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Articles

Probing the Ligand-Induced Conformational Change in HLA-DR1 by Selective Chemical Modification and Mass Spectrometric Mapping[†]

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ABSTRACT: Peptide binding induces conformational changes in class II MHC proteins that have been characterized using a variety of hydrodynamic and spectroscopic approaches, but these changes have not been clearly localized within the overall class II MHC structure. In this study, empty and peptide-loaded complexes of HLA-DR1, a common class II MHC variant, were chemically modified using the side chain-specific chemical modifiers *p*-hydroxyphenylglyoxal (arginine), tetranitromethane (tyrosine), *N*-bromosuccinimide (tryptpophan), and NHS-biotin (lysine). Modified proteins were subjected to in-gel digestion with trypsin and subsequent analysis by MALDI/MS. Three arginine residues and two lysine residues were differentially reactive, modified in the empty form but not the peptide-loaded form of the protein, indicating that the chemical reactivity of these regions differs in the two conformations. Three of the differential modifications were located on a single lateral face of the protein, indicating that this region is involved in the conformational change. Additionally, a number of lysine and tyrosine modification sites were present in both protein conformations. Overall, the pattern of reactivity is inconsistent with the idea that empty MHC molecules exist as molten globules or other partially unfolded intermediates, and suggests that the peptide-induced conformational change is localized to only a few regions of the protein.

Major histocompatibility complex (MHC)[1] molecules are heterodimeric cell-surface proteins that bind peptides derived from extracellular, endosomal, and internalized cell-surface antigens, and present them at the cell surface for inspection by $CD4^+$ T cells (*1*). Three-dimensional structures have been determined for peptide complexes of several polymorphic variants of both human and murine class II MHC molecules (reviewed in ref *2*). Both the α and β chains contribute to the peptide binding domain. Each chain also contributes an immunoglobulin-like domain as well as short transmembrane and cytoplasmic domains. Peptides bind in a groove made up of a β sheet floor topped by two α helices. Within the binding groove, the peptide places a number of side chains in pockets burying approximately two-thirds of its total surface area, leaving the remainder available for interaction with antigen receptors on T cells (*3*). The residues lining these pockets vary between allelic variants, providing different peptide−sequence binding specificity.

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[1] Abbreviations: DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; MHC, major histocompatibility complex; HPG, *p*-hydroxyphenylglyoxal; NBS, *N*-bromosuccinimide; NHS, *N*-hydroxysuccinimido; MALDI/MS, matrix-assisted laser desorption ionization mass spectrometry; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNM, tetranitromethane.

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Although the structures of class II MHC−peptide complexes visualized by X-ray crystallography all are similar, a number of studies have suggested that alternate conformations of class II MHC molecules can exist under certain conditions (*4*−*7*). Kinetic studies of the peptide binding reaction indicate a multistep binding pathway, suggesting that peptide binding is accompanied by a conformational change in the MHC, although other interpretations of these kinetic analyses are possible (*8*−*13*). Conformational transitions between such isomers have been measured by NMR (*14*). Other studies have shown that at low pH, MHC molecules shift their equilibrium from a "closed" to an "open" or "peptide-receptive" state (*15*−*17*). These alternate conformations have been suggested to play an important role during the peptide binding reaction, and also in the interaction with the endosomal peptide exchange factor HLA-DM (*5*, *18*−*20*).

Little structural information is available for the empty form of the protein. Spectroscopic and hydrodynamic studies on HLA-DR1, a common human class II protein and the subject of this study, have shown that a distinct conformational change occurs upon peptide binding, suggesting that the conformation of the empty protein is different from that of the peptide-loaded form (*7*, *21*). The conformational change results in a decrease in the hydrodynamic radius from 35 to 29 Å, together with a small increase in helical content as observed by circular dichroism (*7*). The change can be induced by binding any of a large variety of peptides, including a capped dipeptide (*4*), and also by filling the P1 side chain binding pocket through mutagenesis (*6*). No high-resolution structural information is available for the empty protein.

Recently, antibodies which are specific for the empty conformation of HLA-DR1 were used to identify a number of amino acid residues which change their accessibility upon peptide binding (*22*). These include residues both nearby and distant from the peptide binding site. That study was limited by the fact that each of the antibodies was raised against the β chain; therefore, no data pertaining to the structural rearrangement of the α chain could be obtained. Thus, a more comprehensive study which can simultaneously analyze both the α and β chains of DR1 was needed.

Side chain-specific chemical modification has often been used to elucidate the role of specific amino acids in enzyme active site reactivity (reviewed in refs *23* and *24*), protein−protein interactions (*25*), and structural studies of protein conformation (*26*). Mass spectrometric peptide mapping has been shown to be an unequivocal method for the characterization of chemical modifications in proteins. The combination of limited tertiary structure-selective chemical modification with mass spectrometric mapping allows molecular characterization of protein function when other methods are unreliable or unavailable (*27*).

In this study, we have identified regions involved in the HLA-DR1 peptide-induced conformational change using a number of side chain-specific chemical modifiers. The arginine-specific modification reagent *p*-hydroxyphenylglyoxal (HPG) has been used previously in experiments to identify functional arginine residues (*25*, *28*−*32*). Tetranitromethane (TNM) has similarly been used to identify reactive tyrosine residues (*33*, *34*), and *N*-hydroxysuccinimide esters (in this case NHS-biotin) have been used to label amine nitrogens of lysine with a number of different functional groups (*35*, *36*). Here, these chemical modification reagents were used to evaluate conformational differences between empty and peptide-loaded HLA-DR1 in an attempt to examine in more detail the structural changes involved in the ligand-induced conformational change in HLA-DR1.

EXPERIMENTAL PROCEDURES

Chemical Reagents

Proteomics grade trypsin, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), NBS, TNM, and trifluoroacetic acid were obtained from Sigma (St. Louis, MO). HPG and NHS-biotin were obtained from Pierce (Rockford, IL).

Recombinant MHC Molecules

Empty and peptide-loaded DR1 were prepared by expression in *Escherichia coli* and folded in vitro, as previously described (*37*). Briefly, HLA-DR1 extracellular domains were expressed individually as insoluble inclusion bodies, isolated by denaturing ion exchange chromatography, and refolded in vitro without peptide. DR-Ha−peptide complexes were prepared by incubating immunoaffinity-purified empty HLA-DR1 (1−5 μM) with an at least 5-fold molar excess of peptide (PKYVKQNTLKLAT-NH_2) for 3 days at 37 °C in PBS. The resultant peptide−DR1 complexes or unloaded HLA-DR1 that had been stored at 4 °C was purified by gel filtration to remove aggregates and unbound peptide, and stored at 4 °C.

Chemical Modifications

Arginine. Empty or peptide-loaded DR1 samples were exchanged into 25 mM ammonium bicarbonate (pH 8.0) using a centrifugal filter device (Amicon Ultra 10 000 molecular weight cutoff), and were incubated at a final protein concentration of 0.5 μg/μL (10 μM) with HPG ranging from 0 to 10 mM in 25 mM $NaHCO_3$ for 15 h at 25 °C in the dark. Unmodified DR1 was prepared and treated similarly without incubation with HPG. After modification, each sample was exchanged into PBS to remove excess unreacted HPG.

Lysine. A 10 mM stock solution of *N*-hydroxysuccinimidobiotin (NHS-biotin) was prepared in 100% dimethyl sulfoxide (DMSO). Empty or peptide-loaded DR1 samples in PBS (0.5 μg/μL) were mixed with NHS-biotin in a 5- or 20-fold molar excess over protein amine groups (the final concentration of DMSO was <5%). The reaction was allowed to proceed for 15−60 min at room temperature. All reactions were stopped by addition of 10 mM Tris (pH 7.8). Samples of unmodified DR1 were prepared under identical conditions as a negative control.

Tryptophan. Empty or peptide-loaded DR1 samples (0.5 μg/μL) were mixed with a 5−20-fold molar excess of *N*-bromosuccinimide for 1 min in PBS (pH 7.0). The extent of the reaction was measured in a spectrophotometer. The reaction was stopped by the addition of 1 mM tryptophan, and samples were exchanged into PBS using a centrifugal filter device.

Tyrosine. A stock solution of 6 mg/mL TNM was prepared in 95% ethanol. Empty or peptide-loaded DR1 samples (0.5 μg/μL) were mixed with a 10−100-fold molar excess of TNM in PBS. The reaction was allowed to proceed for 1 h at room temperature. All reactions were stopped by addition

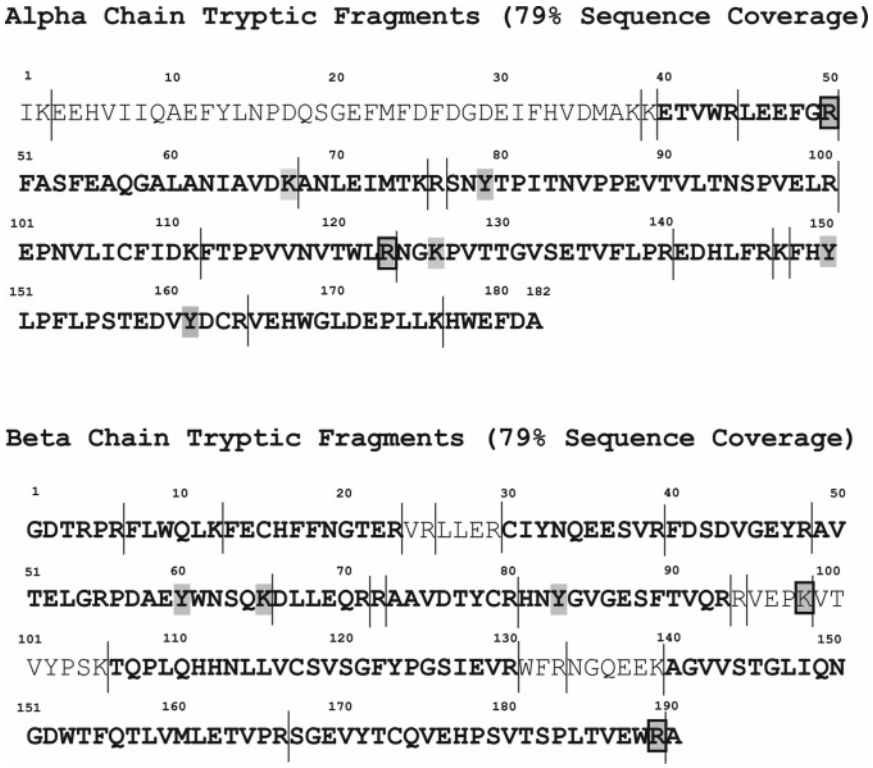


FIGURE 1: Sequence coverage of DR1 tryptic digest: α chain (top) and β chain (bottom). Trypsin-cut sites are marked with a line. Peptides which were observed for unmodified DR1 are shown in bold. Sequence coverage for modified proteins was similar. Modified residues are highlighted in gray. Modified residues which were observed in digests of empty, but not peptide-loaded DR1, are boxed.

of β-mercaptoethanol at a final concentration of 5% (v/v). Samples of unmodified DR1 were prepared under identical conditions as a negative control.

SDS−Polyacrylamide Gel Electrophoresis (PAGE)

Aliquots (150 pmol/protein) of modified DR1 or unmodified control DR1 were applied to a 12% polyacrylamide gel (Bio-Rad). SDS−PAGE was performed according to the manufacturer's instructions. Subsequently, gels were stained with colloidal Coomassie (GelCode Blue, Pierce) for 30 min and destained with deionized water until all background staining was removed.

In-Gel Protein Digestion

Gel slices were excised and cut into small fragments, transferred into a 0.6 mL siliconized microcentrifuge tube, and washed with 100 μL of deionized water. After being destained with a 50% acetonitrile/50 mM NH_4HCO_3 mixture, the gel was washed with 100 μL of 50 mM NH_4HCO_3 and then dried by vacuum centrifugation. The gel was rehydrated in buffer containing 25 mM NH_4HCO_3 and 12.5 ng/μL trypsin. Following digestion for 15 h at 37 °C, the peptides were concentrated and desalted using a C18 monolithic sorbent tip (OMIX, Varian) and cocrystallized on a stainless steel MALDI target in 4 μL of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid [in a 49/50/1 (v/v) acetonitrile/deionized water/trifluoroacetic acid mixture] according to the dried-droplet method (*38*).

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI/MS)

Proteins and peptides were analyzed by MALDI/MS under positive ion conditions on a Micromass MALDI L/R system (Waters, Milford, MA), equipped with a nitrogen UV laser (337 nm). Peptides were analyzed in reflectron mode from *m*/*z* 675 to 5000. An external calibration was used, and 50 shots were summed for each spectrum. Ten to twelve spectra were averaged for each sample. Relative abundances of peptides were calculated from their peak height (mean value of three measurements), assuming similar characteristics for the desorption and ionization of a specific ion in the samples for modified and unmodified DR1. All mass spectra were recorded in the Proteomics and Mass Spectrometry Core Facility at the University of Massachusetts Medical School.

RESULTS

Enzymatic Digestion and Mass Spectrometry of Unmodified DR1. The human class II MHC protein HLA-DR1 can be obtained in well-characterized empty and peptide-loaded forms by separately expressing the α and β subunits in *E. coli* and subsequently refolding the subunits together in vitro in the absence or presence of peptide ligand (*37*). The soluble α chain construct contains 182 residues, and the β chain contains 190 residues. These sequences correspond to the mature extracellular region of the protein not including the membrane-proximal "connecting-peptide" region.

For analysis by trypsin digestion and mass spectrometry, unmodified empty HLA-DR1 complexes were separated by SDS−PAGE under reducing conditions. Bands corresponding to α and β chains were excised and digested with trypsin, and the resulting peptides were analyzed by MALDI/MS. For the α chain, 14 of 15 expected peptide fragments were observed in the mass spectrum in the range of *m*/*z* 675−5000 (Figure 1 and Table 1). For the β chain, 12 of the expected 14 tryptic peptides were observed (Figure 1 and Table 2). During electrophoresis, all cysteine residues were observed to have been modified by acrylamide and the

Table 1: α Chain Sequence Coverage

				observed mass (Da)						
					hydroxyphenylglyoxal		NHS-biotin		tetranitromethane	
theoretical mass[a] (Da)	peptide	MC[b]	sequence	unmodified DR	empty DR	DR-Ha	empty DR	DR-Ha	empty DR	DR-Ha
690.36	40−44	0	(K)ETVWR(L)	690.34	690.35	690.39	690.37	690.38	690.39	690.37
750.38	45−50	0	(R)LEEFGR(F)	750.36	n/o[c]	750.63	750.50	750.50	750.63	750.61
804.33	177−182	0	(K)HWEFDA(−)	804.31	804.36	804.33	804.35	804.32	804.34	804.32
816.40	141−146	0	(R)EDHLFR(K)	816.44	816.44	816.45	816.42	816.42	816.45	816.43
818.45	39−44	1	(K)KETVWR(L)	818.43	818.43	818.43	818.43	818.43	818.43	818.43
919.49	68−75	0	(K)ANLEIMTK(R)	919.47	919.57	920.66	919.56	919.55	920.66	920.64
1361.71	101−111	0	(R)EPNVLICFIDK(F)	1361.83	1361.88	1361.85	1361.76	1361.74	1361.85	1361.83
1428.80	112−123	0	(K)FTPPVVNVTWLR(N)	1428.67	1428.75	1429.65	1429.75	1429.73	1429.65	1429.63
1435.76	165−176	0	(R)VEHWGLDEPLLK(H)	1435.74	1435.68	1435.80	1435.80	1435.80	1435.80	1435.80
1751.90	51−67	0	(R)FASFEAQGALANIAVDK(A)	1751.90	1751.90	1751.87	1751.88	1751.89	1751.43	1751.43
1801.98	124−140	0	(R)NGKPVTTGVSETVFLPR(E)	1801.94	1801.97	1801.92	1801.91	1801.92	1801.92	1801.92
2173.01	148−164	0	(K)FHYLPFLPSTEDVYDCR(V)	2173.03	2173.01	2173.01	2173.03	2173.03	2173.02	2173.05
2301.10	147−164	1	(R)KFHYLPFLPSTEDVYDCR(V)	2301.10	2301.10	2301.11	2301.10	2301.10	2301.10	2301.12
2640.39	77−100	0	(R)SNYTPITNVPPEVTVLTNSP-VELR(E)	2640.39	2640.39	2640.39	2640.39	2640.39	2640.39	2640.39
4262.90	3−38	0	(K)EEHVIIQAEFYLNPDQSGEFM-FDFDGDEIFHVDMAK(K)	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]

[a] Monoisotopic mass $[MH]^+$. Mass window of 675−5000 Da. [b] Missed cleavage. [c] Peak not observed.

Table 2: β Chain Sequence Coverage

				observed mass (Da)						
					hydroxyphenylglyoxal		NHS-biotin		tetranitromethane	
theoretical mass[a] (Da)	peptide	MC[b]	sequence	unmodified DR	empty DR	DR-Ha	empty DR	DR-Ha	empty DR	DR-Ha
701.3695	1−6	0	(−)GDTRPR(F)	701.22	701.38	701.37	701.33	701.32	701.35	701.33
704.3215	134−139	0	(R)NGQEEK(A)	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]
773.4157	66−71	0	(K)DLLEQR(R)	773.44	773.38	773.39	773.41	773.40	773.38	773.37
793.4460	99−105	0	(K)VTVYPSK(T)	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]	n/o[c]
834.4878	7−12	0	(R)FLWQLK(F)	834.49	834.48	834.47	834.48	834.47	834.46	834.45
969.4464	73−80	0	(R)AAVDTYCR(H)	969.45	969.43	969.41	969.43	969.43	969.42	969.40
1087.4696	40−48	0	(R)FDSDVGEYR(A)	1087.47	1087.45	1087.44	1087.46	1087.45	1087.44	1087.43
1311.6003	30−39	0	(R)CIYNQEESVR(F)	1311.60	1311.59	1311.59	1311.58	1311.58	1311.56	1311.56
1457.6272	13−23	0	(K)FECHFFNGTER(V)	1457.60	1457.61	1457.60	1457.59	1457.59	1457.64	1457.65
1493.7137	81−93	0	(R)HNYGVGESFTVQR(R)	1493.71	1493.71	1493.70	1493.69	1493.68	1493.73	1493.73
1963.9514	49−65	0	(R)AVTELGRPDAEYWNSQK(D)	1963.95	1963.94	1963.94	1963.93	1963.93	1963.91	1963.91
2675.2775	167−189	0	(R)SGEVYTCQVEHPSVTSPLTV-EWR(A)	2675.28	n/o[c]	2675.28	2675.22	2675.19	2675.20	2675.25
2852.4518	106−130	0	(K)TQPLQHHNLLVCSVSGFYP-GSIEVR(W)	2852.45	2852.45	2852.45	2852.43	2852.43	2852.42	2852.41
2932.5243	140−166	0	(K)AGVVSTGLIQNGDWTFQT-LVMLETVPR(S)	2932.52	2932.52	2932.52	2932.50	2932.51	2932.50	2932.51

[a] Monoisotopic mass $[MH]^+$. Mass window of 675−5000 Da. [b] Missed cleavage. [c] Peak not observed.

theoretical mass values were calculated accordingly. Generally, under the conditions used in this study, fragments containing fewer than five or greater than 35 amino acid residues were difficult to detect. Small peptides are hidden by ions resulting from the MALDI matrix. Larger peptides typically remain in the gel after digestion. Overall, the sequence coverage was greater than 75% for each chain.

Modification of DR1 with HPG and Localization of Derivatized Residues by MALDI/MS. The extracellular domains of DR1 contain 25 arginine residues on both the α and β chains (eight on the α chain and 17 on the β chain). Hydroxyphenylglyoxal was used to investigate the differential accessibility/reactivity of arginine residues in empty and peptide-loaded HLA-DR. HPG can react with the guanidino group of arginine via different mechanisms to yield different final products (Figure 2, I and II) (*39*, *40*). At the mildly alkaline pH used in this study, equimolar amounts of HPG are incorporated. At high pH (>12), reaction yields incorporation of two HPG molecules per arginine residue.

Exposed arginine residues on empty and peptide-loaded DR were derivatized using 5 mM HPG. Modification was monitored using UV−visible absorption spectroscopy, and the number of modified arginine residues was calculated using a molar extinction coefficient of 18 300 M^{-1} cm^{-1} (*41*) (Figure 3). This concentration of HPG was sufficient to differentially modify the two forms of the protein. Moderate increases in either the concentration of HPG or the time of reaction did not change the extent of modification. Empty DR1 incorporated 1.5 HPG molecules per αβ heterodimer, while peptide-loaded DR1 incorporated approximately 0.5 molecule per protein.

Modified empty and peptide-loaded HLA-DR1 complexes were separated by SDS−PAGE and digested with trypsin. Digestion by trypsin results in hydrolysis of the peptide backbone after arginine and lysine residues, and modification

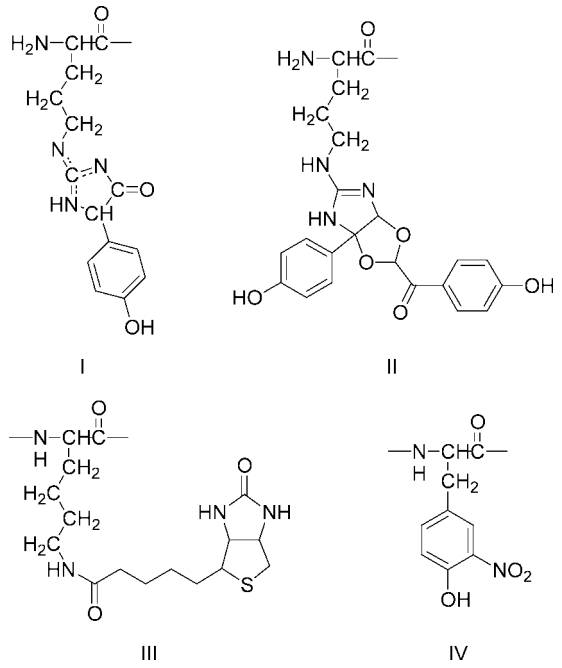


FIGURE 2: Side chain-specific chemical modifications. Product of reaction of arginine with *p*-hydroxyphenylglyoxal. (I and II) Reaction at pH 8 yields equimolar HPG incorporation as proposed by Wood et al. (*39*) (I); reaction above pH 12 incorporates two HPG molecules per residue (II). Biotinylation of lysine yields biotinyllysine (III). Reaction of tyrosine with tetranitromethane yields nitrotyrosine (IV).

of either arginine or lysine disrupts the ability of trypsin to cut the peptide backbone and results in a different spectrum of tryptic peptides. This change allows easy identification of incorporation sites of the arginine modifier.

No differences were observed in the mass spectra of the unmodified sample and the peptide-loaded preparation which had been modified with HPG (Figure 4A,B). This is consistent with the small amount of incorporation measured by absorbance: any modification of the peptide-loaded form that is present appears to be at a concentration too low to be measured by mass spectrometry, or possibly located on one or more of the tryptic peptides which were not observed in the experiment.

Empty DR1 was modified by HPG at three arginine residues, indicating that the regions around these residues are likely to have a different accessibility to solvent and are therefore more reactive than the same residues in the peptide-loaded conformation.

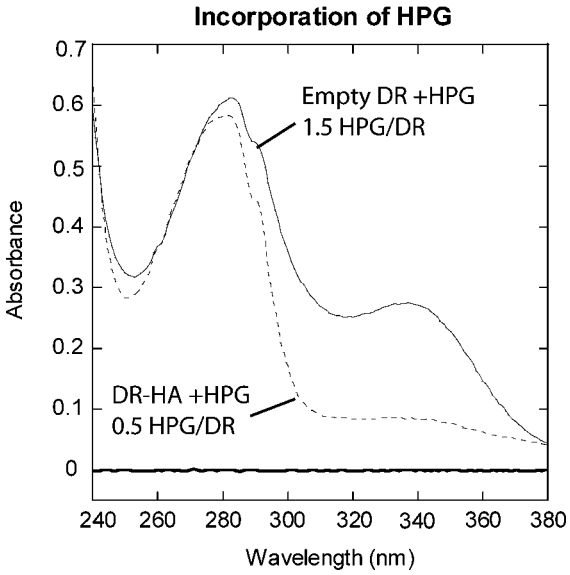


FIGURE 3: Incorporation of HPG. UV−visible absorption spectra of empty and Ha-loaded DR modified by 5 mM HPG. Empty DR (—) incorporates 1.5 molecules per protein complex. DR-Ha (− − −) incorporates approximately 0.5 HPG molecule per protein.

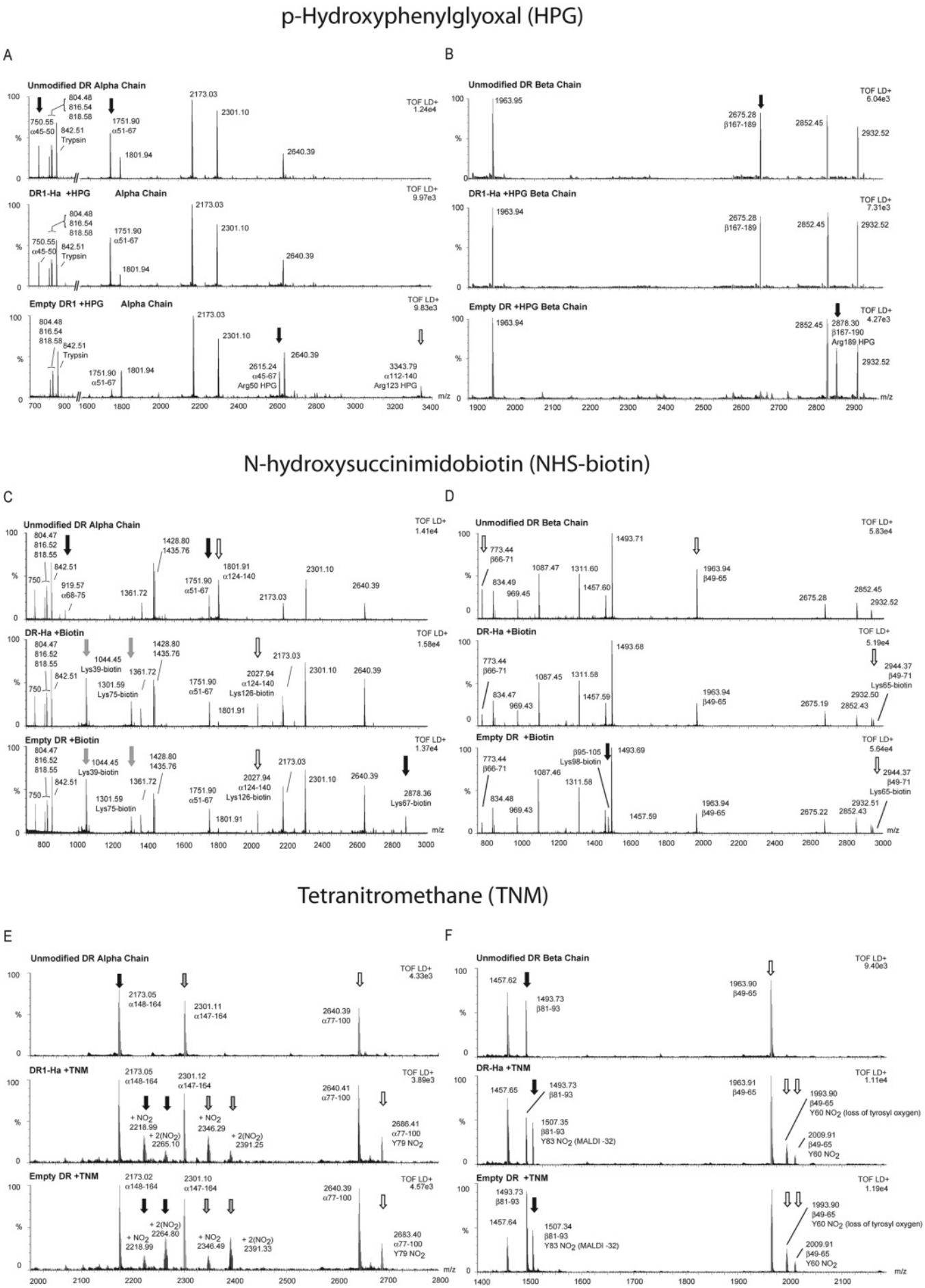
On the α chain, two modifications to empty DR1 were identified (Table 3). A mass peak of 2615.24 Da corresponding to a 132 Da shift for the HPG-labeled peptide α-(45−67) (LEEFGR*FASFEAQGALANIVDK) was observed (Figure 4A, solid arrows). This longer peptide contains a modified arginine at position 50, and results from the inability of trypsin to cut the peptide backbone at this site. Additionally, there was a loss of the peak at 750.38 Da [α-(45−50)], and a marked decrease in the relative abundance of the peak at 1751.90 Da [α-(51−67)], consistent with modification at Arg50α. A second modification site was observed at position Arg123 on the α chain. A mass peak of 3343.79 Da corresponding to a 132 Da shift for the peptide DRα-114−140 (FTPPVVNVTWLR*NGKPV-TTGVSETVFLPR) was observed (Figure 4A, empty arrow). This was not, however, accompanied by a reduction in the abundance of the peptide containing unmodified Arg123 [mass peak of 816.40 Da for α-(141−146)] or of the peptide generated by cutting the backbone at that position [mass peak of 1801.98 for α-(124−140)]. This would indicate only a small degree of modification of empty DR1 at this position.

One site of modification was identified on the *β* chain of empty DR1. A new peak at 2878.29 Da was observed (Fig-

Table 3: Relevant HPG-Modified Arginine Mass Peaks

DR subunit	peptide	arginine	observed mass[a] (Da)	calcd mass[a] (Da)	MC[b]	modification	relative abundance[c]		
							unmodified DR	empty DR	DR-Ha
α	45−50	50α	750.55	750.38	0	none	=1	0[d]	0.95 ± 0.04
α	51−67	50α	1751.90	1751.90	0	none	=1	0.08 ± 0.02	1 ± 0.05
α	45−67	50α	2615.24	2615.28	1	Arg50α HPG		=1	0[d]
α	112−123	123α	1428.37	1428.80	0	none	=1	0.99 ± 0.04	0.97 ± 0.03
α	124−140	123α	1801.94	1801.98	0	none	=1	1.16 ± 0.08	0.73 ± 0.03
α	112−140	123α	3343.79	3343.78	1	Arg123α HPG		=1	0[d]
β	167−189	189*β*	2675.28	2675.28	0	none	=1	1.05 ± 0.05	0[d]
β	190	189*β*	−	89.04	0	none	N/A[e]	N/A[e]	N/A[e]
β	167−190	189*β*	2878.30	2878.30	1	Arg189*β* HPG		=1	0[d]

[a] Monoisotopic mass $[MH]^+$. [b] Missed cleavage. [c] Peak height relative to the same peak in the unmodified sample ± the standard deviation; the standard deviation for nonrelevant peptides is 0.05. [d] Mass not present. [e] Not within the mass window.


p-Hydroxyphenylglyoxal (HPG)
A
Unmodified DR Alpha Chain
DR1-Ha +HPG Alpha Chain
Empty DR1 +HPG Alpha Chain
B
Unmodified DR Beta Chain
DR1-Ha +HPG Beta Chain
Empty DR +HPG Beta Chain
N-hydroxysuccinimidobiotin (NHS-biotin)
C
Unmodified DR Alpha Chain
DR-Ha +Biotin
Empty DR +Biotin
D
Unmodified DR Beta Chain
DR-Ha +Biotin
Empty DR +Biotin
Tetranitromethane (TNM)
E
Unmodified DR Alpha Chain
DR1-Ha +TNM
Empty DR +TNM
F
Unmodified DR Beta Chain
DR-Ha +TNM
Empty DR +TNM

ure 4B, solid arrows, and Table 3). This corresponds to the peptide β-(167−190) (SGEVYTCQVEHPSVTSPLTV-EWR*A). This peptide includes a modified arginine at position 189 and is the result of a missed cleavage due to the modification. This arginine residue is located at the next to last position in the soluble DR1 construct. Modification resulted in the complete loss of the peak at 2675.28 Da (β-167−189, the peptide which includes R189), indicating near-complete labeling. The corresponding mass for the C-terminal alanine which would be generated by cleavage at this site in unmodified DR cannot be detected by MALDI/MS in this system. Interestingly, Arg189 was one of the peptide residues to be identified as being important to the conformational change as measured by a change in antibody reactivity (*22*).

In all, three arginine modifications were observed which were present in only the empty conformation of DR. On the basis of the relative intensities of the unmodified tryptic fragment peaks, Arg50α and Arg189β were modified to a large extent, while Arg123α was modified to a much smaller extent.

Modification of HLA-DR1 with NHS-Biotin and Localization of Derivatized Residues by MALDI/MS. The amino group of lysine is a prime target for chemical modification. NHS esters can be used to add functional groups or other modifiers to lysine residues. In this case, NHS-biotin was used to label lysine residues in empty and peptide-loaded preparations of DR1 (Figure 2B). The soluble DR1 construct contains 14 lysine residues (nine on the α chain and five on the β chain). These residues are located throughout the protein complex and are mainly solvent-accessible.

Empty and peptide-loaded HLA-DR1 complexes were modified with NHS-biotin and separated by SDS−PAGE. Gel bands were excised and digested with trypsin. As with arginine, modification of lysine changes the pattern of tryptic peptides, making identification of incorporated biotin moieties relatively easy. A concentration of 50 μM NHS-biotin was sufficient to differentially modify the two forms of the protein. Empty DR was modified at six lysine residues, while DR-Ha was modified at only four (Figure 4C,D). Moderate variation of incubation time (15−60 min) and NHS-biotin concentration (50−200 μM) yielded similar patterns of reactivity.

Three α chain modifications were observed to be present in modified preparations of both empty and peptide-loaded DR1 (Figure 4C and Table 4). Modification at Lys126α was evident as a new peak observed at *m*/*z* 2027.64. This corresponds to the peptide DRα-(124−140) (NGK*PVTTGVS-ETFLPR) with a modified lysine (Figure 4C, empty arrows). The longer peptide is observed regardless of modification because Lys126 is followed by proline and is therefore unable to be cleaved by trypsin. A corresponding decrease in the intensity of the unmodified peptide peak at *m*/*z* 1801.91 was observed, indicating approximately 80% modification.

Similar modifications were observed at positions Lys39α and Lys75α (Figure 4C, gray arrows). These correspond to new peaks observed at *m*/*z* 1044.45 and 1301.59, respectively, and are present in modified preparations of both empty and peptide-loaded DR1. Both modifications yield peptide fragments that are extended by one amino acid: DRα-(39−44) (K*ETVWR) and DRα-(68−76) (ANLEIMTK*R).

On the α chain, one differential modification was observed only in NHS-biotin-treated preparations of empty but not peptide-loaded DR1 (Figure 4C, solid arrows). A new peak (*m*/*z* 2878.36) was observed that corresponds to the peptide DRα-(51−75) (FASFEAQGALANIAVDK*ANLEIMTK) and contains biotinylated lysine at postion 67. A reduction in the peak intensities corresponding to the unmodified tryptic peptides α-(51−67) and α-(68−75) was also noted. The larger peak, α-(51−67), is reduced by approximately half, indicating that the extent of modification at this site was approximately 50%.

The β chain of DR1 was also differentially modified by NHS-biotin. In empty DR, modification of the β chain occurred at position Lys98. This is indicated by a new mass peak at *m*/*z* 1472.72 (Figure 4D, solid arrow), and corresponds to the β-(99−105) peptide (VEPK*VTVYPSK). The extent of modification at this site is difficult to ascertain as both of the short tryptic peptides that make up the longer peptide in the modified sample were not observed.

Finally, a new peak at *m*/*z* 2944.36 was observed in digests of both empty and peptide-loaded DR1 that had been treated with NHS-biotin (Figure 4D, empty arrows, and Table 4). This peak corresponds to the β-(49−71) peptide (AVTEL-GRPDAEYWNSQK*DLLEQR) with a modified lysine at

FIGURE 4: MALDI mass spectra of α chain peptides after HPG modification and in-gel trypsin digestion. Peptides derived from unmodified complex (top) or modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. (A and B) Modification by HPG. (A, α chain) Modification at Arg50α in empty DR1 yields a new peak at *m*/*z* 2615.24 and a reduction in the intensities of peaks corresponding to unmodified peptides α45−55 and α51−67 (solid arrows). Modification at Arg123α yields a new peak at *m*/*z* 3343.79 (empty arrow) without a reduction in peak intensity for corresponding unmodified peptides α112−123 and α124−140. No modification was observed for DR-Ha. (B, β chain) For empty DR1, modification at Arg189β yields a new peak at *m*/*z* 2878.30 (β167−190) and a complete loss of intensity for the peak corresponding to unmodified peptide β167−189 (solid arrows). No modification was observed for DR-Ha. (C and D) Modification by NHS biotin. (C, α chain) In empty DR1, modification at Lys67α yields a new peak at *m*/*z* 2878.36 and a reduction in the peak intensities for corresponding unmodified peptides α51−67 and α68−75 (solid arrows). Modification at Lys126α in both empty and peptide-loaded DR yields a new peak at *m*/*z* 2027.94 (α124−140) and a reduction in the intensity of the peak corresponding to unmodified peptide α124−140 (empty arrows). Modifications at Lys39α and Lys75α yield new peaks at *m*/*z* 1044.45 and 1301.59, respectively (gray arrows). (D, β chain) In empty DR1, modification at Lys98β yields a new peak at *m*/*z* 1472.72 (solid arrow). The peaks corresponding to unmodified peptides β95−98 and β99−105 were not observed in the experiment. Modification at Lys65β in both empty and peptide-loaded DR yields a new peak at *m*/*z* 2944.36 (β49−71) and a reduction in the intensities of the peaks corresponding to unmodified peptides β49−65 and β66−71 (empty arrows). (E and F) Modification by TNM. (E, α chain) Residues Tyr150α and Tyr161α were observed on two tryptic peptides, α148−164 (solid arrows) and α147−164 (gray arrows), due to a missed cleavage at position 147. Modification at these sites yields new peaks corresponding to mass shifts of 45 Da (one nitrotyrosine) and 90 Da (two nitrotyrosines). Modification at position Tyr79α yields a new peak at *m*/*z* 2683.40 (empty arrows). Modification was similar in both empty and peptide-loaded DR. (F, β chain) Modification at Tyr83β yields a new peak at *m*/*z* 1507.35 (solid arrows). Modification at Tyr60β yields two new peaks corresponding to mass shifts of 29 and 45 Da (*m*/*z* 1993.90 and 2009.91, respectively) (empty arrows). Modification was similar in both empty and peptide-loaded DR.

Table 4: Relevant Biotinylated Lysine Mass Peaks

DR subunit	peptide	lysine	observed mass[a] (Da)	calcd mass[a] (Da)	MC[b]	modification	relative abundance[c]		
							unmodified DR	empty DR	DR-Ha
α	40−44	39α	690.34	690.36	0	none	=1	0.11 ± 0.03	0.07 ± 0.04
α	39−44	39α	1044.45	1044.45	1	Lys39α biotin		=1	1.14 ± 0.08
α	68−75	75α	919.49	919.47	0	none	=1	0[d]	0[d]
α	68−76	75α	1301.59	1301.59	1	Lys75α biotin		=1	1.34 ± 0.08
α	51−67	67α	1751.88	1751.90	0	none	=1	0.50 ± 0.03	0.83 ± 0.04
α	68−75	67α	919.56	919.49	0	none	=1	0[d]	0[d]
α	51−75	67α	2878.36	2878.38	1	Lys67α biotin		=1	0[d]
α	124−140[f]	126α	1801.91	1801.98	0	none	=1	0.20 ± 0.03	0.50 ± 0.04
α	124−140[f]	126α	2027.96	2027.99	1	Lys126α biotin		=1	0.93 ± 0.06
β	49−65	65β	1963.93	1963.95	0	none	=1	0.46 ± 0.03	0.41 ± 0.06
β	66−71	65β	773.41	773.42	0	none	=1	0.24 ± 0.02	0.18 ± 0.04
β	49−71	65β	2944.36	2944.36	1	Lys65β biotin		=1	0.98 ± 0.06
β	95−98	98β	−	472.28	0	none		N/A[e]	N/A[e]
β	99−105	98β	−	793.45	0	none		N/O[g]	N/O[g]
β	95−105	98β	1472.72	1472.72	1	Lys98β biotin		=1	0[d]

[a] Monoisotopic mass $[MH]^+$. [b] Missed cleavage. [c] Peak height relative to the same peak in the unmodified sample ± the standard deviation; the standard deviation for nonrelevant peptides is 0.05. [d] Mass not present. [e] Not within the mass window. [f] The peptide contains the PK sequence which is not cut by trypsin. [g] Peptide not observed.

Table 5: Relevant Nitrotyrosine Mass Peaks

DR subunit	peptide	tyrosine	observed mass[a] (Da)	calcd mass[a] (Da)	MC[b]	modification
α	77−100	79α	2640.39	2640.39	0	none
α	77−100	79α	2686.41	2686.38	0	Tyr77α NO_2
α	147−164	150α, 161α	2301.11	2301.10	1	none
α	147−164	150α, 161α	2346.26	2347.09	1	Tyr150α or -161α NO_2
α	147−164	150α, 161α	2391.25	2393.09	1	Tyr150α and -161α NO_2
α	148−164	150α, 161α	2173.05	2173.01	0	none
α	148−164	150α, 161α	2218.99	2219.00	0	Tyr150α or -161α NO_2
α	148−164	150α, 161α	2264.80	2264.99	0	Tyr150α and -161α NO_2
β	49−65	60β	1963.90	1963.93	0	none
β	49−65	60β	1993.91	1993.93	0	Tyr60β NO_2 (−O)
β	49−65	60β	2009.91	2009.92	0	Tyr60β NO_2
β	81−93	83β	1493.73	1493.73	0	none
β	81−93	83β	1507.34	1507.7330	0	Tyr83β NO_2 (−2O)

[a] Monoisotopic mass $[MH]^+$. [b] Missed cleavage.

position 65. The modification of this residue occurred to approximately the same extent (∼60−80%) in both preparations.

Overall, two modifications were observed that were present in only the empty conformation of DR (Lys67α and Lys98β). Modification at Lys39α, -75α, -126α, and -65β was observed in preparations of both empty and peptide-loaded DR.

Modification of HLA-DR1 with TNM and Localization of Derivatized Residues by MALDI/MS. Under mild conditions, TNM is an efficient and specific reagent for the nitration of solvent-accessible tyrosine residues (*42*, *43*) (Figure 2C). The soluble DR1 construct contains a total of 13 tyrosine residues (five on the α chain and eight on the β chain) which are distributed throughout each of the three tertiary domains of the protein.

Empty and peptide-loaded DR1 were labeled with TNM and subjected to in-gel tryptic digestion and MALDI/MS. Labeling of tyrosine residues does not interfere with the trypsin digestion, and therefore, the spectrum of expected peaks is not complicated by missed cleavages. Surprisingly, for some modified peptides, in addition to the expected mass shift of 45 Da ($+NO_2$), mass shifts of 29 and 13 Da were also detected. Recently, similar losses of oxygen due to the prompt fragmentation of nitrated tyrosine species have been identified by MALDI/MS (*44*). In that work, the authors suggest that this fragmentation occurs upon laser excitation in the spectrometer. While the fragmentation actually reduces the abundance of signals that can be detected, the overall fragmentation pattern can be used to unequivocally assign mass peaks to peptides containing nitrotyrosine.

Using this approach, we identified five sites of incorporation (Table 5). The extent of labeling appears to be small, and the pattern of reactivity was similar for both chains in the location of nitrated residues as well as for the peak intensity of the observed peptides. Upon digestion of the α chain, three new peaks are observed. The mass peak at *m*/*z* 2686.41 (Figure 4E, empty arrow) corresponds to a 45 Da shift for the α-(77−100) peptide (SNY*TPITNVPPEVTV-LNSPVELR) due to nitration of Tyr79. There is not a significant decrease in the intensity of the peak at 2640.41 Da (corresponding to unmodified tyrosine at that position); therefore, the extent of labeling is likely to be very low. Similarly, new peaks are observed for the nitration of tyrosine at positions α150 and α161. These residues are both located on the same α-(148−164) peptide (FHY*LPFLPSTED-VY*DCR). Mass shifts of 45 and 90 Da are observed, corresponding to the nitration of one and two tyrosine residues, respectively (Figure 4E, solid arrows). This sequence is also represented on peptide fragments which are generated by missed cleavage at position α147. Mass peaks corresponding to α-(147−164) peptides (KFHY*LPFLPST-EDVY*DCR) and their corresponding nitrated peptides are also observed (Figure 4E, gray arrows).

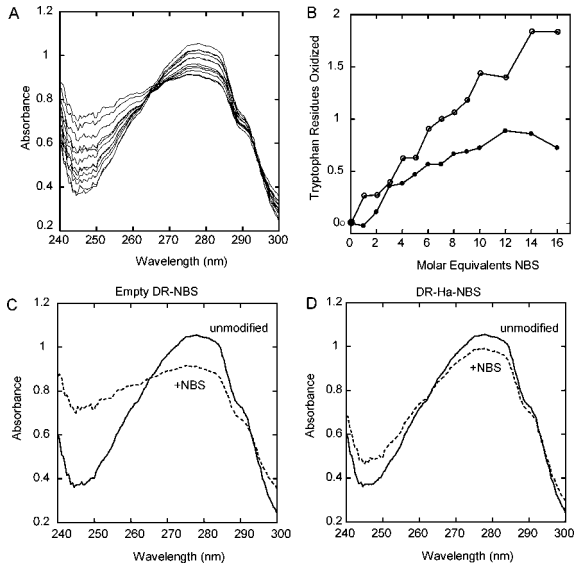


FIGURE 5: Modification of DR1 by *N*-bromosuccinimide. (A) Addition of NBS to empty DR1 causes a decrease in the absorbance at 280 nm and an increase at 260 nm. (B) Oxidation of tryptophan residues in empty (○) and peptide-loaded (●) DR by NBS. (C) Modification of empty DR with 15 molar equiv of NBS (1.8 tryptophan residues oxidized). (D) Modification of DR-Ha with 15 molar equiv of NBS (0.8 tryptophan residue oxidized).

On the β chain, two tyrosine residues become modified (Table 5). Mass shifts of 45 and 29 Da are observed for the β-(49−65) peptide (AVTELGRPDAEY*WNSQK), indicating modification of Tyr60 (Figure 4F, empty arrows). Additionally, a shift of 16 Da is observed for the β-(81−93) peptide (HNY*GVGESFTVQR), indicating modification of Tyr83 (Figure 4F, solid arrows).

Overall, five tyrosine modifications were observed for both empty and peptide-loaded HLA-DR1. These occurred at positions Tyr79α, -150α, -161α, -60β, and -83β. On the basis of the relative intensities of the unmodified tryptic fragment peaks, all of the reactive tyrosine residues were modified to a small extent.

Modification of HLA-DR1 with NBS. At neutral pH, *N*-bromosuccinimide can be used to selectively modify tryptophan residues (*45*−*47*). Reaction with NBS can be used to probe the accessibility and reactivity of tryptophan residues in proteins. The indole side chain of tryptophan absorbs strongly at 280 nm, and oxidation by NBS causes a shift in absorption maxima from 280 to 260 nm (*48*); thus, oxidation by NBS can be monitored in a spectrophotometer.

Empty and Ha-loaded DR were treated with 1−15 molar equiv of NBS. Modification was monitored in a spectrophotometer, and the number of tryptophan residues oxidized was calculated using the method of Spande et al. (*45*) (Figure 5A,B). Addition of 15 molar equiv of NBS resulted in the oxidation of two tryptophan residues in empty DR but only one residue in DR-Ha (Figure 5C,D). It is not known whether all of the differential reactivity occurs at a single tryptophan residue or whether more than one tryptophan is oxidized to a smaller degree.

Attempts to localize tryptophan oxidation by in-gel tryptic digestion and MALDI/MS were unsuccessful as the oxidation was reversible even under nonreducing conditions for SDS−PAGE. Solution digestion and LC−MS analysis were also unable to identify oxidized residues (not shown).

DISCUSSION

The class II MHC protein HLA-DR1 undergoes a distinct conformational change as it binds to its peptide ligand. In previous studies, monoclonal antibodies specific for the empty conformation identified two distinct regions on the β chain which change upon binding peptide; however, little localized structural information is available for the involvement of the α chain.

In this study, we probed the accessibility and/or reactivity of empty and peptide-loaded protein to a variety of chemical modifiers. Differences between the two conformations were observed for three of the modifiers that were used (Figure 6). This information can be used to gain insight into which regions are involved in the overall conformational change. Modifications which were observed in both conformations give information about regions which remain constant in the two conformations. Overall, these data can be used to generate a model for the conformational change.

The soluble DR construct used in this study contains 25 arginine residues (eight on the α chain and 17 on the β chain). Of these, 100% of the arginine residues on the α chain and 13 of 17 arginine residues (76%) on the β chain could be observed by mass spectrometry. The arginine-specific modifier HPG differentially labeled empty and peptide-loaded DR. Three sites of incorporation were identified in the empty conformation that were not present in Ha-loaded DR. Of the 25 arginine residues present in DR, all but two of them are involved in ion pairing or hydrogen bonding interactions in the crystal structure of DR bound to the Ha peptide (*49*). Arg50α and Arg189β have no hydrogen bonding partner and are two of the three sites of HPG incorporation.

One of these sites, Arg50α, is located on the loop between the β sheet floor of the peptide binding site and the α chain helix. In the structure of DR bound to the Ha peptide, the main chain atoms of nearby Ser53α and Phe51α make hydrogen bonds with the N-terminal portion of the bound peptide, holding it in an extended polyproline type II helix. It is likely that in the absence of peptide, this loop becomes more flexible and allows Arg50α to react with the HPG modifier.

The other, Arg189β, is located at the C-terminal end of the immunoglobulin-like domain of the β chain. It is also one of the residues identified as being important for binding to MEM-266, an antibody specific for the empty conformation of DR (*22*), confirming the involvement of this region in the conformational change. The residues in this region are solvent-accessible in the structures of HLA-DR1 peptide complexes, but are located in a cleft between the domains. The observed conformational differences in this region can be due to one of a number of possible structural transitions such as local unfolding or rigid body domain shifting. Either case would result in a change in the accessibility of residues in this region to both antibodies and chemical modifiers. Given the general stability of the immunoglobulin fold, it is unlikely that this region unfolds in the empty conformation. Rigid body shifts of the entire β2 domain, including rotations of up to 15°, are routinely observed in comparisons of HLA-DR1 peptide complexes in different crystal forms (*50*). Such shifts realign the β2 domain relative to the peptide binding

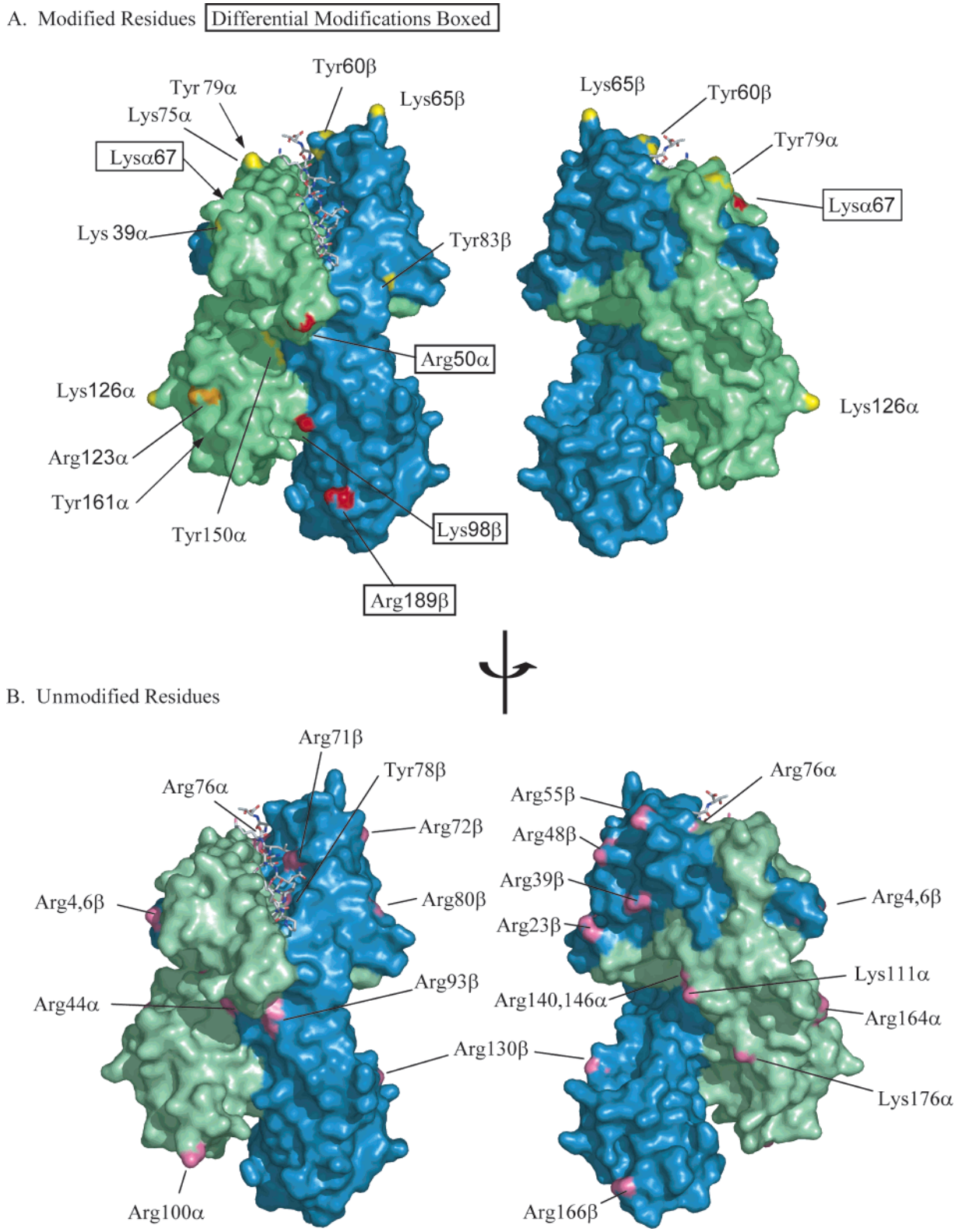


FIGURE 6: Map of chemical modifications on HLA-DR1 structure. The α subunit is colored light green and the *β* subunit blue, and the peptide is modeled as gray sticks. (A) Residues which were identified as modified in this study. Modifications which occur in only empty DR1 are colored red (residue labels are boxed). Modifications which occur in both empty and peptide-loaded DR1 are colored yellow (residue labels unboxed). Arg123α, which is modified only to a small extent in empty DR1 (and not in DR-Ha), is colored orange. (B) Observable arginine, lysine, and tyrosine residues which were not modified in this study (magenta). Only the reactive portion of each residue is highlighted. These unmodified residues are distributed throughout the protein.

domain, and could potentially couple peptide binding to domain rotation via interactions between loop residues at the top end of the Ig domain and the *β* sheet floor of the peptide binding domain.

The third arginine residue which was modified in this study was Arg123α. Peak intensity measurements indicate only a small degree of modification at this site. This residue is located on the α2 immunoglobulin-like domain and is

exposed to solvent. In the crystal structure of DR1 bound to the Ha peptide, this residue is involved in a hydrogen bond with the side chain hydroxyl oxygen of Thr130α. Its involvement in this hydrogen bond interaction could explain the low degree of reactivity at this position.

The soluble DR1 construct contains 14 lysine residues (nine on the α chain and five on the β chain), nine of which can be observed by mass spectrometry. In this study, six lysine modifications were observed in the empty protein, but only four of these were observed for peptide-loaded DR. The three observable lysine residues that were not modified in either case were either buried within the structure and inaccessible to soluble reagents (Lys12β) or involved in an intramolecular ion pair (Lys111α) or hydrogen bonding interaction (Lys176α).

Lys39α, Lys75α, Lys126α, and Lys65β were modified in both conformations (Figure 6, yellow). Analysis of the crystal structure of peptide-loaded DR indicates that these residues are completely solvent exposed and are not participating in any hydrogen bonds within the protein. Lys39α is located on the β sheet floor of the peptide binding domain, but lies outside the α chain helix and is oriented away from the binding site into solution. Lys75α is located on the α chain helix near, but oriented away from, the peptide binding groove. Lys126α is located on the α2 Ig-like domain near Arg123α and Tyr161α which were also chemically modified. Lys65β is located on the β chain α helix near the peptide binding site. In the X-ray structure, this residue is oriented away from the peptide and does not appear to be involved in peptide binding. This region of the β chain helix is located within the binding epitope of antibodies that bind the empty but not the peptide-loaded form of DR1 (*22*). Other evidence for conformational lability in this region can be seen in crystal structures of DR−peptide complexes, which exhibit significant peptide-to-peptide variation in this region, and relatively high thermal *B*-factors, particularly for residues 63−67. Whether the overall secondary or tertiary structure differs in this region between empty and peptide-loaded DR, the chemical susceptibility of Lys65β remains the same for both conformations.

Two lysine residues were differentially reactive, being modified in empty but not in peptide-loaded DR. One of these, Lys98β, is located on the first β strand of the β chain Ig-like domain. It is situated at the interface between the α2 and β2 subunits and in the crystal structure of the peptide-bound form makes a hydrogen bond with Ser95α on the AB loop of the α chain Ig-like domain. Differential reactivity of Lys98β between the empty and peptide-loaded conformations is consistent with a rigid body movement of the β2 domain relative to the α2 domain, opening up the region for modification at this site.

The other differentially modified lysine residue, Lys67α, is located on the α chain helix and oriented away from the peptide binding site. In the structure of peptide-loaded DR, this residue is located at the interface of the α chain α helix and the β sheet floor of the peptide binding domain. It forms a hydrogen bond network with the side chains of Tyr13α, Asn15α, and Gln18α from the β sheet as well as Glu71α from the helix. In the empty protein, this residue is accessible for modification and must rearrange relative to its position in peptide-loaded DR1. Nearby residues Asn62α, Asp66α, and Val65α make up the P6 binding pocket, and other nearby residues (Asn62α, Asp66α, and Asn69α) make hydrogen bonds with the main chain atoms of the bound peptide. The absence of peptide and lack of an MHC−peptide hydrogen bond may cause slight rearrangement of the helix relative to the β sheet floor. Modeling indicates that even a 3−5° rotation along the helical axis would cause Lys67α to disrupt the hydrogen bonds formed between the α helix and the β sheet.

The extracellular domain of DR contains nine tryptophan residues (four on the α chain and five on the β chain). Four of these tryptophan residues are completely buried in the structure of DR-Ha. Two more are located in the peptide binding site and would be blocked by the addition of peptide. Though we did observe differential oxidation of tryptophan in empty and peptide-loaded DR (Figure 5) (two oxidized residues in empty DR relative to one for peptide-loaded DR), we were unable to localize the site of modification by mass spectrometry. Other evidence for the involvement of tryptophan in the conformational change has been shown in the literature. Using intrinsic fluorescence studies, Kropshofer and co-workers show that empty DR1, prepared by acid release of endogenous peptide, exposes two Trp residues which are normally buried in the peptide-loaded conformation (*51*). Since there are two tryptophan residues located in the peptide binding site, it is possible that in the absence of peptide, one or both of these residues become accessible. The observed NBS oxidation of tryptophan in empty DR1 would be consistent with the reported exposure of tryptophan residues in the peptide binding groove in the absence of peptide (*51*); however, this remains to be verified by identification of the site of NBS-induced oxidation.

TNM modifies tyrosine to form *o*-nitrotyrosine and related compounds. Under the mild conditions used in this study, labeling of empty and peptide-loaded DR with TNM did not result in differentially modified residues. There are 13 tyrosine residues present in the DR construct. Seven of these are completely buried. Of the six solvent-accessible tyrosine residues, nitration was observed at five. Modification occurred at the following positions: 79α, 150α, 161α, 60β, and 83β. The similar reactivity of the two protein conformations indicates that the local environments surrounding each tyrosine residue are likely the same in both conformations.

The pattern of nonmodified residues can provide information about the conformational change complementary to that obtained from the sites of differential modification. Overall, approximately 22 observable potential sites of modification (surface-exposed side chains of residues present on tryptic peptides identified by mass spectrometry) were, in fact, not observed to be modified in either the empty or peptide-loaded form (Figure 6B). With few exceptions (Arg100α, Arg4β, Arg23β, and Lys176α), side chains of these residues are involved in salt bridging or ion pairing interactions. These interactions apparently reduce the chemical reactivity to the point where modification was not observed under our conditions. Any substantial conformational change in the empty form relative to the peptide-loaded conformation would be expected to disrupt such interactions and increase the chemical reactivity. Since the large majority of the sites which were unreactive in the peptide-loaded form were also unreactive in the empty form (22 of 26 total), we conclude that peptide-induced conformational changes are limited to the regions of differential modification (Figure 6A). A similar

argument can be made with regard to side chain accessibility. As no modification was observed for the empty protein at sites observed in the peptide-bound structure to be buried in the interior of the protein, we conclude that no large-scale unfolding accompanies peptide release. Overall, the pattern of unmodified residues suggests that the empty form and the peptide-loaded complex are structurally similar.

The structures of class I (*52*) and class II (*53*, *54*) MHC molecules in the absence of peptide have previously been called folding intermediates or molten globules. Molten globules are characterized as containing secondary structure elements of the native state in a less compact form, with many of the interior packing interactions unformed. Also, loops and other elements of the surface structure remain largely unfolded with different conformations. A large unfolding event or a general loosening of the secondary structure elements such as those observed for molten globules or other folding intermediates would appear to be inconsistent with the pattern of reactivity seen in this study for the empty protein (Figure 6). If the empty protein had resembled a molten globule, one would expect many more modifications to the empty (molten globule) form than to the peptide-loaded (native) form, and the differential modifications would be expected to be distributed relatively uniformly around the protein. In contrast, we observed only four modifications to the empty form that were not also observed in the peptide-loaded conformation, and three of those four differential modifications are located together in a region distinct from the shared sites of modification (Figure 6A).

Overall, it is clear from the chemical modification experiments that though the conformational changes are distributed throughout the protein, only a few localized regions are involved. Three of the modifications which were observed in the empty but not the peptide-loaded conformation are located on the same lateral face of the protein. Interestingly, this face has been proposed to be the interaction face of DR with HLA-DM (*55*). HLA-DM helps catalyze peptide binding and release through conformational effects on DR1 (*56*), and several models of interaction have been proposed (*10*, *12*, *17*, *56*−*58*). Many of these models involve HLA-DM recognizing or promoting a structural change in HLA-DR which could facilitate peptide release. The location of three modification sites on this face is consistent with such a model.

In summary, empty and peptide-loaded DR1 were modified using a number of side chain-specific chemical modifiers to probe the solvent-accessible residues present in both conformations. Overall, the pattern of reactivity suggests that the conformational changes, while distributed throughout the protein, involve only a few localized regions, and appears to be inconsistent with the idea that empty MHC molecules exist as molten globules or other partially folded intermediates.

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