A^d molecules or the subfractions that can (10 – 20%) or cannot (80 – 90%) bind exogenously added peptides.

Jensen (23) has shown that pretreatment of MHC class II molecules at mildly acidic pH does not lead to an irreversible change in their ability to interact with peptide, but that simultaneous incubation with peptide and protons is required for enhanced MHC – peptide interaction. Our data are entirely consistent with this observation in that mild acid treatment does not lead to an irreversible change in MHC class II structure. Thus our data are compatible with a loosening of the structure at acidic pH, so as to allow peptide exchange or entry into the site.

Another aspect of model A is that it predicts a second conformational change upon peptide binding. Pre-incubation of the A^d molecules with OVA323 – 339, so as to saturate the binding site, does not alter the initial CD spectrum. Furthermore, pre-incubation with peptide does not change the pH stability of the CD spectrum (Fig. 6). It may be that a peptide with higher affinity for MHC class II molecules, such as those that survive pH 4 – 5 treatment, would be able to stabilize the structure in such a way as to prevent peptide release or acid-induced unfolding. In the case of A^d – OVA323 – 339, the increased on-rate at low pH is offset by an increased off-rate (18,15), so that it is perhaps not surprising that this peptide did not change the pH sensitivity of the CD spectrum. The failure to detect peptide-induced conformational changes may also be due to the low fraction of A^d molecules capable of binding OVA323 – 339 (10 – 20%).

Other investigators have provided evidence for a peptide-induced conformational change in MHC class II proteins. Mellins *et al.* (38) have identified cell lines with a defect in antigen processing that possess normal MHC class II genes but express proteins with altered structure as measured by antibody binding and the instability of the MHC class II dimers in SDS. Sadegh-Nasseri and Germain (39) showed that exposure of MHC class II proteins to peptides at acidic pH followed by neutralization resulted in an MHC II dimer that is resistant to separation in SDS gels. Germain and Hendrix (40) have shown that progress of the MHC class II molecules through the export pathway of a cell during a pulse chase experiment results in conversion of unstable dimers to stable dimers. The inference from this data is that formation of a peptide – MHC II complex leads to acquisition of the stable state. The stable dimers in the Germain and Hendrix experiments were better able to bind some conformationally sensitive MHC class II-specific antibodies, consistent with the idea that occupancy with peptides alters the conformation of MHC class II proteins.

Stern and Wiley (41) have recently shown that HLA-DR1 expressed in insect cells assembles into dimers in the absence of peptide, but otherwise forms aggregates. Addition of a DR1-binding peptide to the DR1 preparation from insect cells prevents the aggregation of the dimers and increases their resistance to SDS-induced chain dissociation, again suggestive of peptide-induced conformational changes.

Although the behavior of the A^d molecules as a function of pH is compatible with a reversible opening up of the structure to accommodate peptide at mildly acidic pH, the E^d molecules were more acid stable. According to Germain and Hendrix (40), the 14-4-4S antibody used in the E^d purification shows a particular preference for stable MHC class II dimers, which are assumed to be already occupied with peptide. In support of this, we found that binding of an E^d-restricted peptide, FITC-labeled myoglobin 108 – 121, to our purified E^d preparations was indistinguishable from binding of the peptide to lipid vesicles. This implied that $<2\%$ of the purified E^d molecules had available peptide binding sites (data not shown). It is also of interest to note that Sette *et al.* (18) found that E molecules have a more acidic pH optimum for peptide binding in detergent than do A molecules.

In conclusion, far UV CD studies suggest that MHC class II molecules undergo conformational changes at acidic pH levels which are known to modulate MHC class II – peptide association and dissociation. These changes may involve a loss of α-helical content in the N-terminal domains. Further interpretation of the spectral properties of MHC class II molecules as a function of pH and peptide occupancy will require a more homogeneous population of empty or fully occupied MHC class II proteins. Recent evidence suggests that in an APC, processed antigen binds primarily to newly synthesized rather than cell surface MHC

class II proteins (42 – 44). This interaction takes place in an acidic compartment of the endosome lysosome pathway (28,29) and involves the dissociation of the invariant chain (45,46). Thus the ideal experiment to investigate the role of pH on peptide – MHC class II association would involve studying the structure of empty MHC class II proteins upon incubation with peptides at acid pH, immediately after the release of invariant chain.

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Abbreviations

APC	antigen presenting cell
CD	circular dichroism
FITC	fluorescein-5-isothiocyanate
OVA	ovalbumin

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