Secondary Structure Composition and pH-dependent Conformational Changes of Soluble Recombinant HLA-DM*

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### Abstract

HLA-DM catalyzes the release of invariant chain fragments from newly synthesized major histocompatibility complex (MHC) class II molecules, stabilizes empty class II molecules, and edits class II-associated peptides by preferentially releasing those that are loosely bound. The ability of HLA-DM to carry out these functions in vitro is pH dependent, with an optimum at pH 4.5–5.5 and poor activity at pH 7. The structural basis for these properties of HLA-DM is unknown. Sequence homology suggests that HLA-DM resembles classical, peptide-binding MHC class II molecules. In this study, we examined whether HLA-DM has a secondary structure composition consistent with an MHC fold and whether HLA-DM changes conformation between pH 5 and pH 7. Far-UV circular dichroism (CD) spectra of recombinant soluble HLA-DM (sDM) indicate that HLA-DM belongs to the α/β class of proteins and structurally resembles both MHC class I and class II molecules. The CD peak around 198 nm increases upon going from neutral to endosomal pH and drops sharply upon denaturation below pH 3.5, distinguishing at least three states of sDM: the denatured state and two highly similar folded states. Fluorescence emission spectra show a slight blue-shift and a ~20% drop in intensity at pH 5 compared with pH 7. Unfolding experiments using guanidinium chloride show that the stability of sDM is somewhat reduced but not lost at pH 5. These results indicate that sDM undergoes a pH-dependent conformational change between neutral and endosomal pH. The change seems to involve both hydrogen bonding patterns and the hydrophobic core of sDM and may contribute to the pH dependence of DM activity.

Major histocompatibility complex (MHC)encoded glycoproteins bind antigenic peptides and display them on the surface of antigen-presenting cells for inspection by T lymphocytes bearing αβ antigen receptors. MHC molecules can be divided into class I and class II molecules, which share a common tertiary fold with a characteristic peptide binding groove but differ in their domain connectivity, specificity requirements for peptide, and ability to stimulate selectively CD8+ and CD4+ lymphocytes, respectively (1). These highly polymorphic “classical” MHC molecules belong to a larger family, which includes more distantly related and less polymorphic molecules encoded in the class II and class Ib regions of the MHC and elsewhere in the genome (2). Among the functions identified for such “nonclassical” MHC molecules are antigen presentation to unconventional T cells, accessory functions in MHC class II antigen presentation, and other, unrelated functions.

Classical MHC class II molecules load peptides in endosomal compartments, and this process is regulated by at least three additional molecules: invariant chain (Ii), HLA-DM, and HLA-DO (3, 4). Ii, which lacks homology to MHC molecules, associates with class II molecules in the endoplasmic reticulum, facilitates their assembly, and targets them to endosomes. Here, Ii is degraded, leaving Ii-derived peptides in the antigen binding groove (class II-associated Ii peptides, CLIP). CLIP must be released from class II molecules to permit normal binding of endosomal peptides, a process that is accelerated by HLA-DM (5–17). In addition, DM-catalyzed peptide release is not limited to CLIP (15, 18, 19), so that distinct sets of peptides are loaded onto class II molecules in DM− and DM+ cells (20, 21). These effects do not seem to be due to any influence of HLA-DM on MHC class II trafficking (22–24). Finally, DM stabilizes class II peptide binding sites against inactivation (25, 26). The function of HLA-DO has been studied less extensively, but there is some evidence that it modulates DM function (27–29).

The intron-exon structure and promoter regions of the DMA and DMB genes that encode DM αβ dimers show similarities to class II α and β chain genes (30). Both DMA and DMB cDNAs show weak sequence homology to classical class I and class II MHC molecules (31, 32). The lowest degree of homology is seen in the membrane-distal domains, which contain the antigen-binding site of classical MHC proteins. Consistent with an MHC-like fold, a Cys residue (Cys-79) in the p1 domain of DM, which is conserved among MHC molecules, is important for proper DM folding (33). Unlike classical class II molecules, however, no evidence for peptide binding activity of HLA-DM has been found, raising the possibility that it lacks a ligand binding groove (16, 34).

The mechanism by which DM alters peptide loading of class II molecules is starting to be elucidated. HLA-DM associates directly with MHC class II molecules (35); the most stable association seems to be with empty molecules (25, 26), but peptide-loaded molecules also may have some affinity for DM (35). DM accelerates peptide dissociation from MHC class II molecules (15–17, 36) without changing peptide specificity (37). This result suggests that DM stabilizes an “open” class II...
conformation without altering interactions between class II specificity pockets and side chains of the bound peptide, perhaps by breaking hydrogen bonds between class II and the peptide (38). Antibody-blocking experiments and the characterization of a HLA-DR mutant defective for DM binding suggest that a specific face of the class II molecule is involved in DM interactions (16, 24, 39). Based on hydrophobic dye binding studies, it has been proposed that the interaction involves hydrophobic sites that are buried at the DM-class II interface (40).

Peptide loading of class II molecules occurs in acidic (pH 4.5–5.5) late endosomal compartments (23, 24). Structural features of both MHC class II molecules and HLA-DM may contribute to ensuring efficient peptide loading under these conditions. In some instances, titratable side chains at the peptide-MHC class II interface may provide optimal interactions at endosomal pH (41). In addition, MHC class II molecules change conformation between pH 7 and pH 5, as shown by spectroscopy and by differential hydrophobic dye binding (42–45). The most pronounced rearrangements may occur at sites that are distant from the peptide binding groove (45); nevertheless, they are associated with accelerated peptide binding and release at low pH. Not all peptide/MHC class II combinations behave like this; examples of pH-independent peptide binding are not uncommon. Even in these cases, however, peptide binding becomes pH dependent upon adding HLA-DM (15). DM binding to class II molecules and its catalytic activity are greater at pH 4.5–5.5 than at neutral pH (15, 17, 25, 35).

Hydrophobic dye binding of HLA-DM also increases at pH 5, but the effects seem modest compared with those previously seen for classical class II molecules (28, 40). We therefore wished to determine the nature and extent of any changes in HLA-DM conformation between neutral and endosomal pH. Here, we have used far-ultraviolet circular dichroism and intrinsic fluorescence spectroscopy to analyze both the secondary structure composition of the luminal domains of HLA-DM and the effect of pH on their conformation and stability.

**MATERIALS AND METHODS**

**Cells—**Drosophila melanogaster-derived S2 cells expressing recombinant soluble HLA-DM (sDM) have been described (15). Briefly, expression vectors were constructed that contained truncated DMA*0101 and DBB*0101 cDNAs under the control of a metallothionein promoter. For both chains, the transmembrane regions and cytoplasmic tails were replaced by epitope tag sequences, which are recognized by the monoclonal antibodies, M2 and RT3, respectively. A similar strategy was used to generate transfectants expressing full-length DM, as well as full-length and soluble HLA-DR1, all without epitope tags. After transfection and antibiotic selection, highly expressing subclones were isolated, and expression of transfected cDNA was induced in serum-free spinner cultures by adding 1 mM CuSO4. For full-length molecules, cells were harvested after 24 h, washed in Dulbecco’s phosphate-buffered saline (without Ca2+ and Mg2+), and stored at −80 °C for soluble molecules, culture supernatant was harvested after 7 days.

**Purification of Recombinant Molecules—**Insect cell-derived recombinant DR1 was purified by immunoaffinity chromatography as described (46). Soluble DR1 was purified similarly, except that concentrated culture supernatants were loaded onto the column and detergent was omitted. For purification of sDM, culture supernatants were centrifuged (5000 > g, 20 min), and 1 mM phenylmethylsulfonyl fluoride and 10 mM iodoacetamide were added. Filtered (<0.2 μm) supernatants were passed over a glycine-coupled CNBr-Sepharose column and an M2 monoclonal antibody column (Eastman Kodak) connected in series. After washing in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, specifically bound material was eluted from the M2 column using 100 mM FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Eastman Kodak) in wash buffer, and the column was reequilibrated by alternating 20-min washes in 10 mM glycine-HCl, pH 3.5, and wash buffer. The column eluate was concentrated by centrifugal ultrafiltration (Centricon-30 and Centriprep-30) and loaded onto a 2 × 30-cm Sephacryl S200-HR column (Amersham Pharmacia Biotech), which was eluted in phosphate-buffered saline. Fractions containing sDM dimer without contaminating proteins were pooled and again concentrated. After a buffer exchange into water, sDM was stored in aliquots at −20 °C. The final yield of pure sDM dimer was approximately 4 mg per liter of culture supernatant.

Full-length recombinant DM was affinity purified from S2 cell transformants. A previously described polyclonal antiserum raised to sDM (11323; see Ref. 33 for a description of its reactivity) was affinity purified using sDM covalently coupled to CNBr-activated Sepharose 4B (Pharmond eluted using 100 mM glycine-HCl, pH 3.0. The affinity-purified immunoglobulin, in turn, was coupled to CNBr-Sepharose 4B (2 mg of IgG per ml of beads). A CHAPS (1% w/v) extract from 1010 S2 transformants was applied to the anti-DM affinity column. The column was washed with 10 mM Tris-HCl, pH 7.5, 1% CHAPS, 150 mM NaCl, and then with 10 mM Tris-HCl, pH 7.4, 1% CHAPS, 500 mM NaCl. DM was eluted with 50 mM glycine-HCl, pH 3.8, 1% CHAPS and immediately neutralized with 2 M Tris-HCl, pH 8.0. Fractions containing DM were pooled and further purified by gel filtration chromatography on a Superdex 200 column (Pharmacia). Protein was quantified by Bradford assay and by a DM-specific competitive enzyme-linked immunosorbent assay using purified sDM as a standard, and silver staining of purified full-length DM failed to detect any contaminating proteins (data not shown). The final yield was approximately 0.5 mg of full-length DM per 1010 cells.

**Protein Characterization and Quantitation for Spectroscopy—**Fractions from various stages of the purifications were subjected to SDS-PAGE (47). Total proteins were visualized by Coomassie Blue staining, and large amounts of protein (up to 20 μg lane) were loaded to assess purity. Soluble DM α and β chains were identified by immunoblotting using epitope tag-specific mAbs, using previously described protocols (15, 33). To confirm the stability of sDM heterodimers following storage, samples were rerun on a Sephacryl S200-HR column in 20 mM sodium phosphate (pH 7.0) or sodium acetate (pH 5.0) buffers containing 150 mM NaCl. Amino-terminal sequencing by Edman degradation was performed by the Stanford University Protein and Nucleic Acid Facility.

The derived amino acid sequences encoded by the sDMA and sDMB were used to calculate an extinction coefficient ε280 = 72670 M−1 cm−1 in 20 mM sodium phosphate buffer, pH 6.0, 8 mM guanidinium chloride (48, 49). The extinction coefficient of sDM was not affected measurably by the presence or absence of denaturant (data not shown). The calculated extinction coefficient agreed to within 3% with an empirical value derived from quantitative amino acid analysis (performed by the Stanford University Protein and Nucleic Acid Facility).

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**Circular Dichroism Spectroscopy—**Far-ultraviolet circular dichroism spectra were acquired on an Aviv 62DS spectropolarimeter equipped with a thermostatted cuvette, using previously described conditions (44, 50). Samples contained 100–250 μg/ml sDM in 5 mM sodium phosphate buffer, pH 7.0, or 5 mM sodium acetate buffer, pH 5.1. A 1-mm path length cuvette was used, and samples were recovered after analysis and quantitated by UV absorption spectrophotometry as described above. Five CD spectra were acquired using previously described parameters (44), averaged, and smoothed using a 9-point (2.25 nm) sliding window; the buffer background was then subtracted. For pH titrations, samples were made up in appropriate mixtures of 5 mM sodium phosphate, 50 mM sodium acetate, and HCl, at an iced plate, and the mixture was stirred after each experiment. A single spectrum was acquired at each pH in 0.5-nm steps with a 5-s averaging time. Estimates for α-helix and β-sheet content were obtained using the program, k2d, (available at http://columbia.ebi.ac.uk:8765/andrade/k2d.html) (51, 52).

**Fluorescence Spectroscopy—**Fluorescence spectra were acquired at room temperature, using a Hitachi F-4010 spectrofluorimeter with ex-
For spectroscopic characterization, recombinant-soluble HLA-DM (sDM) was purified from supernatants of transfected Drosophila cells, using epitope tag affinity chromatography and gel filtration (Fig. 1A). Gel filtration separated heterodimeric sDM from high molecular weight aggregates containing contaminating proteins, as shown by Coomassie Blue staining of overloaded native (data not shown) and SDS-PAGE gels (Fig. 1A). The SDS gels were overrun to resolve sDM chains as a closely spaced multiplet of bands, which were assigned as either α or β chains by Western blotting with mAbs to the C-terminal epitope tags (Fig. 1B). Thus, all the material in sDM dimer fractions could be accounted for by sDM α and β chains. N-terminal sequencing of sDM dimers yielded two si-
ultaneous sequences, VPEAPTPMWPDDLQ and FVAH-
VESTXLLDDAG, consistent with signal sequence cleavage after residue 26 of immature DM α and after residue 20 of immature DM β, respectively (counting from the initiating Met). Note that the mature β chain N terminus is two residues downstream from the beginning of the β1 domain predicted by Kelly et al. (31). This may be due to incorrect prediction of signal sequence cleavage or limited proteolysis during or after secretion. As the N termini seemed homogeneous by sequencing and all bands resolved by SDS-PAGE contained the C-terminal epitope tags, the size heterogeneity of both chains seen by SDS-PAGE likely was a result of heterogeneous covalent modifications, such as glycosylation. By gel filtration, sDM remained an αβ heterodimer after storage at −20 °C, both at pH 7 (Fig. 1C) and pH 5 (data not shown). In conclusion, our sDM preparations were of sufficient purity and quality for spectroscopic analysis.

We have shown previously that sDM catalyzes release of CLIP and a subset of other peptides from MHC class II molecules (15). sDM also promotes association of labeled peptides with class II molecules, likely by catalyzing dissociation of full-length DM purified from Epstein-Barr virus-transformed B cell lines are catalytically active (16, 17). To assess the role of the transmembrane and/or cytoplasmic domains in the interaction between DM and class II molecules, full-length and soluble, insect cell-derived DM molecules were compared for their ability to catalyze peptide loading of full-length and soluble DR1 (Fig. 2). In this experiment, between 4 and 8 μM soluble DM were required for half-maximal loading of either soluble or full-length DR1 molecules. Even though we were unable to test the activity of high concentrations of full-length
DM, at low concentrations similar amounts of full-length and soluble DM were needed to load soluble DR1 molecules, suggesting that the transmembrane region and cytoplasmic tail of DM were unimportant for peptide loading of soluble DR molecules. Interestingly, when full-length DR1 was used, full-length (but not soluble) DM was active at about 200 times lower concentrations (half-maximal loading between 20 and 40 nM DM), a potency comparable with that seen for affinity-purified DM from B cell lines.3 These results showed that optimal catalytic activity required the transmembrane domains and/or cytoplasmic tails of both DM and DR1, either because these domains interact specifically during catalysis or because the transmembrane regions help to align DM with DR through interactions with detergent micelles. However, because peptide loading of soluble DR1 was not influenced by the presence or absence of the DM transmembrane region or cytoplasmic tail, these domains did not seem to be required for correct folding and activity of the extracellular domains of DM. This finding justified the use of soluble molecules for spectroscopic studies investigating the folding and conformational dynamics of DM.

Far-UV CD spectroscopy was used to analyze the secondary structure composition of sDM (Fig. 3). The CD spectrum at 25 °C and pH 7.0 revealed a pronounced minimum at 218 nm and a maximum at 198 nm, placing sDM within the αβ class of proteins with a significant amount of β-pleated sheet and a moderate α-helical content (Fig. 3A, Table I). The spectrum changed little upon heating to 37 °C (Fig. 3B) and underwent little change up to about 50 °C (data not shown). However, further heating to 80 °C resulted in drastic changes in the far-UV CD spectrum; the heat-denatured material gave a minimum at 205 nm and a maximum at 188 nm (Fig. 3B). These spectral changes showed that sDM adopted a highly stable, folded structure at neutral pH.

The qualitative features of the native sDM far-UV CD spectrum resembled those of classical MHC class I and class II proteins, but the spectra were not superimposable (Fig. 3A). The overall shape of the sDM spectrum was somewhat closer to typical class II (I-Ek) than class I (HLA-A2) CD spectra, as reflected for instance in the ratio of the 218- and 198-nm peaks and the presence of a shoulder in the 222–230-nm range. However, the intensity of this shoulder for sDM was intermediate between I-Ek and HLA-A2. The secondary structure composition of sDM, as estimated using a neural net algorithm, was within the range previously determined by crystallographic or spectroscopic analysis of a set of MHC-like proteins (Table I). The differences between the CD spectra of distantly related molecules of the MHC family might reflect differences in the details of tertiary and quaternary structure, or they could reflect non-peptide bond contributions to the far-UV CD spectrum (for discussion of such contributions, see Refs. 44 and 53).

In conclusion, overall the CD properties of sDM are consistent with an MHC-like fold, but the precise tertiary structure may differ from that seen for classical MHC class I and class II proteins with peptide-hinding function.

To study the effect of exposure to low pH on the conformation of sDM, CD spectra were recorded in a series of buffer mixtures ranging from pH 7.3 to pH 2.5. Fig. 4A shows far-UV CD spectra of sDM at pH 7.3, 4.8, and 2.5, recorded at room temperature. Dropping the pH from 7.3 to 4.8 resulted in a slight but reproducible increase in the intensity of the 198-nm peak of the far-UV CD spectrum, with little (if any) change in estimated secondary structure content (Table I). In particular, no changes were observed in the 222-nm region of the spectrum, indicating that helix content was not altered at endosomal pH. A further drop in pH to 2.5 resulted in drastic spectral changes; the pH 2.5 spectrum closely matched that of heat-denatured material at pH 7 (cf. Fig. 3B). This result indicated that the increase of the 198-nm peak at pH 4.8 was not due to

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Fig. 2. Peptide loading of full-length and soluble DR molecules in the presence of full-length or soluble DM. Full-length (triangles) and soluble (circles) DR1 molecules were purified from insect cells and incubated at 2 nM with 1 μM biotinylated HA307–319 peptide in the presence of varying amounts of either full-length (filled symbols) or soluble (open symbols) DM as shown. Peptide binding was quantitated by an antibody capture assay as described under “Materials and Methods.”

Fig. 3. Far-UV CD spectroscopic analysis of sDM. A, far-UV CD spectrum of sDM (continuous line) at pH 7 and 25 °C. Error bars represent standard deviations from three independent measurements. Shown for comparison are spectra of a recombinant soluble form of the murine class II molecule, I-Ek (dashed line; from Ref. 44), and the papain-solubilized extracellular domains of the human class I molecule, HLA-A2 (dotted line; from Ref. 50). B, far-UV CD spectrum of sDM at 25 °C (continuous line), 37 °C (dashed line), and 80 °C (dotted line).

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3 D. M. Zaller, R. C. Doebele, and R. Busch, unpublished observations.
Structure and Conformation of HLA-DM

Table I

| Molecule | Conditions | α-Helix | β-Sheet | Method | Ref. |
|----------|------------|---------|---------|--------|------|
| sDM | pH 7.3, 25 °C | 18 | 34 | CD | This paper |
| | pH 4.8, 25 °C | 16 | 36 | CD | |
| HLA-DR1 | pH 7–8 | 17–23 | 38–42 | CD | 53 |
| HLA-A2 | Crystal | 10 | 53 | PTIR | 56 |
| | pH 7.75, 20 °C | 8–13 | 74–77 | CD | 53 |
| FcRn | pH 8.0 | 15 | 85 | CD | 57 |

* For sDM, the native far-UV CD spectra from Fig. 4A were analyzed by the neural net algorithm, k2d (51, 52). Estimates of helix content were robust, varying by ±1% between repeat spectra acquired under the same conditions. However, given the quality of the curve fit provided by the k2d algorithm, the small increase in β-sheet content and corresponding decrease in α-helix estimates for sDM at pH 5 may not be significant. For other MHC proteins, included for comparison, spectrally and crystallographically derived secondary structure estimates reported in the literature are shown. In previous studies, CD analysis has frequently overestimated the β-sheet content of classical MHC class I but not class II molecules (53, 57). Crystal structures of FcRn and the class II molecule, HLA-DR1, show similar tertiary folds and secondary structure content as HLA-A2 (55, 58). FTIR, Fourier transform infrared spectroscopy.

\[ a \] Papain cleaved native molecules from Epstein-Barr virus-transformed B cells.

\[ b \] Phosphatidylinositol linked recombinant molecule from Chinese hamster ovary transfectants.

\[ c \] FcRn, Phosphatidylinositol-linked recombinant molecule from Chinese hamster ovary transfectants.

\[ d \] X-ray crystallography.

\[ e \] FTIR, Fourier transform infrared spectroscopy.

\[ f \] Estimated secondary structure composition of sDM and MHC-related molecules of known structure

![Image](image-url) Fig. 4. Effect of pH on CD spectrum of sDM. A, far-UV CD spectrum of sDM at pH 7.3 (continuous line), pH 4.8 (dashed line), and pH 2.5 (dotted line) at 25 °C. B, pH dependence of the CD signal at 198 nm (filled circles) and 218 nm (open triangles). Error bars are standard deviations estimated from several independent experiments for both neutral (pH range 6.7–7.3; n = 5) and endosomal pH (pH range 4.7–5.2; n = 4); for the 198-nm signal, the error bar at neutral pH is too small to be visible. Variations in the intensity of the 218-nm peak at pH ≥3.5 were within experimental error, whereas the intensity of the 198-nm peak was reproducibly higher at endosomal pH. As a concentration-independent measure of spectral shape, ratios of the intensities of the 198-nm to the 218-nm peak (\( \frac{-[\theta]_r}{[\theta]_r + 218} \)) were 0.82 ± 0.06 in the neutral and 1.01 ± 0.03 in the endosomal pH range.

incipient acid denaturation because a further drop in pH decreased the CD signal at this wavelength. The intensity of the 218- and 198-nm peaks was measured for a range of pH values between pH 7 and 5 and decreased below pH 3.5; in contrast, the 218-nm peak did not change in intensity until the pH was decreased below 3.5. Together, these observations define three spectroscopically distinct states of sDM: the denatured state (similar for both heat and acid denaturation) and two states differing in abundance between pH 7 and 5.

Changes in far-UV CD spectra tend to be dominated by changes in the chiral environment surrounding peptide bonds (54). We wished to investigate whether other aspects of sDM conformation also were influenced by pH. To this end, we used fluorescence spectroscopy, which is highly sensitive to the environment of aromatic residues in proteins, particularly tryptophans and to a lesser extent tyrosines. The sDM molecule has a large number of aromatic residues, including 11 tryptophans, dispersed throughout both the α and β chain. Spectral changes between neutral and endosomal pH were readily seen using this technique (Fig. 5A). At pH 7.0, the emission spectrum had a maximum at 338 nm; at pH 5.0, the maximum was reproducibly blue-shifted to 337 nm, and the maximum fluorescence intensity was decreased by about 20%. These results indicated that a majority of tryptophans were buried in native sDM and suggested that their average environment became slightly more nonpolar at endosomal pH. These changes could involve subtle adjustments in the environment of numerous residues or substantial movements of one or a small number of them. The spectral changes were reversible on a time scale of minutes or less (Fig. 5A), implying that the underlying structural changes were rapid and bidirectional. These observations ruled out, for instance, pH-dependent cleavage by contaminating proteases as an explanation for the spectral changes. Both the pH 5 and pH 7 signals were linear between 50 and 1000 nM sDM, indicating that concentration-dependent differences in oligomerization state were not responsible for the spectral differences (data not shown).

To assess the extent to which Trp residues are buried within the folded structure of sDM, we examined spectral changes upon unfolding in the presence of 6 M guanidinium chloride (Fig. 5B). Under these conditions, the emission maximum was red-shifted to 353 nm, and a substantial increase in fluores-
cence intensity was observed. Given that fully buried Trp residues generally emit maximally at about 330 nm and fully exposed residues at around 350 nm (54), these results confirmed that sDM was substantially folded and indicated that an average Trp (or the majority of Trp residues) in sDM was likely to be shielded from aqueous solvent within the folded structure at both neutral and endosomal pH levels. In contrast to the drastic effects of high denaturant concentrations, addition of 150 mM NaCl had little (if any) effect on the fluorescence spectra (Fig. 5B). This result suggested that the conformational changes measured by far-UV CD in low ionic strength buffers resemble those at more physiological salt concentrations.

The changes seen by both CD and fluorescence spectroscopy between neutral and endosomal pH levels were rather subtle and could have arisen from relatively minor structural perturbations. To obtain additional information on the extent of the pH-dependent changes, the stability of sDM was compared at pH 7 and pH 5. Guanidinium chloride was used to unfold sDM at room temperature, low sDM concentrations were used to avoid aggregation of denatured sDM at pH 5 (data not shown), and unfolding was monitored using intrinsic tryptophan fluorescence at 350 nm (Fig. 6). At both pH 7 and pH 5, unfolding occurred over a narrow range of guanidinium chloride concentrations, with greater stability at pH 7 (half-maximal unfolding at 1.8 M guanidine-HCl) than at pH 5 (1.3 M guanidine-HCl). At both pH values, unfolding was irreversible (data not shown), precluding analysis of unfolding thermodynamics using simple reversible two-state models. Nevertheless, these results confirmed the stability of sDM at both pH 7 and pH 5 in the absence of denaturant and showed that the conformational stability of sDM was somewhat decreased at pH 5. We concluded that the pH-dependent structural change was sufficiently extensive to perturb protein stability.

**DISCUSSION**

In this study, we have used recombinant soluble HLA-DM molecules to explore the secondary structure composition and conformational dynamics of HLA-DM. The choice of analyzing only the luminal domains as a soluble molecule, rather than full-length material containing the transmembrane and cytoplasmic domains, was based on two considerations. First, it permitted comparisons between our CD spectra and published spectra of a number of soluble derivatives of classical MHC molecules analyzed using similar techniques. Use of soluble molecules also obviated the need for detergent to solubilize transmembrane proteins, which interfere with far-UV CD measurements by UV absorption. Despite the requirement for relatively high concentrations of sDM to observe activity in vitro, there is no detectable difference between the amounts of full-length and soluble DM required to catalyze peptide exchange of soluble DR molecules, implying that the absence of the transmembrane anchor in soluble DM does not cause mal-folding. Although it is conceivable that the folding of sDM is heterogeneous, the spectroscopic and stability studies reported here do not show evidence for contamination with denatured material and provide direct evidence for pH-dependent conformational changes. Thus, even though it is difficult to rule out minor structural differences between native and recombinant
full-length DM, it is unlikely that the structural and conformational properties of sDM differ substantially from those of the native molecule.

Secondary structure estimates for sDM based on far-UV CD spectra suggest that the $\alpha$-helix and $\beta$-sheet content of sDM are within the range seen for classical and nonclassical MHC class I and classical class II molecules. Substantial helix content likely is limited to the membrane-distal, N-terminal domains that comprise the peptide-binding groove of classical MHC proteins. Both of the C-terminal epitope tags are widely charged, proline-containing sequences that are quite unlikely to form $\alpha$-helices, and the membrane-proximal domains have unambiguous homology to immunoglobulin superfamily domains that are known to lack helices (32). Together with our recent finding that the conserved Cys-79 of the DM $\beta$ chain participates in disulfide bonding (33), the spectroscopic data reported here are consistent with an MHC-like fold. Nevertheless, spectra of representative MHC class I and class II molecules differ significantly from one another, and the sDM spectrum, while being somewhat more class II-like in overall shape, was clearly not superimposable with that of E$\alpha$. The spectral differences could be due to minor differences in secondary or tertiary structure or in non-peptide bond contributions. Thus, our results allow for the possibility that the tertiary fold of DM may be different from that of classical MHC molecules in important aspects, for instance with regard to the presence of an antigen-binding groove. A precedent for this possibility has been found upon crystallographic analysis of the rat neonatal Fc receptor (55). The secondary and tertiary structures of this molecule are MHC-like, but the helices that flank the bound antigenic peptide in classical MHC proteins are clearly opposed, leaving no room for a ligand.

Previous work has shown differences in binding of the hydrophobic fluorescent dye, anilinonaphthalenesulfonic acid, to sDM at pH 7 and pH 5 (28, 40). However, the amounts of protein required to observe substantial dye binding differed 10-fold between the published studies, and the pH effects were relatively small, raising questions about the nature and extent of the conformational change. Our far-UV CD (Fig. 3) and fluorescence spectra (Fig. 5) show that sDM exists in spectroscopically distinguishable, rapidly interconvertible states at pH 7 and pH 5, which are distinct from acid-, chaotrope-, or heat-denatured conformations. These observations could reflect conformational changes or changes in the local dielectric constant near side chains that become protonated at pH 5. That the conformation of sDM does change significantly at endosomal pH is indicated by the observation that the stability of sDM to unfolding by guanidinium chloride is decreased at pH 5. Some destabilization at pH 5 is consistent with the results of experiments using hydrophobic fluorescent dyes that indicate an increased exposure of hydrophobic sites in sDM to solvent at endosomal pH (40). However, the small extent of the pH-dependent changes in the CD and fluorescence spectra indicates that the structural alterations are not drastic; indeed, CD-based secondary structure estimates change minimally, if at all, between pH 7 and pH 5. Clearly, sDM maintains stability and has a long half-life in protease-rich endocytotic compartments (7, 33); it therefore must remain sufficiently rigid at endosomal pH to remain resistant to proteolytic attack in acidic peptide loading compartments.

How the pH-dependent conformational change identified by our experiments contributes to improved function of DM at endosomal pH remains unclear. A previous report showed evidence for masking of hydrophobic sites within DM-class II complexes, implicating hydrophobic interactions in maintaining affinity (40). However, the global structural changes indicated by the effect of pH on protein stability and the ability of diverse spectroscopic techniques to detect the conformational change raise the possibility that multiple structural rearrangements in DM contribute to optimal interactions with class II molecules. In addition, previously identified pH-dependent conformational changes in class II molecules may contribute to the interaction (see Introduction). Further work will be needed to evaluate the relative contributions of different types of intermolecular interactions to the ability of DM both to bind class II molecules and to catalyze their peptide exchange reactions.

The reversibility of the conformational change is compatible with models in which DM release from class II molecules is controlled by pH changes, which occur upon recycling between endosomal compartments and the cell surface (35). However, the alternative view that binding of stable peptides causes DM release within endosomes (25, 26) is not ruled out by our data. In some respects, the conformational dynamics of sDM seem to be intermediate between classical class I and II molecules (cf. Ref. 44). For several classical class II molecules, exposure to endosomal pH causes slight CD changes in the near-UV region (which probes aromatic residues) and even subtler changes in the far-UV region, but no changes in protein stability as measured by thermal unfolding. In contrast, for classical class I proteins, conformational stability tends to be greatly reduced or lost at pH 5; this may be a functional adaptation that prevents aberrant loading of class I molecule recycling through endosomes with exogenous peptides. In our studies, HLA-DM seems to be intermediate with respect to the effect of pH on spectral properties and stability to unfolding. Whereas the conformational dynamics of sDM revealed by our studies may readily be viewed as an adaptation for its function in vivo, it is also possible that the differences between the conformational properties of DM and classical MHC class I and II molecules relate to their early divergence in evolution (32).

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