Two Structural States of Complexes of Peptide and Class II Major Histocompatibility Complex Revealed by Photoaffinity-labeled Peptides*

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The complex of the murine class II histocompatibility molecules I-Ak with high affinity binding peptides were resistant to denaturation when examined by SDS-polyacrylamide gel electrophoresis at various pH levels. In contrast, complexes made with low affinity binding peptides were highly sensitive to denaturation by SDS. This effect was more pronounced at low pH. Placing a photo-activatable probe at the amino terminus of the peptides resulted in their covalent linkage to soluble I-Ak molecules. We found an inverse relationship between the capacity of peptides to form SDS-stable complexes with I-Ak and their extent of covalent association with either the α or β chain. The relationship held true for three different peptides in which the main anchor residues were changed so as to affect their binding affinity for I-Ak molecules. Thus, high affinity peptides generate a complex in which the motion of their amino termini was restricted, whereas complexes of low affinity peptides are more flexible. In agreement with this observation, complexes of I-Ak with high affinity peptides were highly resistant to proteolysis, in contrast to those formed with weakly binding peptides, which were more likely to be cleaved. Complexes with low affinity peptides generate a structure with enhanced flexibility as compared with complexes with high affinity peptides.

We have been examining the interaction of peptides with class II major histocompatibility complex (MHC) molecules, attempting to relate binding interactions to biological responses. Here, we examine the degree to which a peptide has conformational flexibility when bound to a class II MHC molecule. This aspect can be important to its fate during intracellular traffic and during its interaction with a T cell. To examine this point, we analyzed peptide-MHC complexes using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), evaluating the effects of pH on the resistance or susceptibility to denaturation. Importantly, we used photoactivatable peptides, to examine their restriction or freedom to covalently link to MHC molecules. Finally, we also examined the protease sensitivity of various peptide-MHC complexes.

We, as well as other laboratories, examined the behavior of the ternary complexes of peptides with the α plus β chains of class II MHC molecules under various physicochemical conditions, such as pH, temperature, and denaturing agents such as SDS. Early studies indicated that class II molecules were resistant to denaturation under SDS-PAGE conditions, if they contained bound peptides of high binding affinity (1-4). Studying the I-Ak class II molecules, we found that peptides resulted in either stable or unstable complexes with I-Ak, i.e. SDS-resistant or SDS-sensitive complexes (4, 5). The complexes of peptide-class II molecules resist or are sensitive to dissociation if subjected to the SDS-PAGE at room temperature, i.e. stable and unstable complexes, respectively; at 100 °C the components of the complex always dissociate.) The conditions that determined stable or unstable SDS complexes were related to the presence of high affinity binding peptides in which, first, a critical P1 anchor residue was represented by an appropriate amino acid side chain (with I-Ak, that of aspartic acid or glutamine) and, second, the polypeptide chain was of appropriate length (~16 residues). Actually, polyalanine peptides containing only one aspartic acid residue bound to I-Ak proteins and formed stable SDS complexes, but the length of the chain was highly critical (6). Thus, binding affinity depended on two critical interactions, that between the peptide backbone and conserved residues on the α and β chains of I-Ak and that between the amino acid side chain with the allele-specific site in P1 (7). (Similar results have been found for the human HLA-DR molecules (8, 9).) The correlation, however, between binding affinity and SDS stability was not absolute (5, 10). In our experience, we found peptides that bound with good affinity but were highly SDS-unstable as a result of unfavorable residues interacting with the other pockets of the I-Ak peptide-binding site (for a complete review, see Nelson et al. (5)).

The biological significance of the two sets of SDS complexes is uncertain. We correlated the time of persistence of the complexes in the antigen-presenting cell (APC) with the SDS state (11, 12). For example, using metabolically labeled I-Ak, the time of persistence of a peptide-MHC complex was the result of two components: a long t1/2 was represented in the SDS-stable fraction, whereas a short t1/2 (about ½ less) was represented in the SDS-unstable component. The average t1/2 in APCs (~20 h) was accounted by the ratio of SDS-stable and SDS-unstable complexes. Curiously, the koff of peptides bound to I-Ak in solution did not correlate strictly with the time of persistence of the complexes in APCs.

We now describe further analysis of a dominant antigenic peptide, the peptide at residues 48-61 of hen egg white lysozyme, when bound to I-Ak molecules. This peptide (DGST-DYGLQLQINSR) binds strongly to I-Ak as a result of the interaction of Asp-52 (underlined above) with the P1 binding site of

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‡ The abbreviations used are: MHC, major histocompatibility complex; APC, antigen-presenting cell; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; IASA, iodoazidosalicylic acid; MES, 4-morpholineethanesulfonic acid.
I-A\(^{\text{a}}\) (6, 7). We examined peptides in which we changed this P1 anchor residue from aspartic acid to other amino acids that reduced their binding strength and SDS-PAGE behavior. These peptides were bound to I-A\(^{\text{a}}\), and the complexes were tested first, for the effects of the pH on their SDS resistance or susceptibility and, second, for their interaction using photoactivatable 48–61 peptides. These analysis suggest that the complexes of peptide-I-A\(^{\text{a}}\) have two structural states of interaction, as was revealed by the extent of freedom of the amino terminus of the peptide bound to I-A\(^{\text{a}}\). In the peptide is bound in a more fixed structure resistant to SDS denaturation, in which the amino terminus of the peptide binds poorly to the I-A\(^{\text{a}}\) class II molecule. In the second, the peptide is bound in a structure sensitive to SDS denaturation in which the peptide has freedom of its amino terminus and efficiently binds the I-A\(^{\text{a}}\) molecule. The I-A\(^{\text{a}}\) peptide complexes resistant to SDS denaturation are likewise highly resistant to proteolytic enzymes, in contrast to those sensitive to SDS denaturation.

**MATERIALS AND METHODS**

I-A\(^{\text{a}}\) Purification—The class II molecule I-A\(^{\text{a}}\) was purified from the cell line T-2 expressing I-A\(^{\text{a}}\). I-A\(^{\text{a}}\) molecules were isolated from cell lysates by affinity chromatography, using Sepharose 4B coupled with 40F (anti-I-A\(^{\text{a}}\)) antibody as described previously (13). Eluted material was purified by reverse phase high pressure liquid chromatography (HPLC) with a Bio-gel TSK 250 filtration column (Bio-Rad) equilibrated with phosphate-buffered saline (PBS) containing 20 mM Mega 8, 20 mM Mega 9, 5 mM lysophosphatidyl-choline, 300 mM 4-morpholineethanesulfonic acid (MES) buffer, pH 5.5, in a final volume of 25 μl (14). Complexes were recovered as described above, buffered at pH 5.5 or 7.5 with 40 mM MES, incubated with SDS at a final concentration of 0, 0.1, 0.5, 1, or 2% for 30 min or 6 h, neutralized, and analyzed by SDS-PAGE as described above.

**Peptides**—Peptides were synthesized by Fmoc (N-(9-fluorenyl)me-thoxy carbonyl) technology on an Applied Biosystem peptide synthesizer, purified by reverse phase HPLC using a C18 Vidac column and analyzed by mass spectrometry (6). Peptides were labeled with Na\(^{125\text{I}}\) to a specific activity of 2–4 × 10\(^{4}\) cpm/μmol by the chloramine T method and purified on reverse phase HPLC using a C18 Vidac column.

**Preparation of IASA-Peptide—**N-Hydroxysuccinimidyld-4-azidosalicylic acid (NHS-ASA) (Pierce) was labeled with Na\(^{125\text{I}}\) by the chloramine T method. The radiolabeled peptide was covalently conjugated at the peptide amino terminus, as described previously (13–16) and the complex (IASA-peptide) was purified by reverse phase HPLC using a C18 Vidac column. The main radioactive peak was collected, dried, dissolved in PBS, and used for binding assays.

**Assays to Test Effect of pH—**Purified I-A\(^{\text{a}}\) (25 nmol) was incubated for 48 h at room temperature with 25 pmol of radiolabeled peptide in a solution of PBS containing 20 mM Mes, 20 mM Mes 9, 5 mM lysophosphatidylcholine, 300 mM 4-morpholineethanesulfonic acid (MES) buffer, pH 5.5, in a final volume of 25 μl (14). Complexes were recovered by gel filtration using a Bio-Spin P6 column (6-kDa cut-off) (Bio-Rad) equilibrated with PBS, pH 7.4, containing 20 mM Mes 8, 20 mM Mes 9. Then, 10 μl of the complexes (usually 1000–1500 cpm/μl) were incubated with 10 μl of a solution containing 40 mM MES at the indicated pH (from 5.5 to 7.5) for 15 min at room temperature; 20 μl of a 2% SDS aqueous solution was then added, and the mixture was incubated for 45 min at room temperature. Finally, complexes were neutralized with 40 μl of a solution containing 250 μl Tris, pH 7.0, 1.44 \(\mu\)M 2 β-mercaptoethanol, 20% glycerol, 0.002% bromphenol blue and run under SDS-PAGE conditions at either 100 °C or to room temperature. Gels were fixed for 5 min, dried, and exposed to x-ray film.

**Assays to Test the Effects of Proteolytic Enzymes—**Purified I-A\(^{\text{a}}\) (25 nmol) was incubated for 48 h at room temperature with 25 pmol of IASA-peptide in a solution of PBS containing 20 mM Mes 8, 20 mM Mes 9, 5 mM lysophosphatidylcholine, 300 mM MES, pH 5.5, in a final volume of 25 μl. The complexes (10\(^{10}\) cpm) were recovered by gel filtration on a column equilibrated with PBS, pH 7.4, containing 20 mM Mes 8, 20 mM Mes 9, ultraviolet light-irradiated for 2 min at 340 nm, and then immunoprecipitated with 40F antibody (11, 13) followed by protein A-Sepharose. The Sepharose-bound I-A\(^{\text{a}}\)-peptide complexes were eluted at room temperature for 1 h with 62.5 mM Tris, pH 6.8, 0.77 mM 2 β-mercaptoethanol, 2% SDS, 10% glycerol, 0.001% bromphenol blue. Half of the sample was boiled at 100 °C for 2 min and half of the sample was kept at ambient temperature before SDS-PAGE analysis. Bands were analyzed using a PhosphorImager system densitometer to quantify the percentage of photolabeled peptide under boiled and nonboiled conditions.

Under nonboiling conditions, a photolabeled peptide that is covalently linked to an SDS-stable complex will migrate at around 50 kDa, the position of the αβ-peptide complex. When boiled, the covalently linked peptide will remain associated with the free α or β chain or with both. However, a covalently bound peptide that forms part of an SDS-unstable complex will migrate with the free α or β chain under both nonboiling and boiling conditions. A peptide not covalently linked to the α or β chain will migrate as part of an SDS-stable complex in the position of αβ dimer when not boiled, but it will appear at the front of the gel under boiling conditions (i.e. the peptide dissociates from the complex and migrates freely). A peptide not covalently linked to an SDS-unstable complex will appear at the front under either condition.

**Assays to Test the Effects of Proteolytic Enzymes—**Three assays were set up. First, 125I-labeled 48–61 peptides were incubated with I-A\(^{\text{a}}\), as described above. After 48 h, the I-A\(^{\text{a}}\) peptide complex was separated from free peptide using a Bio-Rad P6 column and placed at 37 °C in an appropriate buffer solution with or without the following enzymes: chymotrypsin (Sigma product C7762), in 0.5 μl Tris buffer with 1% Triton X-100, at pH 7.8; Cathepsin B (Sigma product C6286) in 50 mM sodium acetate, 1% Triton X-100, 1 mM dithiothreitol, pH 5.5, and cathepsin D (Sigma product C31381) in 50 mM sodium acetate, 1% Triton X-100, 1 mM EDTA, pH 5.5. The final incubation was done in 100–150 μl of solution that contained 50 μg of the I-A\(^{\text{a}}\) molecule per ml with the enzymes at the concentrations stated in the figures. At various

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**TABLE I**

| Peptide       | Binding strength | SDS-PAGE | Photocross-linking | Sensitivity to acid pH | Sensitivity to proteases |
|---------------|------------------|----------|--------------------|------------------------|-------------------------|
| HEL (48–61): (DGTDYGLIGNSR) | Wild type: Asp-52 | <95 | 9 | Resistant | Very resistant |
|               | Score: Asp-52    | 48       | 40                | 8 | Sensitive | Partially resistant |
|               | Thr at 52        | 41       | 33                | 47 | Sensitive | ND |
|               | Leu at 52        | 46       | 5                 | 49 | ND        | Sensitive |
|               | Ala at 52        | 150      | 6                 | 76 | ND        | Sensitive |
| Hsp-70 28–41: (IANDQGNRTPPS) | Wild type: Asp-32 | 19 | 66 | 12 | ND |
|               | Ala at 32        | 32       | 9                 | 50 | ND |
| Ryudocan 84–101: (EPLVPLDNHIPENAQPG) | Wild type: Asp-90 | 21 | 91 | 6 | ND |
|               | Ala at 90        | 257      | 7                 | 65 | ND |
that expresses both I-Ak molecules and hen egg white lysozyme (5) were incubated with purified I-Ak for 48 h at room temperature. The complexes were recovered by gel filtration, and an aliquot was incubated with a solution (1:1 (v/v)) containing 40 mM MES at the indicated pH (from 5.5 to 7.5) for 15 min at room temperature, followed by addition of an equal volume of 2% SDS solution for 45 min. Samples were neutralized with 250 mM Tris, pH 7.0, and analyzed by SDS-PAGE. Left panels represent the quantitation of the radioactive bands expressed as the percentage of stable complexes (●) of the SDS-PAGE gels represented in the right panels. The percentage of stable complexes for these two peptides did not change with the pH. For the wild type 48–61 Asp-52 peptide, the percentages varied from 91% at pH 5.5 to 96% at pH 7.5 (top panels). For 48–61 Leu-52 peptide, the percentages ranged from 2% at pH 5.5 to 3% at pH 7.5 (bottom panels).

Fig. 1. Influence of the pH on the SDS-PAGE stability of peptide-I-Ak complexes. Radiolabeled 48–61 peptides (having either Asp-52 or Leu-52) were incubated with purified I-Ak for 48 h at room temperature. The complexes were recovered by gel filtration, and an aliquot was incubated with a solution (1:1 (v/v)) containing 40 mM MES at the indicated pH (from 5.5 to 7.5) for 15 min at room temperature, followed by addition of an equal volume of 2% SDS solution for 45 min. Samples were neutralized with 250 mM Tris, pH 7.0, and analyzed by SDS-PAGE. Left panels represent the quantitation of the radioactive bands expressed as the percentage of stable complexes (●) of the SDS-PAGE gels represented in the right panels. The percentage of stable complexes for these two peptides did not change with the pH. For the wild type 48–61 Asp-52 peptide, the percentages varied from 91% at pH 5.5 to 96% at pH 7.5 (top panels). For 48–61 Leu-52 peptide, the percentages ranged from 2% at pH 5.5 to 3% at pH 7.5 (bottom panels).

In the second assay, the complexes were isolated after 2 h of incubation in chymotrypsin using the monoclonal antibody 40F-bound to Sepharose 4B. (To 500 μl solution containing about 2 nmol of I-Ak was added 50 μl of a 50% (v/v) slurry of the 40F antibody bound to Sepharose 4B. The beads had been conjugated using cyanogen bromide with 10 mg/ml protein to 1 ml of Sepharose 4B slurry.) After 2 h, the Sepharose particles were washed twice and placed in 500 μl of 0.1% trifluoroacetic acid. Beads were removed, and the radioactive peptide examined by reverse phase HPLC in a Waters system with a C18 reverse phase column (Vidar 2187P54, Hesperia, CA), at a flow rate of 1 ml/min and a gradient of acetonitrile in trifluoroacetic acid (from 0 to 80% acetonitrile in 0.05% trifluoroacetic acid, for 60 min).

Third, the I-Ak molecules from APCs were labeled with [35S]methionine and then examined by SDS-PAGE. Cells from a B lymphoma line which expresses both I-Ak molecules and hen egg white lysozyme (5) were suspended at 10⁷ cells/ml in Dulbecco’s minimal essential medium lacking methionine and cysteine and containing 5% dialyzed fetal calf serum. The cells were incubated with 200 μCi of [35S]-labeled methionine and cysteine (Trans-35S-label from ICN, Irvine, CA) in 1 ml for 1 h, after which the culture was made in 1 mg/ml methionine, 0.5 mg/ml cysteine, and 10% fetal calf serum and incubated for 4 h more. Cells were harvested and lysed in PBS containing 20 mM MgA, 20 mM Mega 9, 10 mM iodoacetamide, 25 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation and immunoprecipitated with the antibody 40F-Sepharose 4B. The I-Ak molecules were eluted with a buffer solution of 0.1 M diethylamine, 0.15 M sodium chloride, and 20 mM each of Mega 8 and Mega 9 at pH 10.65. The eluate was concentrated in a Centricon 30 tube (Amicon, Inc., Beverly, MA) and incubated with chymotrypsin at 37 °C for 2 h. The material was then subjected to SDS-PAGE, as described above. The intensity of each band was quantitated in a PhosphorImager.

RESULTS

Most of the results shown in this study were made using the 48–61 peptide of hen egg white lysozyme (DGSTDYGIL-QINSR) bound to I-Ak, using variants in which the main anchor residue for the P1 pocket was changed in order to weaken the affinity of the peptide for the I-Ak class II molecule (i.e., Asp at 52). In general, peptides with a weak anchor residue yielded a complex with I-Ak that was less stable under SDS-PAGE conditions. A summary of our previous results (4, 5), as well as those obtained in this study, is shown in Table I. We first report on the SDS-PAGE stability of the various I-Ak-peptide complexes and the influence that pH has on this assay. We next consider the use of peptides containing a photoactivatable probe: we examine the extent of covalent linkage of the peptides to I-Ak using the SDS-PAGE readout. Finally, we examine the protease sensitivity of the complexes.

Effect of pH on SDS-PAGE stability of I-Ak-Hen Egg White Lysozyme Peptide Complexes—Purified I-Ak molecules were incubated with radiolabeled peptides at pH 5.5, the optimal pH for their binding (17), for 48 h; the complexes were isolated by gel filtration, incubated at the indicated pH solution for 15 min and then for 45 min with 1% SDS, and finally subjected to the SDS-PAGE. (Note that in all these assays, the complex of I-Ak with peptides was not subjected to a 100 °C temperature. Boiling the complex resulted in the dissociation of the α and β chains and the peptide. Unless otherwise stated, the results reported with SDS-PAGE represent protein complexes not subjected to 100 °C.)

Fig. 1, top panels, shows that the peptide 48–61 bound to...
I-A^k forms highly SDS-stable complexes, independent of the pH of incubation. In other experiments, we found that the stability of these complexes persisted in 2% SDS for as long as 6 h. In contrast, complexes formed with 48–61 or 46–61 having a substitution of leucine for aspartic acid at residue 52 (Table I) (4, 5) were unstable (0–31%). At pH 7.0 or 7.5, the complexes became unstable (0–31%). At mildly acidic pH (5.5–6.5), the complexes were mostly unstable (0–31%). However, after boiling, the peptide will be found associated with the α or β chain if covalently bound to it, and in the front of the gel if not bound. Thus, when the peptide bound to I-A^k was 48–61 Ala-52 (which is unstable on SDS-PAGE) (Table I), most of the complexes migrated at the position of α or β chain (when not boiled). This indicates that the unstable complexes were extensively cross-linked by the amino-terminal bound photoprobe. After boiling, the photoprobe remained attached to the α or β chain, confirming its covalent linkage. The same results were found with 48–61 Thr-52.

A photolabeled-bound peptide that forms an SDS-unstable complex runs by SDS-PAGE in the position of the α or β chain if covalently bound to it, and in the front of the gel if not bound. Thus, when the peptide bound to I-A^k was 48–61 Ala-52 (which is unstable on SDS-PAGE) (Table I), most of the complexes migrated at the position of α or β chain (when not boiled). This indicates that the unstable complexes were extensively cross-linked by the amino-terminal bound photoprobe. After boiling, the photoprobe remained attached to the α or β chain, confirming its covalent linkage. The same results were found with 48–61 Leu-52.

A photolabeled-bound peptide that forms an SDS-stable complex (i.e. with 48–61 or 46–61) will run at the position of the stable αβ dimer regardless of whether it is covalently bound by the photoprobe (under conditions in which the complex is not boiled). However, after boiling, the peptide will be found associated with the α or β chain if covalently bound, or at the front if not bound. As it is shown in Fig. 3 (NB (nonboiled)) peptide 48–61 or 46–61 formed stable complexes with I-A^k, but most of them were not covalently bound to the α or β chain, and appeared in the front of the gel (Fig. 3, lanes B (boiled)).

This different capacity between the stable and unstable complexes to cross-link was more dramatically shown with the 48–61 Ser-52 or 48–61 Thr-52 peptides. These peptides were subjected to SDS-PAGE as a mixture of stable and unstable complexes (Fig. 3). Only a percentage of the complexes were covalently associated with the β chain. These corresponded mostly with the unstable complex (i.e. the percentage remaining in the position of the α or β chain did not increase after boiling: see legend to Fig. 3). (The 48–61 Ser-52 peptide despite being pure showed some interaction with the gel as indicated by the extra band. Note that when the sample was boiled a fraction of the peptide from the stable complex again had an abnormal migration (Fig. 3.) Confirming the above results with 48–61 Asp-52 and 48–61 Ala-52, most of the peptide covalently associated with class II molecule (β-chain) was accounted for by the unstable complexes, whereas only a small fraction of stable complexes were covalently bound (Fig. 3). Table I summarizes all the experiments.

We also examined two other peptides that bind to I-A^k (4–6). A peptide from hsp70 residues 28–41 is found normally bound to I-A^k molecules extracted from APCs. This peptide also had
an aspartic acid residue anchoring it to the P1 pocket, at residue 32, and when bound to I-A\(^k\) molecules ran stable by SDS-PAGE. As before the stable complexes were poorly cross-linked (Fig. 4). Substituting an alanine at the P1 anchor site of residue 32 resulted in weak binding, and an increase in SDS unstable complexes, that was covalently linked to the \(\alpha\) and \(\beta\) chain. The same findings applied to another self-peptide, that from ryudocan residues 84–101; the wild type peptide with an aspartic acid at residue 90 was SDS-stable but showed a poor covalent linkage to I-A\(^k\). Having the peptide with an alanine substitution at residue 90 resulted in an SDS-unstable complex that was covalently associated with I-A\(^k\) molecules.

**Fig. 5.** 48–61 peptides show differential sensitivity to chymotrypsin. The six panels show a representative result taken from three to five different experiments. The \(^{125}\)I-labeled peptides were bound to I-A\(^k\), and the complex was purified and incubated with chymotrypsin at the indicated concentrations. The amount of label remaining associated with I-A\(^k\) was determined at the various times. A wide range of chymotrypsin were used in these sets of experiments. Indicated with 48–61 Asp-52 and 48–61 Ser-52 are the highest amounts used. Indicated with 48–61 Ala-52 are the results with 0.2 and 0.5 mg/ml. Other experiments examined the sensitivity of the 48–61 Ala-52 complexes with a higher concentration of chymotrypsin. For example, in one experiment, treating the 48–61 Ala-52 complexes for 3 h with 1 and 2.5 mg/ml resulted in 24 and 16% of peptide associated with I-A\(^k\) at 3 h, respectively. The untreated complex showed 48% of the peptide bound to I-A\(^k\). Indicated in the bottom panels are the results with the two peptides resistant to chymotrypsin cleavage (discussed in the text).
Sensitivity to Proteolysis of the Peptide-MHC Complexes—Considering the possible biological consequences of the two states of peptide-MHC complexes described above, we examined their degree of sensitivity to proteolytic enzymes. We reasoned that the SDS-stable complexes, which were assumed to have a more fixed or rigid structure, could be more resistant to proteolysis. In contrast, those with the SDS-unstable conformation, i.e. with a more flexible conformation, would be sensitive. If so, these states could reflect in the fate of the complexes in the proteolytic-rich vesicles of the APC.

Radiolabeled peptides were bound to I-\(\text{A}^\text{k}\), after which the complexes were isolated and then incubated at 37 °C with chymotrypsin or the lysosomal enzyme cathepsin B or D. The time of dissociation of the peptide from I-\(\text{A}^\text{k}\) varied, in part dependent on the binding strength of the peptide, an issue studied before (5). The complex of I-\(\text{A}^\text{k}\)-48–61 was highly resistant to each of the enzymes. A representative experiment is shown in Fig. 5. Even the very large concentration of 10 mg/ml chymotrypsin did not change the time of dissociation of the peptide from the MHC molecule. Confirming previous results (18, 19), 48–61 bound to I-\(\text{A}^\text{k}\) was highly stable with prolonged time of dissociation. We proceeded to recover the peptide from I-\(\text{A}^\text{k}\) after 2 h and to examine it by HPLC: the I-\(\text{A}^\text{k}\)-peptide complex was recovered, isolated using antibodies bound to Sepharose beads, and washed; then, its components were dissociated, and the 125I peptide was examined by reverse phase HPLC. The peptide was not affected, eluting in the same fraction as the untreated peptide (Fig. 6). Thus, in agreement with previous reports, the 48–61 peptide, which can be cleaved by chymotrypsin in free solution, is now resistant to the enzyme during the time that it is bound to I-\(\text{A}^\text{k}\) molecules. (Likewise, cathepsin B and cathepsin D, even at 10 units/ml, did not affect the amount of peptide bound after 3 h; i.e. relative to control, the amounts of 48–61 lost were 2.5 and 1.9%, respectively.

The complex formed with 48–61 Ser-52 was slightly more sensitive to chymotrypsin and to the cathepsins (Fig. 5). Even at 5 mg/ml chymotrypsin, the amounts lost at 6 h did not exceed 25% of the bound peptide. As with 48–61, the peptide bound to I-\(\text{A}^\text{k}\) was shown not to be cleaved when recovered from I-\(\text{A}^\text{k}\) molecules (Fig. 6). (The amounts lost after 3 h in 10 units/ml cathepsin B or cathepsin D were 7.8 and 5.2%, respectively.)

The complexes formed with 48–61 Leu-52 and 48–61 Ala-52 were highly sensitive to chymotrypsin (Fig. 5). Note in Fig. 5 that the loss of 48–61 Leu-52 was affected at a 10 mg/ml concentration of enzyme. Also to note is that the peptide that remained bound to I-\(\text{A}^\text{k}\) was still protected from chymotrypsin cleavage (Fig. 6). (The complexes were likewise sensitive to cathepsin B and cathepsin D. Thus, about half of the complexes at 3 h were lost with a 10 unit/ml concentration of either cathepsin.)

All of the peptides were protected from chymotrypsin action during the time that they remained associated with I-\(\text{A}^\text{k}\), yet the enzyme dissociated the complexes, particularly with 48–61 Leu-52 and 48–61 Ala-52. Similar results were obtained with two synthetic peptides based on the 48–61 sequence but modified so as to make them resistant to chymotrypsin. Peptide AGSTDAGAAQANSKY bound to I-\(\text{A}^\text{k}\) with about the same strength as 48–61 (relative inhibitory capacity \(2^{1}\) of 2) (see Table I), because of the aspartic acid at the fifth residue from the amino terminus. The complex of it with I-\(\text{A}^\text{k}\) was highly resistant to chymotrypsin, even at a 10 mg/ml concentration of enzyme. In contrast, a peptide that was similar but had a

![Fig. 6. Peptides bound to I-\(\text{A}^\text{k}\) are resistant to chymotrypsin.](image-url)
Fig. 7. I-A<sup>k</sup> molecules represented in the SDS-unstable component are more sensitive to chymotrypsin. <sup>35</sup>S-Labeled I-A<sup>k</sup> molecules from APCs were isolated, treated with the indicated concentrations of chymotrypsin, and subjected to SDS-PAGE. Each lane shows the I-A<sup>k</sup> molecule after incubation in the sample buffer at room temperature (not boiled (NB)) or at 100 °C (boiled (B)). There was no loss of stable I-A<sup>k</sup> molecules (for example, area = 5778 without treatment, compared to 5579 at 1 mg/ml chymotrypsin). The percentage recovery after chymotrypsin, in the NB lanes, were 48, 29, 18, and 20% at 0.1, 0.5, and 1 mg/ml chymotrypsin, respectively. The percentages of stable molecules were 58% in the untreated sample and 77, 82, 87, and 85% in samples treated with the concentrations of chymotrypsin described above. Note that some splitting of the β chain occurred at the 0.5 and 1.0 mg/ml concentrations.

The differences in SDS denaturation at low pH between the two sets of complexes might reflect a higher or lesser exposure of hydrophobic residues brought about by the pH change (20–23). Our results indicate that the nature of the P1 anchor residue of the peptide profoundly influences the SDS susceptibility to low pH, probably because the free energy of unfolding depends on the binding affinity between a peptide side chain and the corresponding I-A<sup>k</sup> site. Complexes with the high affinity 48–61 peptide with a strong P1 anchor were highly resistant to pH and denaturation, in marked contrast to the complexes with substitution that have a weak P1 anchor site. These results are in agreement with previous reports in showing that the susceptibility to denaturation by SDS-PAGE increased considerably at low pH (24, 25) The transition along the pH from unstable to stable complexes was interpreted as a conformational change from a protonated class II at low pH to an unprotonated class II at neutral pH based on its increase uptake of aminonapthalene (24, 25).

The stable complexes of I-A<sup>k</sup> bearing the strong binding peptides were highly resistant to proteolysis. In contrast, those with a weaker anchor residue were highly sensitive. It was previously shown that peptides bound to class II molecules were protected from cleavage by proteolytic enzymes. We confirm this effect but add two important new features. The resistance or sensitivity of the peptide bound to I-A<sup>k</sup> was independent of its susceptibility to enzyme cleavage. Rather, the binding strength of the peptide with I-A<sup>k</sup> dictated whether the MHC molecules in the complex were sensitive to enzymatic degradation; this result agrees with the studies on pH and photocross-linking. (A previous study on class I MHC molecules reported that peptide-empty molecules were sensitive to proteolytic enzymes (26).)

We agree here that the propensity for SDS denaturation truly reflects a conformational state found with complexes in solutions or in APCs. The two conformational states may reflect in the biology of peptide presentation by APCs. We previously showed the marked differences in the time of persistence of SDS-stable or SDS-unstable forms of the I-A<sup>k</sup> molecule in APCs (11, 12). Thus, the cell sensed the two conformational states, one of which was retained longer and translated in higher immunogenicity than the other (12). Knowing now that the SDS test truly reads out a physiological state adds much credence to these results. Indeed, the survival and time of persistence of a MHC-peptide complex in the proteolysis rich environment of an APC may well depend on the state of the molecule dictated by the binding features of the peptide. Finally, another issue to consider is that the more open conformation may well favor peptide exchange. A peptide bound with low affinity, and in the loose, or SDS-unstable, state will maintain the integrity of the dimer but allows for exchange and reutilization by a new peptide (27). In contrast, the high affinity, highly SDS-stable complex is not interchangeable.

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