Sodium Dodecyl Sulfate Stability of HLA-DR1 Complexes Correlates with Burial of Hydrophobic Residues in Pocket 1[1]

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Certain class II MHC-peptide complexes are resistant to SDS-induced dissociation. This property, which has been used as an in vivo as well as an in vitro peptide binding assay, is not understood at the molecular level. Here we have investigated the mechanistic basis of SDS stability of HLA-DR1 complexes by using a biosensor-based assay and SDS-PAGE with a combination of wild-type and mutant HLA-DR1 and variants of hemagglutinin peptide $HA_{306–318}$. Experiments with wild-type DR1 along with previously published results establish that the SDS-stable complexes are formed only when the hydrophobic pocket 1 (P1) is occupied by a bulky aromatic (Trp, Phe, Tyr) or an aliphatic residue (Met, Ile, Val, Leu). To further explore whether the SDS sensitivity is primarily due to the exposed hydrophobic regions, we mutated residue βGly^{86} at the bottom of P1 to tyrosine, presumably reducing the depth of the pocket and the exposure of hydrophobic residues and increasing the contacts between subunits. In direct contrast to wild-type DR1, the peptide-free mutant DR1 exists as an α/β heterodimer in SDS. Moreover, the presence of a smaller hydrophobic residue, such as alanine, as P1 anchor with no contribution from any other anchor is sufficient to enhance the SDS stability of the mutant complexes, demonstrating that the basis of SDS resistance may be localized to P1 interactions. The good correlation between SDS sensitivity and the exposure of hydrophobic residues provides a biochemical rationale for the use of this assay to investigate the maturation of class II molecules and the longevity of the complexes. ***The Journal of Immunology,*** **1999, 162: 3463–3470.**

Certain MHC class II/peptide complexes resist dissociation into constituent α- and β-chains in the presence of SDS, as determined from SDS-PAGE experiments performed without prior boiling of the samples (1–4). This characteristic of MHC class II complexes has been used as a convenient and sensitive assay to determine the efficiency of in vitro as well as intracellular peptide binding. This assay has been pivotal in determining the influence of peptide binding on class II MHC structure (3) as well as in establishing a distinct structural state associated with stable complexes (5). Formation of SDS-stable compact dimers has been suggested to indicate completion of MHC class II folding upon binding to peptide molecules and thus gaining maturity (6). Making use of SDS stability as a peptide binding marker, events in intracellular trafficking of MHC from synthesis, folding and assembly, sorting to vesicular compartments, association of invariant chain, peptide loading, and the following transport to cell surface have all been characterized (7–21). Furthermore, it has been documented that immunodominant peptide epitopes often form SDS-stable complexes with MHC molecules, and peptides that form SDS-stable complexes increase the longevity of class II molecules (22, 23).

Despite the benefits of using the SDS stability assay, a few recent reports have suggested that the SDS stability of the complex is not strictly correlated to the peptide/MHC affinity, thereby questioning the very basis of this assay (22, 24, 25). Two structures with contrasting properties, long-lived SDS-stable $DR1/HA_{306–318}$[3] complex (26) and short-lived SDS-unstable DR3/CLIP complex (27), also did not provide sufficient information about the basis of SDS stability. Contradictory interpretations of this assay stem from the lack of a clear understanding of the mechanisms involved at the molecular level.

In this report we describe a study of the roles of specific peptide-MHC interactions and SDS in the behavior of class II complexes in SDS using the well-characterized HLA-DR1 molecule. Pocket 1 (P1) of DR1 plays the most important role in the peptide interactions, as shown by binding studies (28) and x-ray crystal structures of $DR1/HA_{306–318}$ (26) and DR1/A2 (29). P1 is a deep pocket lined with a series of hydrophobic residues that constitute about 85% of the solvent accessible area. The P1 anchor residues, Tyr^{308} in $HA_{306–318}$ peptide and Trp^{307} in A2 peptide, are almost completely buried in pocket 1. A strong preference of pocket 1 for the aromatic side chain residues (Tyr, Trp, Phe) has been reported (28, 30). Long aliphatic side chains (Met, Leu, Ile, Val) also bind, although less efficiently (30). In SDS-PAGE experiments with HLA-DR1, only the peptides that had aromatic or long aliphatic side chains as the P1 anchor were able to form SDS-stable complexes (24, 31, 32).

In this work we investigated the biochemical basis of SDS stability of peptide-class II complexes using specific peptide interactions with soluble DR1. Our experiments with several $HA_{306–318}$-derived peptides indicate that hydrophobic interactions between the P1 residues and the bulky P1 anchor and the resulting burial of these residues are primarily responsible for SDS stability. SDS stability was determined

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[3] Abbreviations used in this paper: $HA_{306–318}$, peptide containing 306–318 residues of influenza virus hemagglutinin; CLIP, class II major histocompatibility complex-associated invariant chain peptides (residues 81–105); P1, pocket 1; AMCA, 7-amino-4-methyl-coumarin-3-acetic acid; ETEC, a peptide corresponding to 111–123 of CS6 α subunit of enterotoxigenic *Escherichia coli* pili protein.

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by a new biosensor-based assay we have developed and by SDS-PAGE. To further investigate this idea from a different perspective, a site-specific mutation, $\beta Gly^{86} \rightarrow Tyr$, in pocket 1 was constructed to increase the contacts between the subunits and make the pocket more shallow, thereby reducing the contribution from the P1 anchor required to stabilize the complex. Results with the mutant DR1 help to reaffirm the correlation between exposed hydrophobicity and SDS sensitivity. This correlation provides a biochemical rationale for using the SDS stability assay as a probe for determination of peptide affinity.

Materials and Methods

*Construction of G86Y mutant soluble DRB1*0101*

cDNA encoding the extracellular domain of DRB1*0101 (4) was mutated to change the β86 glycine residue to tyrosine. The wild-type gene was cloned into pBluescript II KS vector (Stratagene, La Jolla, CA). The desired mutation was introduced in this clone using two synthetic primers, complementary to opposite strands, containing the nucleotides to be changed. The sequence of oligonucleotide used (only the coding strand is shown, with the altered nucleotides in boldface type and the new silent *Hin*dIII restriction site underlined) was 5′-C TAC GGG GTT TAT GAA AGC TTC ACA GTG C-3′.

The primers were extended by thermal cycling with *pfu* DNA polymerase to generate the entire plasmid. The clones containing the mutant gene were screened using the new *Hin*dIII site. One such clone was sequenced throughout the entire coding region to verify the absence of undesired mutations. The wild-type gene in the baculovirus transfer vector PVL1393 (4) was replaced with the mutant gene. Baculovirus vector containing both the α and mutant β genes with two polyhedrin promoters was constructed as described previously (29).

Production of recombinant soluble DR1 proteins

Soluble DR1 proteins were expressed and purified as originally described (4). Baculovirus DNA (BaculoGold; PharMingen, San Diego, CA) and transfer vectors carrying the wild-type or mutant genes were cotransfected into Sf9 insect cells to produce recombinant viruses. Hy5 cells were infected with these recombinant viruses, and DR1 proteins were purified from the culture supernatant using anti-DR1 mAb (L243) immunoaffinity chromatography columns (33). Purified wild-type and mutant DR1 proteins migrated similarly in SDS-PAGE at the expected sizes of α and β subunits when samples were boiled before electrophoresis.

Gel filtration

A Superdex 200 fast protein liquid chromatography column (Pharmacia, Piscataway, NJ) was used to analyze wild-type and βG86Y DR1 protein preparations as well as separate peptide-loaded complexes. The samples were eluted in PBS at a flow rate of 0.4 ml/min. The column was calibrated using a protein m.w. standard (Bio-Rad, Richmond, CA), and the parameters (slope and intercept) of the linear plot between log (m.w.) and elution volume were determined. Using this linear equation and the experimentally determined elution volumes of DR1 and DR1 complexes, their apparent m.w. were calculated.

Biosensor assay to study SDS stability

A new method to study SDS stability was developed using the IAsys Auto+ resonant mirror biosensor (Affinity Sensors, Cambridge, U.K.). A carboxymethyl dextran cuvette (Affinity Sensors) was activated to produce *N*-hydroxysuccinimide esters using *N*-hydroxysuccinimide and 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (34). HLA-DR conformation-specific mAb, L243, was immobilized to this activated surface via primary amine groups. Ab was immobilized in both chambers of the cuvette to a response level of 3000–3500 arc seconds. Neither wild-type nor mutant DR1 showed significant binding to the unmodified surface under experimental conditions. Either 1 μM wild-type or 1 μM mutant DR1 was incubated at 37°C without or with 100-μM concentrations of different peptides for 24 h in PBS buffer (pH 7.4) with 1 mM PMSF and 0.025% NaN_3. The reactions were then mixed with SDS such that the concentration of SDS was 0.5% and that of PBS was 80% of the original concentration. After 15 min of incubation at room temperature, 15 μl of the samples were injected using the automated sampler into both cuvettes containing 45 μl of PBS buffer. All samples were allowed to bind to the Ab for 15 min, the time determined to be sufficient for maximum binding. Unbound DR1 was then washed with PBS, and the amount of bound DR1 was determined. The same experiment was performed in two cuvettes side by side, and the results of these independent observations were averaged. Increasing the binding time to more than 15 min did not result in any significant change in the net bound DR1 signal remaining after the wash. Bound DR1 was stripped by exposing the Ab surface to 50 mM 3-(cyclohexylamino)propane sulfonic acid at pH 11.5 for 3 min. The binding capacity of the surface was monitored using the DR1/$HA_{306-318}$ complex as the first and the last sample in the absence of SDS. No significant decrease in the binding capacity was observed during the course of the experiments. Of note, the concentration of monomeric SDS significantly decreases with increasing sodium ion (counter ion) concentration. Consequently, the concentration of sodium chloride during incubation of samples with SDS significantly influenced SDS stability.

Table I. *Amino acid sequences of peptides used in this study*

Peptide	Sequence
$HA_{306-318}$	PKYVKQNTLKLAT
Y308A	--A----------
N312Q	------Q------
Anchorless HA	--A--A-GA-A--
YAK	AA-AAAAAA-AAA
CLIP (Ii_{81-105})	LPKPPKPVSKMRMATPLLMQALPMG
ETEC	IIYQIVDEKGKKK

SDS-PAGE

SDS-PAGE experiments were performed essentially as previously described (2); 0.5 μM wild-type or βG86Y DR1 was incubated without any additional peptide or with 100 μM of different peptides at 37°C for 24 h in PBS buffer (pH 7.4) with 1 mM PMSF and 0.025% NaN_3. The reaction samples mixed with equal volumes of SDS-PAGE sample buffer containing 0.2% SDS (final concentration) were either incubated for 15 min at room temperature or boiled for 3 min. These samples were then applied to 12.5% PAGE gels, and the gels were silver stained according to standard protocols. With gels for which density measurements had to be made, the gel was scanned on a Agfa Arcus laser scanner, and the intensities of the different protein bands were analyzed using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Peptide dissociation kinetics

For this study, we synthesized HA peptide containing 306–318 residues of influenza virus hemagglutinin, $HA_{306-318}$ variants, and human $CLIP_{81-105}$ (Table I). The peptides were purified to apparent homogeneity of >95% by reverse phase preparative HPLC, and their identities were confirmed by mass spectrometry. The concentrations of the peptide stock solutions were determined by ninhydrin assay. For use in kinetic studies, the peptides were labeled with the fluorophore 7-amino-4-methylcoumarin-3-acetic acid (AMCA) at the N-terminus using AMCA-*N*-hydroxysuccinimide (Pierce, Rockford, IL). Excess unreacted free label was removed on Sephadex G-10 columns. The labeled peptides exhibited absorption and emission spectra characteristic of AMCA. Labeling did not modify the binding characteristics, since inhibition of binding of labeled peptide by unlabeled peptides correlated linearly with the concentration of unlabeled peptides. The labeled peptide complexes migrated similarly to the unlabeled peptide complexes in both the SDS and nondenaturing gels, further confirming that the fluorescent labels were nonintrusive.

Concentrations of DR1 protein solutions were determined using an extinction coefficient of 77,000 M^{-1} cm^{-1} at 280 nm (4). Dissociation experiments with wild-type and mutant complexes were performed essentially as previously described (5). The amounts of complexes containing the labeled peptide were determined by measuring the AMCA fluorescence in a spectrofluorometer with microcuvettes. The optimal excitation and emission wavelengths for each DR1/AMCA-labeled complex were 350 and 445 nm, respectively. Experiments performed with the labeled peptides alone and DR1 with nonbinding peptide (AMCA-labeled ETEC) indicated that the Sephadex G-50 spin columns separated the peptides with at least 99% efficiency under the experimental conditions. The fluorescence measurements were made in a Perkin-Elmer LS50B luminescence spectrometer (Norwalk, CT).

Results

Investigation of SDS stability by substitutions of $HA_{306-318}$ peptide at anchor positions

We studied the SDS resistance of DR1 complexes with different $HA_{306-318}$-derived peptides and CLIP (Table I). The $HA_{306-318}$

variant peptides used fell into two categories based on substitutions in single (Y308A) or multiple (anchorless HA, YAK) anchor positions. The anchor residues of $HA_{306–318}$ were identified using the DR1/$HA_{306–318}$ crystal structure (26). Radical substitutions were introduced to study the extreme effects of side-chain interactions on SDS stability and binding. Anchorless HA peptide was designed with the aim of constructing a ligand with side chains that would have no positive interactions with the principal pockets that accommodate the peptide side-chain anchors. Along with alanine substitutions at P1, P4, P7, and P9, threonine at P6 was changed to a glycine instead of an alanine, because alanine substitution at that position increases the binding affinity by 3- to 10-fold (35, 36). We also used N312Q, with a substitution at a nonanchor position, as a positive control. As a negative control, we used ETEC, a peptide corresponding to 111–123 of CS6 α subunit of pili protein of enterotoxigenic *Escherichia coli*, which did not bind to DR1.

Biosensor assay to determine the SDS resistance of complexes

We have developed a new biosensor-based assay that can be used under controlled conditions to study the effects of different detergents, such as SDS, on MHC class II/peptide complexes without the use of an extrinsic label. This assay relies on the availability of the anti-class II Ab that will recognize only the intact α/β heterodimer and not the individual subunits. The assay consists of allowing the MHC/peptide complexes to react with the desired denaturant (SDS) for a predetermined amount of time (see *Materials and Methods*). At this stage, the reaction will contain different amounts of α/β heterodimers, heterodimeric aggregates, and the dissociated subunits. The reaction mixture is then injected onto the Ab-coupled biosensor surface for capture of the SDS-resistant heterodimers. Once maximal stable binding is reached, a PBS wash removes the dissociated subunits, leaving behind only the intact heterodimer bound to the Ab. The amount of complex remaining bound to the Ab, which is determined from the difference of the optical signal before binding and after the buffer wash, is a measure of SDS stability. The time necessary to reach maximal binding of the reaction mixtures to the Ab surface was empirically determined by correlating the binding time to the amount of the bound α/β heterodimers remaining after the buffer wash.

Effect of peptide anchor residues on the SDS stability of DR1 complexes

Representative examples of the biosensor assay using DR1 alone, DR1 with nonbinding ETEC, and DR1 with $HA_{306–318}$ are shown in Fig. 1*A*. The SDS stability of DR1 complexes with different peptides as determined by this assay is shown in Fig. 1*B*. The background level of binding in these experiments was determined with DR1 in the absence of any peptide and in the presence of ETEC. Wild-type DR1 complexes with $HA_{306–318}$, YAK, and CLIP showed binding significantly above background. The positive control N312Q/DR1 complex showed binding similar to that of $HA_{306–318}$/DR1, whereas the binding of Y308A/DR1 and that of anchorless HA/DR1 were similar to that observed at the background level. The samples of DR1 without any added peptides showed a detectable amount of binding to the L243 Ab. This result is probably due to the presence of strong multimeric aggregates that do not dissociate into individual subunits under the conditions used here. At present, the nature of these aggregates is not clear. The ability of L243 Ab to recognize these aggregates, however, has been reported previously (4). Silver-stained SDS-PAGE gels confirm the presence of such molecular aggregates.

SDS-PAGE results with different DR1 complexes, shown in Fig. 1*C*, are consistent with those obtained from the biosensor assay. Among the $HA_{306–318}$ variant peptides, $HA_{306–318}$ and YAK formed SDS-resistant complexes, while complexes of DR1 with Y308A and anchorless HA peptides were SDS sensitive. CLIP/DR1 also formed SDS-stable complexes with DR1, which, despite higher m.w., migrated more rapidly than the rest of the complexes. However, the complex with short CLIP peptide ($Ii_{90–104}$) migrated similarly to typical antigenic peptide/DR1 complexes S.K.N. and S. S-N., unpublished observation). The different electrophoretic mobility of the CLIP/DR1 complex has been attributed to a difference in protein conformation induced by this peptide (37). Yet, in the SDS-free nondenaturing gel, these complexes migrated significantly more slowly than did the complexes of DR1 with $HA_{306–318}$ variants (data not shown). Opposite trends in the nondenaturing and SDS gels indicate that the different migration patterns do not simply reflect differences in the hydrodynamic radii (compactness) of the CLIP peptide complexes. We attribute this unusual migration of CLIP complexes to electrophoretic artifacts, such as SDS binding to the N-terminal region of the peptide or differences between the overall charge on the peptides.

Effect of P1 residue mutation on the SDS stability of complexes with $HA_{306–318}$ peptide variants

Biosensor and SDS-PAGE results with the wild-type DR1 suggested that both the strong hydrophobic interactions between the P1 anchor and P1 and the resulting burial of these residues are necessary and sufficient to result in SDS stability. To account for the dependence of SDS sensitivity on the exposed hydrophobic regions in the complex, we hypothesized that the SDS-induced dissociation is initiated by SDS interacting with P1. To investigate this hypothesis, we mutated βGly^{86} to Tyr, which would potentially reduce the depth of P1 and the solvent accessibility of hydrophobic residues. Furthermore, this mutation can be expected to increase hydrophobic contacts between α and β subunits. Crystal structures of DR1/$HA_{306–318}$ (26), I-E^k/Hsp, and I-E^k/Hb (38) peptide complexes were used as a basis for designing mutant DR1. We chose to mutate βGly^{86} to Tyr for the following reasons. βGly^{86} is the most prevalent nonconserved residue in P1 among all DR alleles. I-E^k, with P1 residues that are the same as DR1 except for βPhe^{86} and βLeu^{90}, suggested that introducing an aromatic residue at β86 position of DR1 would not cause global structural changes. The DR1/$HA_{306–318}$ crystal structure (26) suggested that a tyrosine residue can fill P1. A molecular model of empty βG86Y DR1, energy-minimized to relieve bad contacts, also showed that the tyrosine could be accommodated at β86 without grossly perturbing the other residues in the pocket (model not shown). The hydroxyl group of tyrosine, which is likely to be pointing away from P1 assuming the aromatic ring is accommodated just like βPhe^{86} in I-E^k, could potentially hydrogen-bond with the solvent, making the pocket more hydrophilic.

βG86Y DR1 was expressed in insect cells and was purified using the same procedures as those used for wild-type DR1. The gel filtration profiles of the wild-type and mutant DR1, under nondenaturing conditions, were qualitatively the same, with the predominant species at an apparent molecular mass of 55 kDa and the aggregates, present in significantly smaller amounts, at higher molecular masses of 150 and >700 kDa (Fig. 2*A*). The predominant species was the α/β heterodimer but behaved like a protein of a higher molecular mass, reflecting the lack of compactness in the structure in the absence of a peptide. The amount of the highest molecular mass aggregate was lower in the mutant than in the wild type, suggesting that the reduced hydrophobicity of the mutant decreases the aggregate formation. As expected, this decreased aggregate formation in the mutant was accompanied by the increased amounts of the other peaks.

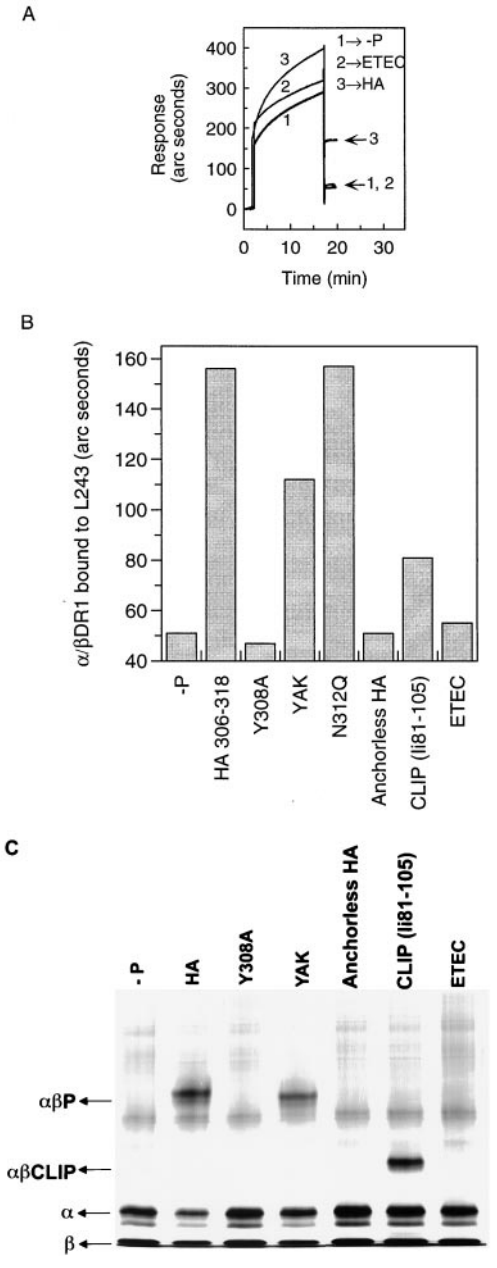

FIGURE 1. Biosensor assay and gentle SDS-PAGE to determine the SDS stability of class II complexes. *A*, A resonant mirror biosensor was used to study SDS resistance and is illustrated here using either peptide-free DR1 or DR1 in complex with peptides. Briefly, L243 Ab was immobilized in a carboxymethyl dextran cuvette to an extent of about 3000 arc seconds using standard amine coupling methods. After incubation of sDR1 (1 μM) without any added peptide and with 100 μM $HA_{306-318}$ and ETEC for 24 h at 37°C, the reaction mixture was incubated with SDS (0.5% final concentration) for 15 min at room temperature. This sample was injected onto the Ab surface and washed with PBS after 15 min of binding at 25°C. The amounts of HLA-DR1 that remained bound after the surface was washed with buffer were 51, 156, and 55 arc seconds for DR1 samples without peptide and with $HA_{306-318}$ and ETEC, respectively. *B*, SDS stability of HLA-DR1 complexes with different peptides determined by biosensor assay. The data presented are an average of two simultaneous independent experiments performed in side-by-side cuvettes. *C*, SDS resistance of HLA-DR1 complexes determined using gentle SDS-PAGE. HLA-DR1 complexes with different peptides were incubated with Laemmli buffer with a final SDS concentration of 0.2%, for 15 min at room temperature and subjected to electrophoresis in a 12.5% polyacrylamide gel. The gel was silver stained by standard protocols. This gel is representative of at least 10 independent experiments performed with different protein preparations.

Qualitatively, all the $HA_{306-318}$-derived peptides and CLIP bound to βG86Y DR1, as evident from the altered migration on a nondenaturing protein gel (data not shown). However, only the complexes of Y308A and anchorless HA peptides with mutant DR1 were SDS stable as determined by the biosensor assay (Fig. 2*B*). This behavior of the mutant DR1 is in direct contrast to that of wild-type DR1 that forms SDS-stable complexes with $HA_{306-318}$, YAK, and CLIP.

Additional information was obtained from SDS-PAGE experiments with the mutant protein (Fig. 2*C*). In the absence of any additional peptide, the mutant DR1 migrated as a band of approximately the molecular mass expected for an intact α/β heterodimer and dissociated subunits. Only Y308A and anchorless HA peptides gave rise to SDS-stable α/β complex bands that migrated more slowly than did the heterodimer-like band observed when no additional peptide was added. We further investigated the identity of the protein in the band that migrated a little more rapidly than did the peptide complex bands. In the presence of excess peptide (Y308A) there was a significant decrease in the intensity of that band in SDS with an accompanying increase in the Y308A complex band (Fig. 2*D*), suggesting that the protein in this band consists of α/β heterodimers. To further investigate whether this band represents peptide-free protein or whether it contains α/β heterodimers in complex with endogenous peptides that may have copurified, we incubated the mutant protein with a 100-fold molar excess of Y308A peptide and isolated the α/β complexes by gel filtration. The protein that had an apparent molecular mass of 55 kDa, upon Y308A binding exhibited a peak characteristic of a 51-kDa species (Fig. 2*E*). This shift of peaks in the gel filtration profile, although small, was very consistent and reproducible, suggesting that the protein achieves a more compact structure on peptide binding. The formation of mutant/DR1 complex, as expected, was also accompanied by a decrease in the higher order aggregates. Half of these isolated complexes were dissociated at 37°C in the absence of any additional peptide, while the other half were maintained in an ice-water bath for 1 day. Then both samples were passed through Sephadex G-50 spin columns to remove any dissociated peptide, and the samples were further incubated at 37°C for 1 day. The SDS-PAGE showed that the dissociated sample contained a significantly increased amount of this band along with a concomitant decrease in the Y308A complex compared with the undissociated sample (Fig. 2*F*). Densitometric analysis of this gel showed that the 86% of the protein of the dissociated sample was contained in this band, while 83% of the protein was present as the Y308A complex in the undissociated sample. This result clearly suggests that this band is indeed peptide-free mutant α/β mutant DR1, since contaminating endogenous peptide complexes, if any are present, must have decreased on incubation with excess Y308A, and furthermore, there was no possibility for these contaminating complexes to increase during the experiment. Also, upon boiling in SDS buffer the mutant protein migrated solely as α and β bands (Fig. 2*B*), indicating that there were no other foreign proteins present in these preparations. We speculate that the peptide-free α/β heterodimer seen in SDS gels is an intermediate in the dissociation of mutant DR1 to individual subunits. This may be similar to the peptide-free full-length heterodimer (Floppy) shown to exist in SDS gels in the case of murine class II molecules (2, 3). However, the mechanisms involved in the formation of peptide-free mutant soluble DR1 and full-length Floppy MHC in SDS may be different. For mutant DR1, enhanced intersubunit contacts along with the more hydrophilic and shallow P1, which may result in reduced binding of SDS, leads to an increased resistance to subunit dissociation. In contrast, in the full-length murine class II, the hydrophobic transmembrane domain contacts may resist complete

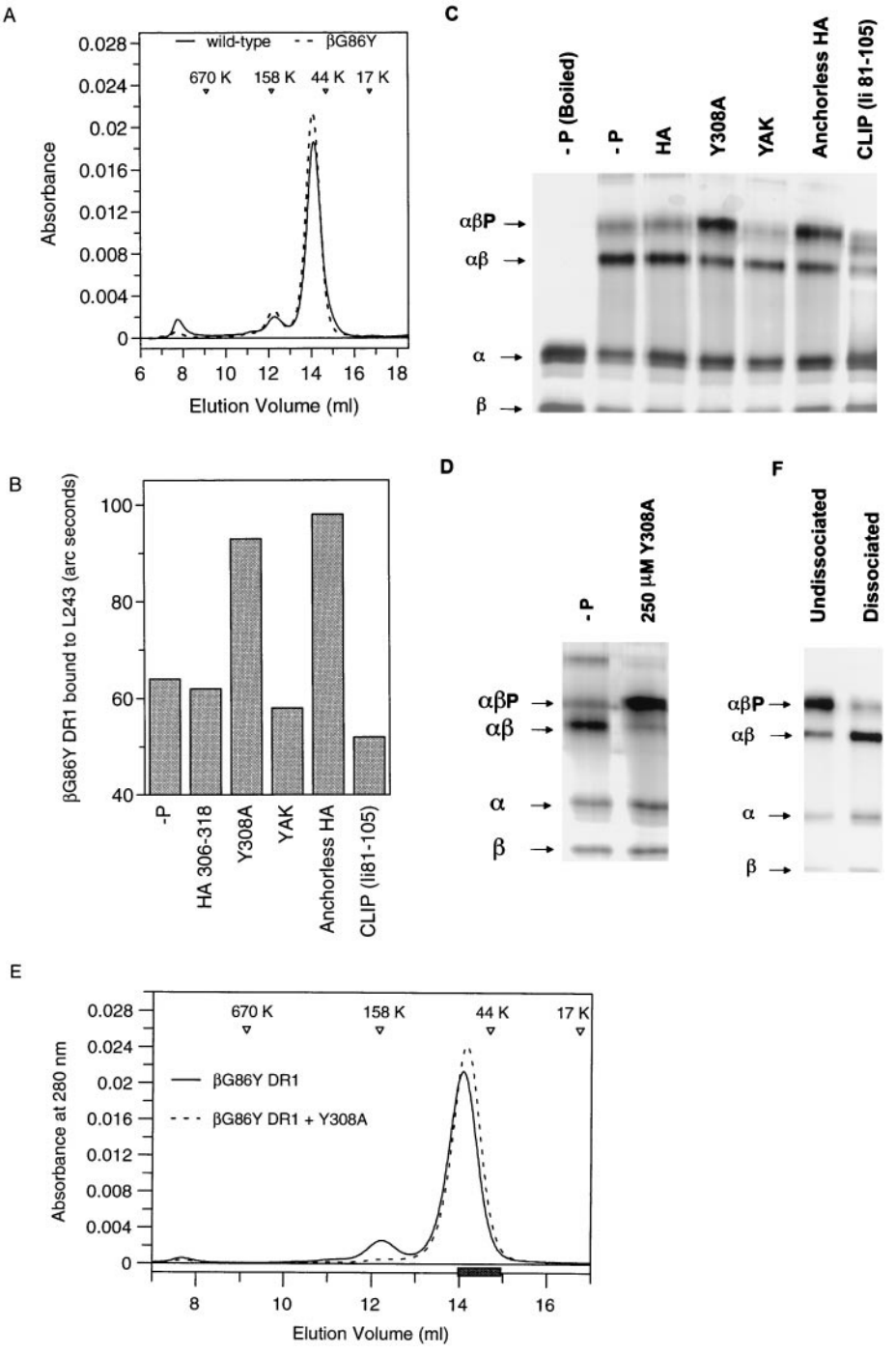

FIGURE 2. Gel filtration, biosensor, and SDS-PAGE assays of βG86Y sDR1 complexes. *A*, Fifty micrograms of wild-type and βG86Y DR1, without any additional peptides, were run on a Superdex 200 column. The elution volumes of the protein standards are shown at the *top*. *B*, SDS stability of mutant HLA-DR1 complexes with different peptides quantified by the biosensor assay as described in Fig. 1. The data presented are an average of two simultaneous independent experiments performed in side-by-side cuvettes. *C*, SDS resistance of mutant complexes analyzed by SDS-PAGE. One sample without peptide was boiled for 3 min before electrophoresis, while the rest of the samples were processed as described in Fig. 1. *D*, SDS-PAGE of samples of βG86Y DR1 reactions without peptide and with 250 μM Y308A. The data in *C* and *D* are a representative of at least 10 independent experiments performed with different protein preparations. *E*, Isolation of βG86Y DR1/Y308A complexes. βG86Y DR1 was incubated with a 100-fold molar excess of Y308A for 30 h at 37°C and was run on a Superdex 200 gel filtration column. The α/β complexes were collected in fractions shown by the gray band on the *x*-axis. *F*, SDS-PAGE of dissociated and undissociated samples of βG86Y DR1/Y308A. Half of these isolated complexes (*E*) were dissociated at 37°C without any additional peptide for 1 day, while the other half were maintained in an ice-water bath. Both samples were run through Sephadex G-50 spin columns to remove any dissociated peptides and were further incubated as before for 1 more day. SDS-PAGE of these samples were run as described in Fig. 1. The gel presented is a representative example of five independent experiments.

dissociation in SDS even though the extracellular domains may have disassembled. This is consistent with the observation that Floppy migrated more slowly than the corresponding compact peptide complex in the SDS gel, whereas the soluble peptide-free mutant DR1 migrated more rapidly than the peptide-bound species.

Dissociation kinetics of wild-type and βG86Y DR1 complexes

In an attempt to relate the SDS stability to longevity of the complexes, we determined the dissociation kinetics of different wild-type and mutant complexes (Figs. 3, *A* and *B*). Dissociation half-lives of $HA_{306-318}$ (5) and YAK complexes with the wild type are 6 and 4.5 days, respectively, at 37°C, pH 7.4. Complexes of Y308A and anchorless HA with DR1 are relatively fast dissociating, with half-lives of 36 and 140 min, respectively. Y308A and anchorless HA complexes with the mutant DR1 also dissociate with half-lives of 43 and 108 min, respectively. Reliable estimates of the half-lives of mutant DR1 complexes with $HA_{306-318}$, YAK, and CLIP could not be obtained because of their fast dissociation rates. The poor binding of these peptides may be due to the significant negative steric interactions that can be expected to exist between the P1 anchor (tyrosine in $HA_{306-318}$ and YAK and methionine in CLIP) and tyrosine at β86 in P1 of the mutant. Thus, among wild-type DR1/$HA_{306-318}$ variants, long-lived complexes are SDS resistant, whereas short-lived complexes are SDS sensitive. Notwithstanding, mutant DR1 in complex with 308A and anchorless HA, although SDS stable, are short-lived.

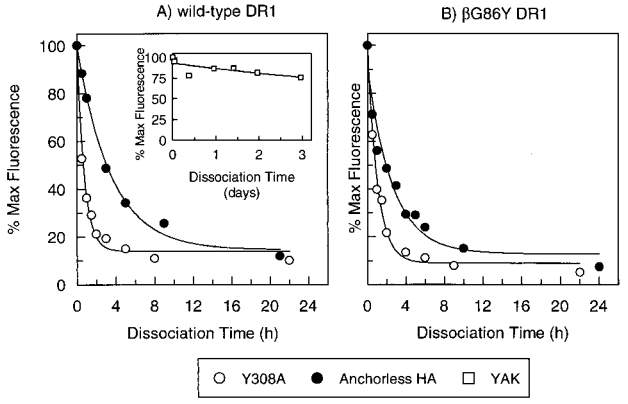

FIGURE 3. Dissociation kinetics of wild-type (*A*) and βG86Y sDR1 (*B*) complexes. HLA-DR1 complexes with AMCA-labeled Y308A, anchorless HA, and YAK (inset) were produced and separated as described in the text. The labeled complexes were dissociated in the presence of an ~100-fold molar excess of relevant unlabeled peptides at 37°C in PBS (pH 7.4) for various times. Then the free peptides were separated by centrifugation on Sephadex G-50 columns. The amount of labeled complex present after dissociation was determined by exciting AMCA at 350 nm and measuring the fluorescence emission at 445 nm in a spectrofluorometer. The fluorescence of the labeled complex before dissociation (f_0) is arbitrarily assigned a value of 100%. The fluorescence of labeled complex after dissociation for various times is expressed as a percentage of f_0. The dissociation data are fitted to a single exponential curve.

Discussion

Our experiments using $HA_{306-318}$-derived peptides and CLIP together with results using MP_{19-31} (24), $A2_{103-117}$ (29), and naturally eluted peptides (31, 32) establish that all peptides that form SDS-stable complexes with DR1 have either an aromatic or an aliphatic residue as their P1 anchor. Such a clear correlation between SDS stability and the nature of the first anchor provides insight into SDS stability at the molecular level. The aromatic and the long aliphatic residues are the most hydrophobic of all the naturally occurring amino acids (39). The combination of hydrophobic P1 and the nature of the anchors, which results in SDS-resistant complexes, strongly suggests that the SDS resistance is predominantly due to extensive interactions between the hydrophobic residues of MHC and peptide in P1 and the resulting sequestration of these residues from the solvent.

Results with βG86Y DR1 further reaffirm the basis of SDS stability. Unlike wild-type DR1, βG86Y DR1 in the absence of peptide exists as an αβ heterodimer in SDS, suggesting that minimization of exposure of hydrophobic residues and improved contacts between the subunits can enhance SDS stability. Furthermore, in direct contrast to the wild type, the SDS stability of the mutant DR1 is enhanced by peptides, Y308A and anchorless HA, that have alanine as their first anchor. Such behavior can be explained by a shallow P1 of the mutant protein that may be filled by the short alkyl side chain of alanine. With wild-type DR1, however, failure to fill the deep P1 would clearly expose the hydrophobic regions, making DR1 heterodimer susceptible to SDS-induced dissociation.

$HA_{306-318}$ and YAK peptides, which have tyrosine at P1 as the only common anchor, result in SDS-stable complexes with wild-type DR1, whereas Y308A and anchorless HA peptides, which have alanine at P1 as the only common anchor, result in SDS-stable complexes with βG86Y DR1. The lack of dependence of SDS stability on anchors other than the first one strongly suggests that P1 along with the main chain interactions are sufficient to result in SDS resistance of DR1 complexes.

Model for SDS stability of DR1 complexes

We propose a model in which the interactions of monomeric SDS with P1 influence the SDS resistance of the complexes. If P1 is already completely occupied by a peptide side chain that can provide better hydrophobic interactions than SDS, then the peptide stays bound, resulting in SDS-stable complexes, but if hydrophobic interactions between SDS and the pocket are more energetically favorable than those between the peptide side chain and the pocket, then SDS preferably binds to the protein, displacing the peptide and triggering subunit dissociation. The consequence of this hypothesis is that the resistance to SDS is a measure of the interaction between the peptide side chain and P1. The binding of SDS and the following dissociation is probably a highly cooperative process, as it is with proteins in general. Notably, under the conditions with which SDS-PAGE is typically performed, the concentration of monomeric SDS is far in excess of that of MHC or the peptide.

The correlation between SDS stability and longevity of the complexes can be understood by examining the molecular bases for each of these properties. It is apparent from the crystal structures of complexes of both human (26, 27, 29, 40) and murine class II alleles (38, 41) that the interactions between the peptide and the MHC pockets are either entirely hydrophobic or consist of a combination of electrostatic and hydrophobic components. For example, the interaction between the Tyr^{308} of HA and P1 of DR1 is predominantly hydrophobic, while some of the better characterized electrostatic charge interactions between the Lys^{76} of mouse heat shock protein and P9 of I-E^k (38) and between the Asp^{51} of HEL peptide and P1 of I-A^k (41) are also aided greatly by the significant hydrophobic contacts made by the aliphatic portion of these peptide side chains. The extent of hydrophobicity in the binding sites of class II molecules, in general, is also reflected by self aggregation in the absence of peptides. Therefore, the hydrophobic interaction between the peptide and the MHC is an important factor in determining the longevity of the complexes. SDS serves as a convenient tool to probe these hydrophobic interactions; hence, it is reasonable to expect the SDS stability to correlate with the longevity of the class II/peptide complexes. Consistent with this viewpoint, with wild-type DR1, the peptides with bulky hydrophobic P1 anchors (HA and YAK) form long-lived complexes as well as confer resistance against SDS-induced dissociation. Radical substitutions of the P1 anchor of $HA_{306-318}$ with alanines result in the loss of complex longevity and SDS stability, as observed from results with Y308A and anchorless HA peptides. With βG86Y DR1, the mutation significantly increases the contacts between α and β subunits as well as presumably decreases the solvent accessibility of the hydrophobic residues, thereby artificially decreasing the typical hydrophobicity of a class II binding site. The mutation also significantly reduces the extent of interactions possible between peptide and MHC, because pocket 1 is already partially filled. Therefore, even though the peptides Y308A and anchorless HA do not make any more positive interactions with the mutant than they do with the wild type, as evident from similar, short lifetimes of these peptide complexes with either protein, the mutant complexes are SDS stable. Mutant DR1 may not represent any naturally occurring MHC protein, since the mutation severely curtails the binding of the peptide with P1, which is seemingly the only critical interaction for stabilizing the DR1 complex, resulting in an MHC molecule incapable of forming a stable complex with any peptide. Therefore, the dissociation data with the mutant can be used to further cement the basis of SDS stability being a measure of buried hydrophobicity and not to be misconstrued as an

exception to the correlation between the dissociation rates and SDS stabilities observed with the natural alleles.

One consequence of the proposed model is that the SDS stability is a measure of the affinity between the peptide and the naturally occurring MHC molecule. Although our experiments have dealt only with DR1, since prominent hydrophobic interactions between the peptide and the MHC seem to be a common feature of all alleles, we speculate that this model may well be applicable to class II molecules in general. Even small differences in this particular interaction are reflected in SDS-PAGE and biosensor assays, although they are not always detected by typical competition binding assays. A few reports, in which competition assays were used to determine peptide affinities, suggest that there is no strict correlation between SDS stability and complex affinity (22, 24, 25). This discrepancy may be due to the complexity of the peptide binding and the failure of the typical competition assays always to reflect the intrinsic affinity of the peptide. The affinity measurements are complicated by several technical and conceptual difficulties related to the measurement of association rates with the heterogeneous pool of MHC complexes isolated from the surface of APCs (42, 43) as well as the soluble molecules made in insect cells without their natural ligand (49). However, the dissociation rates of the class II peptide complexes are a more accurate reflection of the intrinsic affinity of the peptide. Consistent with this viewpoint, the sensitivity of class II complexes to dissociation by HLA-DM, much like SDS stability, also has a stricter correlation with the dissociation rates of the complexes, but exhibits no clear trend with the affinities measured by competition assays (44).

Correlation of SDS sensitivity with exposed hydrophobic residues

The mechanistic basis for the SDS stability of the class II peptide complex can be better understood if SDS is considered as an active participant in this interaction. SDS primarily binds to hydrophobic regions of proteins (45). SDS sensitivity has been suggested to indicate incomplete folding of MHC proteins during synthesis and intracellular transport (6). Incompletely folded proteins, as intermediates in the folding pathway, are often characterized by the presence of exposed nonpolar patches (46). It is possible that SDS binding to these exposed hydrophobic residues leads to subunit dissociation, thus providing a convenient assay for probing the maturity of the MHC molecules.

The result with wild-type DR1 complex with CLIP peptide is seemingly anomalous. CLIP, which has a methionine anchor, forms a SDS-stable complex that dissociates rapidly (47). The SDS stability of CLIP complexes is likely to result only from P1 interactions, whereas the affinity of the entire peptide for MHC can be affected by the interactions with other peptide residues. The N-terminal segment (Ii_{81-89}) has been shown to facilitate rapid release of CLIP (47). The substitution of proline with an alanine at the P6 anchor of CLIP increases binding affinity for DR1 by at least 10-fold (48), indicating that the peptide makes destabilizing interaction at least with one of the pockets. Therefore, the SDS stability of CLIP/DR1 complexes is probably a measure of strong affinity between the methionine side chain and P1. However, significant destabilizing interactions in the other pockets and regions outside the Ag binding site decrease the overall affinity. Interestingly, CLIP complexes of all the other murine or human alleles reported are shown to be SDS sensitive. This anomalous situation with DR1 can be further explained in terms of Ii function in vivo and the molecular basis of SDS stability discussed here. One of the functions of Ii is to prevent empty class II binding sites from self aggregating (14), although with the additional requirement that the CLIP region of Ii should dissociate rapidly. P1 of DR1 contains the most extensive array of exposed hydrophobic residues among any binding pocket of known alleles and hence would be the most vulnerable site for self aggregation. The methionine side chain of CLIP with its strong interactions with the P1 residues buries the otherwise exposed hydrophobic regions, thereby protecting against self aggregation and also consequently resulting in SDS resistance. Unfavorable contacts from other peptide residues, however, help satisfy the requirement of short-lived DR1/CLIP complexes.

Juxtaposition of hydrophobic patches and positively charged residues such as arginine and lysine on the proteins is known to increase SDS binding significantly (45). The combination of both ionic and hydrophobic interactions may have to be invoked to explain the behavior of different class II alleles toward SDS. For example, P1 Asp is an extremely critical anchor in the binding of different peptides to I-A^k (25). Hen egg lysozyme (HEL) peptide 50-62 with an alanine substitution at this position leads to a drastic loss in binding affinity as well as SDS stability, much like the behavior of Y308A peptide with DR1. Consistent with the solution studies, the crystal structure of I-A^k with HEL peptide 50-62 shows that the Asp P1 side chain fits exactly into P1, with the carboxyl group forming a salt bridge with guanidium of Arg α52 while the aliphatic portion of the side chain makes hydrophobic contacts with Phe α24 and Phe α54 (41). Even though there are fewer hydrophobic residues lining P1 in I-A^k than there are in DR1, the presence of positively charged Arg α52 in close proximity makes it a potentially good SDS binding site. Peptides with first anchors that do not fit P1 as well as Asp does can presumably be easily displaced by SDS, resulting in significantly reduced SDS stability.

The biosensor assay introduced here demonstrates that analysis of the behavior of class II complexes in SDS is not restricted to SDS-PAGE gels and can be complemented by more controlled techniques in solution. The study of interactions of class II complexes with various amphiphiles differing in their hydrophobicity and ionic character will further clarify the issues involved.

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