A Biochemical Study of Class II MHC and Cytochrome P-450scc Proteins

By
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B.Sc., Chemistry with Honors, 1993
University of Puget Sound, Tacoma, WA

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY in Biological Chemistry

at the
Massachusetts Institute of Technology June 1999

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ABSTRACT

Class II major histocompatibility complex (MHC) proteins play a key role in the specific immune response. This thesis explores how these proteins function on the molecular level.

HLA-DQ8 (β1*0302) is strongly correlated with Insulin-dependent diabetes mellitus (IDDM) onset. Most IDDM resistant alleles contain Asp at position β57, while most IDDM susceptibility alleles contain a residue other than Asp. Others have proposed that this correlation is due to an instability of these MHC molecules. Using circular dichroism (CD) and thermal denaturation studies, we show that DQ8 is not an unstable molecule and that it adopts a slightly different structure from DQ9 (β1*0303), which has an Asp residue at β57.

A monoclonal antibody (KL-304) specific for the empty conformation of class II MHC molecules revealed the presence of abundant empty molecules on spleen- and bone marrow-derived dendritic cells among various types of antigen presenting cells. The empty class II MHC molecules are developmentally regulated, and can capture peptide antigens directly from the extracellular medium and present them to T-lymphocytes. These results suggest that dendritic cells may utilize an unusual extracellular presentation pathway in which antigen processing and peptide loading occur entirely outside the cell.

As an extension of this cellular study, we also investigated the mechanism of the conformational change between empty and peptide-loaded HLA-DR1. We found that the KL-304 and related KL-295 antibody epitopes are only detected on the empty form of DR1 and are lost when the large, hydrophobic P1 pocket is filled, either with a G86Y mutation of the protein or with a residue from a bound peptide as small as 4 amino acids in length. This immunoassay data is supported by near-UV circular dichroism data that shows that the empty protein has a different secondary structure from the mutant and peptide bound proteins.

Cytochrome P-450scc is a mammalian monooxygenase that catalyzes the conversion of cholesterol to pregnenolone in the steroid hormone biosynthetic pathway. Cholesterol is first hydroxylated at the C-22 pro-R position in a stereospecific manner to form (22R)-22-hydroxycholesterol. This intermediate is then hydroxylated again at the C-20 pro-S of cholesterol to form (20R,22R)-20,22-dihydroxycholesterol. These hydroxyl groups are introduced from two different molecules of oxygen. Finally, the glycol intermediate is cleaved between the hydroxyl carbons to form isocaproaldehyde and pregnenolone. The C-20 oxygen is not disturbed during this step and becomes the ketone oxygen of pregnenolone. In this study, we find that the C-22 oxygen also is not disturbed during the final oxidation step.

Thesis Supervisor: Dr. Lawrence J. Stern
Title: Assistant Professor of Chemistry
DEDICATION and THANKS

I dedicate this thesis to the following people who have helped me professionally and personally over the last six years.

Laura Santambrogio, M.D., Ph.D. I am deeply indebted to you for being my collaborator, friend, and teacher all rolled into one. Through working with you, I learned so much about the value of teamwork. I also learned the importance of placing my biochemical work into a more cellular context.

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Jennifer Schmitke, Ph.D. Thanks for being such a great friend and confidant. I don’t know how I would have survived the last couple years without talking about my fears and problems during our daily coffee breaks.

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To my fiancée, Kyong A. Yi. Kyong Ah, you make my life so complete. Thank you for being so understanding and caring over the last two years. I love you dearly.
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IX. Biographical / CV Note
I. Introduction to Class II Proteins Encoded by the Major Histocompatibility Complex (MHC)

MHC molecules are membrane-bound glycoproteins that play an important role in specific immune responses to foreign antigens (Germain, 1994). Class I MHC proteins are responsible for presenting peptides derived from intracellular antigens and are expressed in all cell types. On the other hand, class II MHC proteins are responsible for presenting peptides derived from extracellular antigens (including membrane-bound antigens) which are brought into specialized antigen-presenting cells (APC), including B-cells, dendritic cells, macrophages, monocytes, via receptor-mediated and/or fluid-phase endocytosis. These protein-peptide complexes bind to T cells with receptors specific for a particular complex of MHC protein and peptide. Once bound to the MHC-peptide complex, the T cell becomes activated and stimulates a specific immune response to the invading antigen (Korman, 1985).

Peptide Binding

MHC class II alleles are non-covalently associated αβ heterodimers (Benacerraf, 1981; Kaufman, 1984; Korman, 1985). Each monomer has an extracellular domain of approximately 200 amino acids and a transmembrane region and cytoplasmic domain of approximately 40 amino acids. Together they form an extracellular region with two immunoglobulin fold domains and a unique peptide-binding site, which is composed of an antiparallel β-sheet and two α helices (Fig. 1A, B). Peptides bind between the α helices in a type II polyproline helix conformation. The MHC protein contacts the peptide through both sequence independent and dependent interactions. The sequence independent interactions consist of hydrogen bonds, electrostatic and Van der Waals interactions with the bound peptide main chain (Fig. 1C). The sequence dependent interactions, however, are mediated between the peptide side chains and several hydrophobic and/or charged pockets (P1, P4, P6, P7, P9; the numbering is derived from N to C terminal numbering of the bound peptide, where P1 is the first N-terminal residue bound in the site) within the site (Fig. 1D). MHC genes are highly polymorphic within the general
population and many of the resulting polymorphic residues in the expressed protein are
concentrated in these binding pockets (see next section). These changes in pocket residues lead
to modified peptide binding preferences for different MHC proteins, or alleles [Fig. 2; For
review, see (Rammensee, 1995, 1997; Stern. 1994b; Sinigaglia, 1994).] In this study, several
human (HLA-DR1, DQ8, DQ9) and murine (I-As) are expressed in recombinant form and
analyzed to see how they may function in the immune system.

Polymorphism

The human MHC class II gene complex consists of many loci, which are designated DM,
DO, DP, DQ, and DR. Within each locus site, there are α and β genes. In the case of DR, there
are several β gene subloci sites, which can express functional subunits. These genes are highly
polymorphic in the general population, with the polymorphic residues concentrated in the
peptide-binding site (Fig. 3; Bodmer, 1991). These genes are labeled using a specific
nomenclature, (species label)-(locus site)*(serology number)(allele number). For example,
HLA-DQB1*0301 is a human class II gene derived from the DQB1 locus with a serology/allele
number of 0301. Before sequencing was as streamlined as it is today, MHC proteins were
identified by their reactivity towards antibodies specific for groups of proteins, or serotypes
(Marsh, 1989). Although the nomenclature numbers refer to cDNA sequences, these proteins are
still grouped together on classification charts and in the above nomenclature by serology number.

As expected by their common reactivity towards the same antibodies, the members of each
serological group contain many of the same polymorphic residues and as a result, may show a
great deal of tertiary structural homology. These allelic variations in a polymorphic pair of α
and β subunits lead to different binding motifs for each particular MHC allotype, and may
contribute to why two individuals can have completely different immune responses to certain
antigens.
Fig. 1. Class II MHC Structure. (A) Side-view ribbon diagram of HLA-DR1 (Stern, 1994a); (B) Top-view ribbon diagram of HLA-DR1 (Stern, 1994); (C) Sequence independent main chain interactions between HLA-DR1 and A2(103-117) peptide (Murthy, 1997); (D) Sequence-dependent pocket interactions between HLA-DR1 and HA(306-318) peptide (Stern, 1994).
Table 4.5 HLA class II motifs

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<td>V</td>
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| Vlassa ligands |                   |        |
| VCGD           |                   |        |
| VCGD           |                   |        |
| VCGD           |                   |        |
| LPNPKPVYV     |                   |        |
| LPNPKPVYV     |                   |        |

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| Examples for ligands |                   |
| ISHQ            |                   |
| ISHQ            |                   |
| IDPV            |                   |
| IDPV            |                   |
| KIQT            |                   |
| KIQT            |                   |
| YPFD            |                   |
| MQM             |                   |
| MQM             |                   |
| LPNPKPVYV      |                   |
| LPNPKPVYV      |                   |

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| Examples for ligands |                   |
| ISHQ            |                   |
| ISHQ            |                   |
| IDPV            |                   |
| IDPV            |                   |
| KIQT            |                   |
| KIQT            |                   |
| YPFD            |                   |
| MQM             |                   |
| MQM             |                   |
| LPNPKPVYV      |                   |
| LPNPKPVYV      |                   |

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| Examples for ligands |                   |
| EPRKPV          |                   |
| EPRKPV          |                   |
| EPRKPV          |                   |
| I2               |                   |
| F               |                   |
| FY               |                   |

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**Fig. 2. Examples of Class II MHC Peptide Binding Motifs with Known Binding Peptides (Ramensee, 1997).**
Fig. 3. HLA-DP, DQ, and DR are highly polymorphic class II proteins. (a) Comparison of DQα1*0101, DQα1*0201, DQα1*0301, and DRα1*0101 Polymorphic Residues; (b) Comparison of DQβ1*0303, DQβ1*0302, DQβ1*0301, and DRβ1*0101 Polymorphic Residues and Their Association with Diabetes; (c) HLA-DQ \( \beta \) polymorphism.

(a) \( \alpha \) allele homology

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(b) \( \beta \) allele homology

|        | 3 | 9 | 13 | 14 | 26 | 28 | 30 | 37 | 38 | 45 | 46 | 47 | 52 | 53 | 56 | 57 | 66 | 67 | 70 | 71 | 74 | 75 | 77 | 84 | 85 | 86 | 87 | 89 |
|--------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| DQβ1 | allele | association | 3 | 9 | 13 | 14 | 26 | 28 | 30 | 37 | 38 | 45 | 46 | 47 | 52 | 53 | 56 | 57 | 66 | 67 | 70 | 71 | 74 | 75 | 77 | 84 | 85 | 86 | 87 | 89 |
| 0302 | positive | Y | G | M | L | T | Y | Y | A | G | V | Y | P | L | P | F | A | V | R | T | E | L | T | Q | L | E | L | T | T |
| 0301 | negative | - | - | A | Y | - | - | - | E | - | - | - | D | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0201 | positive | - | - | - | - | S | S | I | V | - | E | F | L | - | L | - | D | I | K | A | V | R | - | - | - | - | - | - | - | - | - |
| 0303 | negative | - | - | - | - | - | - | - | - | - | - | - | - | D | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0602 | negative | - | F | - | - | - | D | V | - | - | Q | R | P | D | - | G | - | - | E | V | A | F | G |
| DRβ1*0101 | T | W | F | E | L | E | C | S | V | G | E | Y | E | L | R | P | D | D | L | Q | R | A | V | T | G | V | G | F |

(c) Example of Class II Protein Polymorphism, HLA-DQ \( \beta \) Sequences (Ramensee, 1997).
Structure

To date three-dimensional structures have been determined for several human and murine MHC class II proteins. The human protein structures include DR1 bound to endogenous peptides (Brown, 1993), DR1 bound to either HA(306-318) (Stern, 1994a) or A2(103-117) (Murthy, 1997), DR2 bound to MBP(85-99) (Smith, 1998), DR3 bound to CLIP(82-104) (Ghosh, 1995), and DR4 bound to collagen II(261-273) (Dessen, 1997). The murine protein structures include I-Ek bound to either murine hemoglobin(68-76) or Hsp70(238-246) (Fremont, 1996), I-Ad bound to either OVA(323-339) or HA(126-138) (Scott, 1998), I-Ak bound to HEL(50-62) (Fremont, 1998). Although each allele has a different peptide-binding motif, the large number of class II structures has made it easier to understand the general mode of peptide binding and predict peptide-binding motifs for class II proteins of unknown structure. As will be shown in Chapter III, however, future structural efforts should be aimed at determining structures of alleles associated with autoimmune diseases, such as diabetes and celiac disease (Fig. 4A). Several of these alleles associated with diabetes susceptibility contain an Asp to Ala mutation at β57 (Todd, 1987), which destroys a salt-bridge between the α and β subunits (Fig. 4B; Brown, 1993). Since it is difficult to model the effect of such a mutation and how it may affect subsequent peptide binding, the structures of such alleles should be determined to fully understand the structure-function relationship in the development of these diseases.
Fig. 4. Association of HLA-DQ Alleles with Diabetes Susceptibility. (A) Individuals with DQ Alleles containing non-Aspβ57 residues greater risk of contracting diabetes (Todd, 1987); (B) Aspβ57 makes an important salt bridge with Argα76. Loss of this interaction may affect the complexes stability and/or structure (Reizis, 1998).

(A) **HLA-DQ β alleles and IDDM Susceptibility.** Genotype analysis done on 50 unrelated IDDM patients and 73 randomly selected healthy controls.

<table>
<thead>
<tr>
<th>DQβ1 Allele (β57 residue)</th>
<th>Diabetics n (out of 50) / % of Total</th>
<th>Nondiabetics n (out of 73) / % of Total</th>
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<tr>
<td>0501 (V)</td>
<td>10 / 20%</td>
<td>16 / 22%</td>
</tr>
<tr>
<td>0502 (S)</td>
<td>3 / 6%</td>
<td>1 / 1%</td>
</tr>
<tr>
<td>0503 (D)</td>
<td>0 / 0%</td>
<td>5 / 7%</td>
</tr>
<tr>
<td>0601 (D)</td>
<td>0 / 0%</td>
<td>1 / 1%</td>
</tr>
<tr>
<td>0602 (D)*</td>
<td>0 / 0%</td>
<td>19 / 26%</td>
</tr>
<tr>
<td>0603 (D)</td>
<td>0 / 0%</td>
<td>13 / 18%</td>
</tr>
<tr>
<td>0604 (V)</td>
<td>0 / 0%</td>
<td>8 / 11%</td>
</tr>
<tr>
<td>0201 (A)</td>
<td>37 / 74%</td>
<td>31 / 42%</td>
</tr>
<tr>
<td>0301 (D)*</td>
<td>5 / 10%</td>
<td>21 / 29%</td>
</tr>
<tr>
<td>0302 (A)</td>
<td>37 / 74%</td>
<td>12 / 16%</td>
</tr>
<tr>
<td>0303 (D)</td>
<td>0 / 0%</td>
<td>0 / 0%</td>
</tr>
<tr>
<td>0401 (D) + 0402 (D)</td>
<td>1 / 2%</td>
<td>6 / 8%</td>
</tr>
</tbody>
</table>

(B) Model of DQ8 Peptide Binding Site and Location of Aspβ57-Argα76 salt bridge
Processing

Much effort has been directed at understanding the final conformation of the peptide-class II complex, and many studies have tried to understand how bound peptides are derived from foreign proteins and how they are loaded onto empty class II proteins (Germain, 1993a, 1994; Cresswell, 1994). Foreign antigens in the extracellular matrix are brought into endosomal compartments via receptor-mediated and/or fluid phase endocytosis (Fig. 5). Upon uptake, these antigens are proteolyzed into small peptides and loaded onto newly-synthesized or recycled class II molecules. The newly-synthesized class II molecules are transported from the endoplasmic reticulum complexed to invariant chain (Ii), a trimeric protein that binds the empty molecule through the peptide-binding site and other areas and thus prevents unwanted peptide binding during transport (Cresswell, 1994; Cresswell, 1996). Upon reaching the endosomes, the class II-Ii complex is dissociated by Ii proteolysis to yield a DR-CLIP complex (CLIP is a fragment of invariant chain (89-101) that binds within the peptide-binding site). Subsequently, the CLIP peptide is released by interacting with another protein, HLA-DM, a non-polymorphic class II homologue which promotes CLIP peptide release, protects the empty protein from aggregation, and promotes the binding of the antigenic peptides derived from proteolysis (Avva, 1994; Roche, 1995; Sanderson, 1995; Weber, 1996; Kropshofer, 1997; Denzin, 1995). Another non-polymorphic class II homologue, HLA-DO, is a negative regulator of HLA-DM and inhibits DM action, possibly by inhibiting its interaction with other class II molecules (Karlsson, 1991; Kropshofer, 1998; Denzin, 1998). Although this pathway is considered to be the primary class II loading pathway in B-cells, it possible that alternative pathways exist in dendritic cells (Rovere, 1998) and macrophages.

Although not discussed in this thesis, class I molecules obtain their bound peptides through a different mechanism (For review, see Germain, 1993a; Rammensee, 1997). Cytoplasmic proteins (derived from endogenous proteins and/or foreign antigens transcribed within the cell) are proteolyzed by a 20S proteosome complex and the resulting peptide fragments are transported into the endoplasmic reticulum by the TAP transporter (Fig. 5). Upon
binding of these peptides to newly-synthesized class I molecules, the peptide-class I complexes are transported from the endoplasmic reticulum, through the Golgi apparatus, and eventually to the cell surface for possible CD8+ T-cell activation.

Fig. 5. Class I and Class II MHC Antigen Processing and Presentation Pathways.

This majority of this thesis addresses various aspects of class II MHC structure and antigen presentation. Within each chapter, a specific and concise introduction to the subject matter is provided. The references cited within the body of the text are at the end of the thesis (Chapter VII). The final P-450scc chapter contains unpublished data collected in the lab of William Orme-Johnson, Ph.D. during my first year at M.I.T.
II. *In Vitro* Refolding of HLA-DQ Class II MHC Protein for Use in Biophysical and Crystallographic Studies

INTRODUCTION

During my first year in the Stern lab, we attempted to express several HLA-DQ proteins (DQ1, DQ2, and DQ8) using a procedure previously developed to refold HLA-DR1 from *E. coli*-produced subunits. In this procedure, α and β subunits are expressed as shorter extracellular versions of their wildtype form, isolated as insoluble inclusion bodies, and purified to homogeneity using anion exchange chromatography. By removing the connecting peptide, transmembrane, and intracellular regions of each chain, Frayser et al. (1999) refolded soluble peptide-DR1 complexes in high yield and purity. Since the protein was refolded outside of a cell in the presence of an added antigenic peptide, there was no chance of other contaminating peptides within the peptide binding groove, as is often seen in other expression systems that rely on the cellular machinery to fold the molecule. Once the protein was expressed and refolded, we were planning to conduct biophysical studies, as in the next chapter, and crystallization trials for structural determination. In the end, we were able to express but not refold several DQ proteins.

MATERIALS AND METHODS

*Subunit Cloning and Expression of monomers*

The modified versions of the DQα and DQβ genes were amplified (using NEB Vent™ Polymerase) from either Sf9 insect cells, which had been infected with baculovirus carrying each the extracellular gene, or cDNA provided by collaborators and ATCC (see Table I for listing of cloned genes). Each PCR-modified gene terminates at the last homologous residue observed in the HLA-DR1 structure (α184 and β190) and therefore does not contain any of the transmembrane and cytoplasmic domain genes. The forward and reverse primers incorporated

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1 Although not a collaborative effort, Mia M. Rushe played an instrumental role in helping me with the cloning and expression of these class II proteins. These preliminary results have not yet been published.
EcoRI and HindIII sites into the beginning and end of each modified gene, respectively. After the EcoRI site, a ribosomal binding site (AGGAGG) and spacer sequence (AATTTAAA) were added and the cDNA signal sequence was replaced with a Met start codon. Before the HindIII site, a stop codon (TAA) was inserted after the last codon (Fig. 1). PCR products were isolated, purified, and doubly digested with EcoRI and HindIII. These gel purified fragments were then ligated into a previously digested PLM1 vector behind a strong T7 promoter site. The ligations were transformed into DH5α competent cells and grown on LB/Ampicillin (100 μg/ml) plates overnight. All colonies were picked and grown up overnight in LB/Ampicillin (100 μg/ml) media. The plasmid from each culture was isolated using the boiling prep method and EcoRI/HindIII, HincII (only cuts DQA gene inserts), and SmaI (only cuts DQB gene inserts) restriction enzyme digests were conducted. Two positive clones from each allele were transformed into BL21 (DE3) pLysS competent cells and grown on LB/Ampicillin/Chloramphenicol (each antibiotic 100 μg/ml) plates overnight. Single colonies were picked into 5 ml of LB/0.2% glucose/Ampicillin/Chloramphenicol (same antibiotic concentrations) media and grown overnight to saturation. Fifty μl of saturated culture was added to 5 ml of the same media, grown to approximately 0.5 O.D., and then induced with addition of IPTG (0.5 mM). After 1 h of induction, uninduced and induced cell samples were pelleted, lysed in SDS loading buffer at 100 degrees for 3', and then loaded onto an SDS-PAGE gel. All clones expressed well to give a protein product of approximately the correct molecular weight for each subunit (α = 20.8 KD, β = 22.5 KD).

One BL21(DE3)pLysS clone from each allele was grown up and expressed on a 3 liter scale. The insoluble inclusion bodies were isolated from isotonically lysed cells, soluble bacterial proteins and membrane fragments were removed with a deoxycholate wash and a series of Triton X-100/Tris (50 mM, pH 8.0) washes. SDS-PAGE analysis of the washes showed only E. coli endogenous proteins. The inclusion bodies were dissolved in 8M urea, 1 mM DTT, 25 mM MES, and 10 mM EDTA, analyzed on SDS-PAGE, and found to be free of many E. coli endogenous proteins. Some inclusion bodies, however, were partially resistant to urea
solubilization and had to be dissolved in larger volumes of urea. Recombinant proteins were further purified by anion exchange HQ chromatography (Perseptives Biosystems).

**Fig. 1.** Construction of extracellular DQ α and β expression plasmids. (a) PLM1 expression vector with T7 promoter and Amp resistance marker; (b) Truncated gene map; SS, signal sequence; TMD, transmembrane domain; CD, cytoplasmic domain; RIBO, ribosome binding site.

(a)

(b)

**Peptide Synthesis**

Peptides were either synthesized using standard t-Boc chemistry by the MIT biopolymer facility or using Fmoc chemistry by an outside provider (Anaspec). Couplings were checked at each cycle by ninhydrin assay and secondary amino acid couplings were done in the case of incomplete couplings. Transferin receptor (TfR) 334-343 (VQTISRAAAE), MB65KD 243-255 (KPLLIAENVEGE), and FceR 104-122 (SQDLELSWNLNLQADLSS) peptides were biotinylated on the N-terminus using Pierce EZ-link Sulf-NHS-LC-LC-Biotin (equivalent to a two 6-aminocaproic acid linkers and a biotin molecule) (Chicz, 1994; Johansen, 1994). All
peptides were purified by reverse phase chromatography using C4 or C18 columns and were shown by MALDI-MS and amino acid analysis to contain a single peptide species.

Biotinylated Peptide Refolding Assay and Refolding Screen

Using the conditions established by Frayser et al. (1999), E. coli expressed HLA-DQ subunits (5 µg/ml each; 10 mg/ml total protein) and biotinylated peptide (100 µM) were diluted into an optimized buffer system consisting of 50 mM Tris (pH 8.5), 3 mM reduced glutathione, 0.3 mM oxidized glutathione, and 50% glycerol and then allowed to incubate for at least two days. These optimized conditions were originally developed for the refolding of HLA-DR1, another human class II protein. In an attempt to increase the overall DQ refolding yields, the following parameters were varied in microscale refoldings: protein (0.2-20 µg/ml), peptide (1-100 µM), pH (5-9), glutathione [20-1 (reduced: oxidized ratio); keeping total concentration at 3.3 mM], and glycerol (0-50% w/v) concentrations. Since I did not have an antibody capture assay that detected a correctly folded molecule, a specific peptide binding assay was used. In this assay, a polystyrene plate was coated with streptavidin (10 ng/µl) and then blocked with phosphate-buffered saline (PBS), 3% BSA, 0.02% sodium azide. Fifty microliters of each refolding was diluted into 50 µl of dilution solution (PBS, 0.05% Triton-X-100, 0.1% BSA) in the streptavidin plate and allowed to incubate for 1 hour at room temperature. After washing with PBS, 0.05% Triton-X-100 (PBST), the amount of bound DQ was determined by incubating the plate sequentially with 1:10,000 rabbit-anti-DQ polyclonal serum, 1:10,000 goat anti-rabbit polyclonal serum labeled with horseradish peroxidase, and 2,2'-Azido-di-[3-ethylbenzthiazoline sulfonate (ABTS) substrate (Boehringer Mannheim). Between each step, the plate was washed thoroughly with PBST.

RESULTS AND DISCUSSION

We did not have any difficulties expressing the denatured inclusion bodies of the alleles listed in Table I and purifying them to homogeneity on anion exchange chromatography (Fig. 2).
When it came time to refold the molecules, however, we ran into several roadblocks (see Table II for αβ combinations and peptides). Unlike the DR1 system, we had no assays that detected the presence of correctly folded molecule (Fig. 3A). As a substitute, we developed an assay that detected whether biotinylated antigenic peptides were bound by DQ protein molecules (see Materials and Methods) and used it to screen conditions similar to those used by Frayser et al. (1999) [20 mM Tris, 25% glycerol, 3 mM reduced glutathione, 0.3 mMoxidized glutathione, pH 8.5]. As a positive control, we tested HLA-DR1 bound to biotinylated HA 306-318 (a specific DR1 binding peptide) and found that the assay detected the bound complex (Fig. 3B) [Note: The detection DQ polyclonal antibody serum also recognizes DR class II molecules]. Once folding conditions were found that led to high levels of specific peptide binding, we planned to set up larger refoldings with non-biotinylated peptide under the same refolding conditions. For all the α, β-peptide combinations tested (Table II), however, we found no conditions that produced high levels of refolding compared to the DR1 procedure.

For each expressed allele, we have bacterial glycerol stocks of DH5α cells for DNA isolation and BL21 (DE3) pLysS cells for subunit production in the -80°C freezer (Rm. 56-553). Within the same box, any purified plasmid DNA stocks are stored in the -80°C freezer. Finally, all purified DQ subunits, if any, are stored in the -20°C freezer (Rm. 56-567). All of these items are catalogued in the appendix of this thesis. Although we would not recommend refolding any of these DQ molecules, these DNA and protein stocks may prove useful for other class II studies, e.g. expression in the baculovirus system, antibody epitope studies, PCR/sequencing standards.
Fig. 2. Example of HLA-DQ α and β protein expression and purification.

HLA-DQα1*0201 and β1*0302 BL21(DE3)pLys
Induction and Inclusion Body Purifications

| a, α1*0201 not induced; b, α1*0201, induced; c, ion-exchange purified inclusion bodies (pH 7.0); d, β1*0302, not induced; e, β1*0302, induced; f, ion-exchange purified inclusion bodies (pH 9.0) |

Table I: Comprehensive listing of available DQ constructs

<table>
<thead>
<tr>
<th>Allele</th>
<th>Haplotype</th>
<th>cDNA</th>
<th>E. Coli Construct (short versions)</th>
<th>Baculovirus?</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1*0101</td>
<td>DQ1(5)</td>
<td>X</td>
<td>X</td>
<td>X1</td>
</tr>
<tr>
<td>A1*0201</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>A1*0301</td>
<td></td>
<td>X2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>A1*0501</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>B1*0201</td>
<td>DQ2</td>
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<td>X</td>
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<tr>
<td>B1*0501</td>
<td>DQ1(5)</td>
<td>X</td>
<td>X</td>
<td>X1</td>
</tr>
</tbody>
</table>

Notes:
1. Both DQ1 genes cloned into one baculovirus transfer plasmid; both under polyhedrin promoter.
2. A1*0301 was cloned into a periplasmic expression vector (PET27b) under T7 promoter.
3. These baculovirus constructs are not discussed in this chapter, but are included to provide a complete record of all available DQ constructs.
Table II: Subunit combinations tested in refolding screen. See Materials and Methods for peptide sequences.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Allele Combination</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ1(5)</td>
<td>α1<em>0101,β1</em>0501</td>
<td>FcεR 104-122</td>
</tr>
<tr>
<td>DQ2</td>
<td>α1<em>0501,β1</em>0201</td>
<td>MB65KD 243-255</td>
</tr>
<tr>
<td>DQ3(8)</td>
<td>α1<em>0501,β1</em>0302</td>
<td>TfR 334-343</td>
</tr>
<tr>
<td>DQ3(8)</td>
<td>α1<em>0201,β1</em>0302</td>
<td>TfR 334-343</td>
</tr>
</tbody>
</table>

Fig. 3. DQ ELISA Construction – Detecting the formation of folded peptide complex. (A) Problems and solutions to ELISA development; (B) BioHA/HLA-DR1 standard curve using Avidin ELISA as shown in (A).

![DQ ELISA](image-url)

1. Monoclonal antibody HB110 (anti-DQ) recognizes free α and β, as well as complex.
2. HB114 sandwich ELISA is not as sensitive as comparable DR ELISA. The limit of detection greater than 3 ng.
3. Other anti-DQ monoclonal antibodies in lab (HB110, SPVL3) either do not recognize HLA DQ8 or are not sensitive enough.

![BioHA/HLA-DR1 BINDING CURVE](image-url)
INTRODUCTION

Through extensive population studies, the incidence of insulin-dependent diabetes mellitus (IDDM) has been shown to be strongly correlated with the presence of particular MHC class II HLA-DQ genes (Morel, 1988; Todd, 1987). HLA-DQ8 (α1*0301, β1*0302) and HLA-DQ6 (α1*0102, β1*0602), for example, show predisposition and protection against IDDM development, respectively. A comparison of many such pairs revealed that protection alleles have Asp at β57, while susceptibility alleles have Ala, Val, or Ser at β57. Among class II MHC proteins in general, Asp is present at position β57 in ~60% of human alleles [HLA-DR (77%), -DQ (53%), -DP (49%)]. In all class II structures solved to date (Brown, 1993; Dessen, 1997; Fremont, 1996; Fremont, 1998; Ghosh, 1995; Murthy, 1997; Scott, 1998; Smith, 1998; Stern, 1994), this Asp makes a salt bridge with a conserved Arg at position 76 in the α chain. Since there are no structures of non-Aspβ57 alleles, the role that this particular polymorphism plays structurally and in IDDM etiology is still unclear.

Previously, others have observed that DQ8 shows a unique binding preference for peptides with acidic residues near the C-terminus (Kwok, 1995; Kwok, 1996a, Kwok, 1996b; Nepom, 1996), presumably by interacting with Argα76 residue, which would be left unpaired in the absence of an acidic residue at β57, or with other residues within P9 pocket. Modeling of non-Aspβ57 alleles, however, shows that the Argα76 may be too far from the P9 side chain to influence peptide preferences at this position (Reizis, 1998). Previously, others have compared the relative stability of various complexes by comparing their ability to resist αβ chain

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2 In the future, the material contained in this chapter will be submitted as a publication.
dissociation in a non-boiled sample on SDS-PAGE (Gorga, 1987). This property is often referred to as the “SDS-stability” of a particular complex. Complexes of DQ8 (α1*0301, β1*0302) with endogenous peptides are more prone to SDS-induced chain dissociation than for DQ9 (α1*0301, β1*0303), which is identical to DQ8 except having Asp at β57 (Reizis, 1997).

Like DQ9, DQ6 also has an intact salt bridge between Aspβ57 and Argα76 (see MHC Introduction). The majority of DQ6 is SDS-stable, while DQ8 exists in both SDS-sensitive and resistant populations (Reizis, 1997). Unexpectedly, DQ8 and DQ6 persist on cells for similar lifetimes as measured by pulse chase experiments with surface iodinated molecules (t1/2 approximately 24 hours). These results suggest that SDS-stability may not be an accurate measure of overall stability of one complex versus another and that more detailed thermodynamic analysis must be done to address this question.

In this study, we analyze the thermodynamic stability of empty and peptide loaded DQ8 (α1*0201, β1*0302) and DQ9 (α1*0201, β1*0303) complexes using circular dichroism and thermal denaturation studies. We find that DQ8 and DQ9 are both thermodynamically stable molecules, but have somewhat different secondary structures. Since this change is most likely concentrated within the peptide binding site, these results may help to explain why these proteins bind a set of three peptides with different affinities.

MATERIALS AND METHODS

Preparation of HLA-DQ Molecules

Soluble HLA-DQ8 (α1*0201, β1*0302) and DQ9 (α1*0201, β1*0303) were produced in insect cells using separate baculovirus recombinants for α (EDIVAD ... IPAPMS) and β (RDSPED ... WRAQSE) subunits as described in Stern et al. (1992) and Raddrizzani et al. (1997). The recombinant baculoviruses were a gift from Dr. Francesco Sinigaglia (Roche Milano, Italy). For protein production, High Five (BTI-TN-5B1-4) insect cells were grown in 6 liter suspension cultures in Gibco BRL Sf900-II serum-free medium (see Appendix) and were coinfectected with a multiplicity of infection (MOI) between 5 and 10. This optimal MOI was
determined using a SPVL3 capture (anti-DQ complex specific antibody; Spits, 1984) and IVD12 detection (anti-DQ8/9 monoclonal antibody; also known as HB144; Giles, 1983) sandwich ELISA by Raddrizzani et al (1997). This assay can specifically detect folded αβ complex in the presence of denatured subunits and other medium proteins (see Appendix). After 5 days, the cells were removed by centrifugation (4500 x g, 45 min.), and protease inhibitors (1 mM PMSF, 5 mM EDTA, 1 mM iodoacetamide) and sodium azide (0.02%) were added to the supernatant. The supernatant was concentrated to 1/10 the original volume and then precipitated debris was removed by centrifugation. Finally, the DQ protein was purified from the clarified supernatant by immunoaffinity chromatography using mAb IVD12 (anti-DQ3β) coupled to CNBR sepharose as described (Raddrizzani, 1997). Protein was eluted with 50 mM CAPS, pH 11.5 and neutralized with one-third volume of 300 mM sodium phosphate (NaPi), pH 6.0 to give final pH ~ 6.5. The same protease inhibitor cocktail was added to the eluted protein. The theoretical extinction coefficient of 66720 M⁻¹*cm⁻¹ was used for all protein concentration and subsequent θ calculations. This value was verified experimentally by N-terminal sequencing (Fig. 1) and amino acid analysis of empty DQ9 (data not shown). The overall purified yield was approximately 0.5 mg per 6 liter culture for both DQ8 and DQ9.

To prepare peptide complexes, affinity-purified empty DQ protein (~ 1 mg/ml) was immediately mixed with 100 μM peptide and allowed to incubate at 37° C for 3 days. Complexes were isolated using a Pharmacia Superdex 200 HR gel filtration column with a Pharmacia HiTrap Q precolumn (PBS, pH 6.9, 0.4 ml/min). DQ peptide complexes do not bind the HiTrap Q column in this buffer but some non-DQ protein contaminants were removed. DQ complexes separated clearly from aggregates and free peptide on the Superdex column. Any remaining empty DQ protein binds partially to the HQ column and elutes later than peptide complex. For low-affinity peptide complexes, 250 μM excess peptide was immediately added to the size exclusion fractions to maintain the equilibrium towards complex formation.

All empty and peptide loaded proteins were diluted into Laemili loading buffer containing 1% SDS and analyzed on a 12.5% polyacrylamide SDS gel with and without prior
boiling. The percentage of SDS-resistant complex was estimated by scanning the not-boiled (NB) lane and dividing the $\alpha \beta$ complex band intensity by the total band intensity in the same lane. The NIH Image program was used to quantitate the intensity in the scanned gel bands. Using this procedure, we assume that free subunits stain with approximately the same intensity as complex bands.

**Fig. 1.** N-terminal sequencing of empty DQ9. The underlined residues correspond to signal from either the $\alpha$ (EDIVAD ...) and $\beta$ (RDSPEC ...) chains of DQ9. For some sequence positions, both $\alpha$ and $\beta$ contain the same residue (circled residues); so the signal at that position is higher than the previous residue.

**Peptide Synthesis**

Peptides were either synthesized using standard t-Boc chemistry by the MIT biopolymer facility or using Fmoc chemistry by the Roche peptide facility. Couplings were checked at each cycle by ninhydrin assay and secondary amino acid couplings were done in the case of incomplete couplings. TfR (334-343) (VQTISRAAAE) and GAD (253-265) (IARFKMFPVEKKEK) peptides were biotinylated on the N-terminus using Pierce EZ-link Sulf-NHS-LC-LC-Biotin (LC, 6-amino-hexanoic acid; NHS, N-hydroxysuccinimide ester), and GFKA7 (GFKAAAAA) was biotinylated by coupling two 6-aminocaproic acid spacers and one free biotin molecule on the N-terminus, sequentially, using standard amino acid diisopropyl carbodiimide coupling conditions. All peptides were purified by reverse phase chromatography.
using C4 or C18 columns and were shown by HPLC to contain a single peptide species that had the expected molecular weight by MALDI-MS.

Peptide Binding Assays

To determine the Kd for each peptide (TfR, GAD, and GFKA7) in a direct binding assay, DQ was diluted to 50 nM in PBS, 0.2% azide, 1% octylglucoside, protease inhibitor cocktail (same as above) and then 50 µl aliquots were prepared in silanized centrifuge tubes. Various concentrations of each biotinylated peptide were prepared (2 x 10^{-4} to 2 x 10^{-9} M) in the same binding buffer. Fifty µl of each peptide titration sample was added to triplicate DQ aliquots to give a final protein concentration of 25 nM. These samples were incubated at 37° C for 4 days, followed by assay for bound peptides using an antibody capture and streptavidin-Europium detection sandwich ELISA. The ELISA plate was prepared as follows: 100 µl of 10 ng/µl SPVL3 (anti-DQ complex specific; (Spits, 1984)) in PBS, 0.02% azide were added to each well of a 96 well polystyrene plate, followed by incubation at 37° C, 2 hours. The plate was then blocked with PBS containing 3% BSA and 0.02% azide at room temperature for 2 hours. Prior to loading the samples, the blocked ELISA plate was washed with Tris-buffered saline, 0.1% Tween-20 (TBST). Twenty-five µl of each sample was diluted into 75 µl dilution solution (PBS containing 0.05% Triton-X100 and 0.1% BSA) and incubated with the plate at 37° C for 30 minutes. After washing, the DQ bound peptide was detected by adding 100 µl of a 1:1000 dilution of streptavidin-Eu in Wallac DELFIA ™ Assay Buffer to each well. After the plate was incubated at 37° C for 15 minutes, the plate was washed again and developed with 200 µl/well of Wallac DELFIA ™ Enhancement Solution at room temperature for 5 minutes. Bound Europium was detected using a Wallac VICTOR ™ 1420 Multilabel Counter and the default Europium detection protocol (1 second/well counting time; normal aperture).
Circular Dichroism Analysis

For circular dichroism (CD) analysis, empty and peptide loaded complexes (0.5 mg/ml) were dialyzed in a 10K molecular weight cut-off Slide-A-Lyzer cassette (Pierce) into 10 mM sodium phosphate, pH 7. GFKA7 (100 μM), GAD (100 μM) or TfR (250 μM) free peptide was added to the dialyzing buffer of each respective peptide complex to prevent peptide dissociation. Dichroism measurements were made using a 1 mm path length cuvette in an AVIV 60DS CD spectrophotometer. Wavelength scans were obtained using 1.5 nm bandwidth, constant 10° C temperature, and 1 nm sampling with 5 seconds dwell time per point. All experimental scans were adjusted for background signal by subtracting out the signal from a dialyzing buffer scan. CD spectra were reported on a per residue basis; the empty, GFKA7, TfR, and GAD proteins have 382, 392, 392, and 395 residues, respectively.

Thermal denaturation data was obtained using 2 nm bandwidth, 224 nm detection wavelength, 10° C to 90° C range with 1° C intervals, a 1 minute equilibration time and a 1 minute dwell time at each temperature. The relationship of the unfolding transition to an irreversible denaturation that occurs in the same temperature range was investigated by recording the dependence of the midpoint temperature on the rate of the scan for overall scan rates 0.33° C/min to 1.33° C/min (Sanchez-Ruiz, 1992). No dependence was observed over the rates tested, indicating that the two-state approximation can be used at the experimental scan speeds (Fig. 2).

For determination of thermodynamic ΔCp and ΔH, thermal denaturation data were fit to a seven-parameter function that describes a two-state transition:

\[ \theta = (\theta_u + \mu T) + \frac{(\theta_f - \theta_u) + T(m_f - \mu)}{1 + \exp \left[ \frac{\Delta H}{RT} + \frac{\Delta C_p}{R} \left( \frac{Tm}{T} - 1 + \ln \frac{T}{Tm} \right) \right]} \]

where \( \theta_u \) and \( \mu_u \) describe the slope and y-intercept of the unfolded state baseline, \( \theta_f \) and \( m_f \) describe the slope and y-intercept of the folded state baseline, \( Tm \) is the midpoint of the
Fig. 2. The midpoint temperature is independent of the scan rate. The complex tested was DQ8 bound to the GAD peptide. The theta (θ) units are deg*cm²/(dmol residues). The uncertainty in each Tm calculation is approximately +/- 1 degree K.

transition (where ΔG = 0), ΔCp is the heat capacity change upon unfolding, and ΔH is the enthalpy of unfolding at the Tm. For each melt, the Tm was determined separately taking the first derivative of the melt data and selecting the temperature at which the slope is at its highest value. These thermodynamic values derived in this analysis are likely to be dependent upon the concentration at which the equilibrium is measured, and therefore only hold for the concentration ranges tested (0.1-1 mg/ml).

RESULTS

Synthetic Peptide Binding

Previously, Raddrizzani et al (1997) showed that GFKA7 binds to many DR and DQ proteins and can be used as a promiscuous binder in parallel peptide competition assays. Since the peptide bound well to DQ7 (α1*0501, B1*0301), we tested its binding to DQ8 and DQ9 in a direct binding assay using biotinylated peptide. As shown in Fig. 3, bioGFKA7 binds to DQ9 with an apparent Kd of approximately 0.79 μM; whereas the same peptide binds poorly to DQ8 with a Kd of approximately 62 μM. Since their maximal binding signals are approximately the
same (~2x10^5) and the same amount of protein was used for each assay, the binding capacity of both proteins are approximately equivalent.

To further test this differential peptide binding between DQ8 and DQ9, we tested binding of TfR (334-343), a slightly truncated version of the peptide [TfR (332-347)] previously isolated from affinity purified wildtype DQ8 (α1*0301, β1*0302) (Chicz, 1994). Both molecules bound the peptide weakly with Kd's greater than 100 μM, but DQ9 did show a slightly higher binding signal than DQ8 at 100 μM peptide (Fig. 3).
Previously, Kwok et al. identified a GAD peptide (253-265) that showed differential binding between DQ7 (α1*0301,β1*0301), DQ8 (α1*0301,β1*0302), and DQ9 (α1*0301,β1*0303) (Kwok, 1996a). In a whole cell peptide binding assay using 10 μM biotinylated peptide, DQ8 showed the highest relative binding, DQ9 showed approximately 25% of the maximal binding, and DQ7 showed no binding. Using our empty molecules, this peptide was tested in a direct binding assay as described above. Unlike Kwok’s results, our DQ9 (α1*0201,β1*0303) bound the peptide with a three-fold higher affinity relative to DQ8 (α1*0201,β1*0302) (Fig. 3). This difference in peptide binding specificity, however, could be the presence of the α1*0201 chain, instead of α1*0301. On the other hand, the previous results were obtained using peptide-bound DQ on whole cells at only one concentration of peptide. It is likely that our assay gives a closer approximation of the actual binding affinities of this peptide for DQ8 and DQ9.

**SDS-Stability Analysis**

Since the biochemistry of empty DQ molecules has not been described, we first analyzed DQ8 and DQ9 without peptides to determine the intrinsic SDS-stability of the proteins themselves (SDS-stability previously defined on page 24). Surprisingly, DQ9 was partially SDS-stable (as much as 40%); whereas DQ8 was completely SDS-sensitive (Fig. 4). To our knowledge, this is the first instance of an apparently empty MHC class II molecule with SDS-stable character. This property, however, could be due to the presence of bound peptides obtained during insect cell expression. To address these concerns, DQ9 was subjected to N-terminal sequencing (Fig. 1) and amino acid analysis; both conformed to expected values with no extraneous signals from a bound peptide. To check if SDS-stability were dependent on the cell type used for expression, Sf9 insect cells, instead of High Five, were used for expression and again the empty protein was partially SDS-stable (data not shown). We, however, cannot exclude the possibility that a heterogeneous peptide population binds DQ9, where each peptide represents a small percentage of the total bound peptide. For all the peptides tested in the direct
binding assays, none of them affected the above electrophoretic properties of either DQ8 or DQ9 (data not shown). Since all of the peptides bind with moderate (submicromolar) to weak affinities, it is possible that the peptides dissociated from the complexes before a SDS-stable complex could be separated from free subunits in the gel.

Fig. 4. Reduced SDS-PAGE of Empty DQ8 and DQ9. Purified proteins were loaded onto 12.5% gel before (NB) and after boiling for 2 minutes (B) in Laemili buffer. αβ, α, and β represent SDS-resistant αβ complex, free α chain, and free β chain, respectively. The number labels correspond to molecular weight markers in kilodaltons.
Near-UV CD Spectroscopy

The most homologous structures available for modeling HLA-DQ molecules are the murine I-A^k and I-A^d proteins. In these structures, Asp57 makes van der Waals contacts with the bound peptide side chain and in the case of I-Ak, a direct hydrogen bond is formed with the hydroxyl group of the bound HEL (50-62) serine side chain (Fremont, 1998; Scott, 1998). Since the P9 residue in GFKA7 is most likely Ala, Asp57 would not be expected to make extensive Van der Waals or hydrogen bonding contacts with this side chain and therefore loss of this contact should not cause such a drastic decrease in GFKA7 affinity. Thus a plausible explanation for the loss in GFKA7 binding affinity for DQ8 is that removal of the Asp57-Arga76 salt bridge causes a local rearrangement or loosening of the peptide binding groove such that contacts that existed previously between DQ9 and GFKA7 no longer exist in DQ8. To provide more conclusive evidence for this model, we analyzed empty, GFKA7, TfR, and GAD DQ8 and DQ9 complexes using near-UV circular dichroism (CD) spectroscopy to look for differences in secondary structure and overall thermal stability.

Empty and peptide loaded DQ8 molecules showed somewhat different secondary structures from their DQ9 counterparts. It is possible that the absence of the Asp57-Arga76 salt bridge causes an opening or loosening of the P9 end of the binding groove, which may allow for some residue movement in the binding pockets. For the empty molecules, DQ9 showed a significant increase in the negative band centered at approximately 220 nm as compared to DQ8 (Fig. 5A). For all three peptide complexes, DQ8 and DQ9 no longer showed such a large signal difference at 220 nm, but instead the DQ8 peptide complexes showed a significant negative band at lower wavelengths (<210 nm) as compared to DQ9 (Fig. 5B-D). From this, we conclude that the D57A residue change does cause a secondary structure rearrangement within the DQ8 molecule, presumably in the peptide binding site. This rearrangement could change the overall peptide specificity of DQ8 compared to DQ9 and possibly account for the differences in SDS-stability as previously observed when comparing IDDM protective and susceptibility alleles (Reizis, 1997).
Do these differences in secondary structure between DQ8 and DQ9, however, cause changes in the overall thermodynamic stability of each complex? To answer this question, each empty and peptide loaded complex was denatured while monitoring the CD signal at 224 nm. We found that both DQ8 and DQ9 showed similar thermodynamic parameters after fitting each

Fig. 5. Circular dichroism scans from 200 to 260 nm of (A) empty, (B) GFKA7, (C) TfR, (D) GAD complexes, (E) all DQ8 complexes, and (F) all DQ9 complexes. The θ units are deg*cm2/(dmol residues).
curve to a seven-parameter equation for a two-state melt transition. For the empty complexes, DQ8 (323 K) had a slightly higher Tm than DQ9 (317 K). Upon peptide binding, both DQ8 and DQ9 showed at least 10 degree increases in Tm [Table I, Fig. 6]. The GFKA7 and TfR DQ complexes melted at approximately the same Tm regardless of the Asp/Ala mutation [DQ+GFKA7~333-4 K; DQ+TfR~331-2 K]. From these complexes, it appears that the relative differences in binding affinity of each peptide for DQ8 and DQ9 did not produce any dramatic stability differences between the complexes. The GAD peptide, however, increased the DQ9 Tm (338 K) to a much greater extent as compared to DQ8 (333 K). This difference cannot be attributed to a difference in binding affinity as GAD bound well to both complexes.

Both empty complexes also showed a non-cooperative melt with ACp and ΔH values of approximately 500 cal/mol*K and 17000-19000 cal/mol, respectively [Table I, Fig. 6]. For almost all peptide complexes, both DQ8 and DQ9 showed at least two-fold increases in ACp and ΔH compared their respective empty complex melts [Table I, Fig. 6], indicating an increased thermal stability. The only peptide complex that didn’t show these dramatic increases was DQ9+TfR, which showed only slight increases in ACp and ΔH. From this data, therefore, it is clear that DQ8 does not show large differences in overall stability compared to DQ9. The changes in secondary structure, therefore, may only modify the peptide binding specificity and SDS-stability of the DQ molecule and not its intrinsic thermodynamic stability.
Fig. 6. Thermodynamic stability of empty and peptide loaded DQ8 and DQ9 complexes. The CD signal at 224 nm was monitored as the temperature was increased from 283.15 to 363.15 K by 1 K steps. The baseline was adjusted by subtracting the $\theta_{224}$ value at 283.15 K from each data point within each particular data set. The thermodynamic parameters calculated from this data are listed in Table 2.

![Graphs showing Δθ vs Temperature for different complexes](image)

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<th>HLA</th>
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<th>Tm (K)</th>
<th>$\Delta$H (cal/mol)</th>
<th>$\Delta$Cp (cal/mol*K)</th>
<th>Kd (μM)</th>
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Table I. CD Thermodynamic Analysis and Peptide Affinities. $\Delta$H (cal/mol), Tm (K), and $\Delta$Cp (cal/mol*K) were determined for each complex as described in Materials and Methods. Kd values were extracted from Fig. 1A. Two significant figures were used for $\Delta$H and $\Delta$Cp.
DISCUSSION

Since the original publications citing β57 as the key susceptibility residue (Todd, 1987), the relationship between the Asp/Ala dimorphism in DQ alleles to disease development has plagued the field of diabetes immunology. From biochemical analyses of DQ8 from EBV-transformed B-cells, the majority of the isolated complexes were susceptible to SDS-induced chain dissociation on SDS-PAGE (Reizis, 1997). The lack of SDS-stability was proposed to be associated with an unstable molecule. Based on results in the NOD murine system and the observation that both I-Ag7 and DQ8 are sensitive to SDS, many immunologists also went further to speculate that such instability could allow an autoreactive T-cell(s) to evade negative selection in the thymus (Carrasco-Marin, 1996; Kanagawa, 1997). This hypothesis, however, is in striking contrast with publications that isolate DQ8 and sequence the endogenous peptides associated with them (Chicz, 1994). If such complexes were unstable or the peptides had fast off-rates, such experiments could not have been possible.

Since the mechanism of SDS-induced chain dissociation is still unclear, it is possible that the initial SDS-PAGE experiments clouded a more subtle difference between Asp and non-Asp DQ proteins. We show that this dimorphism causes a secondary structural change, presumably in the peptide binding site of DQ8 relative to DQ9 using circular dichroism. A conformational change could allow SDS to penetrate a DQ8-peptide complex and promote chain dissociation (Natarajan, 1999). If so, then SDS-stability would be more of a measure of conformational states rather than intrinsic stability. We substantiate this idea by showing that three DQ8 peptide complexes are just as stable as their DQ9 counterparts bound to the same peptides.

Besides promoting SDS susceptibility, the DQ8 conformational change causes an alteration in peptide binding preferences relative to DQ9. The three peptides tested with DQ8 and DQ9 in the CD analyses show small to large differences in binding affinity for each protein. In particular, GFKA7 binds DQ9 with a sub-micromolar affinity and DQ8 with an affinity nearly 100 times greater, despite the fact that the Aspβ57 substitution is not likely to affect the peptide’s affinity.
So from this data, an alternate model for DQ8 induced IDDM development begins to form. From other more detailed peptide binding studies, it is clear that the Asp to Ala β57 dimorphism causes a dramatic change in the overall DQ8 peptide binding preferences (Kwok, 1995, 1996a, 1996b; Nepom, 1996). *In vivo*, this peptide preference change most likely affects the endogenous peptides bound to DQ8 and thus the overall T-cell repertoire selected in the thymus of a DQ8 patient. Since it does not appear that DQ8 is an unstable molecule, the hypothesis that DQ8 is a dysfunctional MHC molecule seems improbable.

Other mechanisms have been proposed to explain IDDM development without the need to incorporate the possible instability of DQ8. In the cryptic autoantigenic peptide hypothesis, potentially autoreactive T-cells are always present in a patient, but fail to be negatively selected because no endogenous peptide complexes in the thymus sufficiently select out those T-cells (Lanzavecchia, 1995). Only once the ‘autoreactive’ T-cell reaches the periphery does it see a self-antigenic peptide and then become activated. This peptide may only be unveiled upon specific T-cell activation during an unrelated infection or disease in the periphery (Salemi, 1995). In a similar hypothesis, others have shown that a foreign antigen from an infection can stimulate T-cells that can cross-react with self-proteins (Wucherpfennig, 1995). This molecular mimicry hypothesis would predict that autoimmunity would only be induced after subsequent infection by the foreign invader (bacteria, virus, etc.). Following infection, only susceptibility alleles would bind certain peptides derived from foreign antigens, which mimic self antigenic peptides, and present them to the immune system. Some of these peptide-MHC complexes could then go onto to stimulate T-cells that could cross-react with self cells and/or proteins. In summary, we have shown that DQ8 is a stable MHC molecule that can effectively bind peptides. Therefore, the previously described mechanisms should be explored to help explain the onset of IDDM.
FUTURE WORK

Although not discussed in this chapter, the purified DQ proteins were subjected to extensive crystallization screening in the hopes of obtaining a x-ray crystal structure. Since high quality crystals could not be obtained, this phase of the project was left uncompleted. To date, however, no one has solved the structure of a class II molecule containing a non-Asp residue at β57. This goal, therefore, should be pursued in the future.

It is possible that the insect cell protein is glycosylated in such a way as to prevent crystallization and that the E. coli expression system, which produces an unglycosylated molecule, may need to be considered. On the other hand, other purification protocols, such as hydrophobic affinity and IEF chromatographies, may also prove helpful in obtaining more homogeneous protein samples. These alternatives may need to be explored to fully understand the structural role of the β57 residue in diabetes development.
IV. **KL-295 and KL-304:**
Monoclonal Antibodies that Specifically Bind Empty Class II MHC Proteins

A. Antigen Presentation by Empty Cell Surface Class II MHC Molecules on Dendritic Cells

**INTRODUCTION**

KL-295 and KL-304 are monoclonal antibodies that were raised against peptides derived from $\beta_{58-69}$ of murine class II H2-IA$^d$ (AEYWNSQPEILE) and H2-IA$^s$ (AEYYNKQYLE) MHC proteins, respectively (Fig. 2A). This region of the class II molecule is highly polymorphic among murine IA alleles and contains two $\alpha$ helices with a small somewhat disordered, random coil between them (Fig. 2A). Lapan et al. (1992) hypothesized that this region might be accessible to a monoclonal antibody that could discriminate between the two allelic forms, IA$^d$ and IA$^s$. To promote the formation of specific antibodies to this region, the KL-295 and 304 peptides were immunized into mice of s and d haplotypes, respectively. Since the murine immune system will most likely not create an antibody against its own class II molecules, the antibodies derived from such an immunization should be against class II epitopes not present in the mouse, i.e. they may react with the class II molecule which contains the immunizing peptide.

When Lapan et al. tested the antibodies against folded wild-type protein on the surface of EBV-transformed B-cell hybridomas, neither KL-295 nor KL-304 showed any binding. The antibodies only showed binding to denatured protein on a SDS-PAGE/western blot. From this, they concluded that the $\beta_{58-69}$ region is either folded into a conformation that excludes key antibody contact residues from binding, or that the bound peptide interferes with antibody
binding. Since these observations, KL-295 and KL-304 have only been used for detection of denatured protein on a western blot.

H2-IA^8 is a murine class II protein associated with susceptibility to experimental allergic encephalomyelitis (EAE) (Alvord, 1984; Sobel, 1984), a murine model for the human disease multiple sclerosis (MS) in which T cells attack the myelin sheath surrounding neurons. EAE is triggered by the administration of a peptide [PLP(139-151)] derived from proteolipid protein (PLP), a suspected autoantigen in EAE and MS (Satoh, 1987; Tuohy, 1988; Sobel, 1994). To understand how the I-A^8 protein may lead to disease onset, we expressed the extracellular portion of the protein using the baculovirus expression system (Stern, 1992). Since insect cells do not have peptide-loading machinery, the recombinant proteins should be devoid of any bound peptide. During the course of this expression project, we discovered that the ‘empty’ αβ molecules became trapped at the insect cell membrane and were not efficiently secreted as had been seen before for HLA-DR1, a human class II protein (Stern, 1992). These cells displaying the empty protein were tested against a panel of antibodies specific for different forms (denatured, peptide-bound, allele specific) of the murine class II molecule. Unexpectedly, we found KL-304 (IA^8 peptide) stained the cells and that this reactivity was abrogated when the cells were incubated with PLP[139-151] (see data below), a peptide known to bind IA^8 with high affinity (Greer, 1996; Franco, 1994). Later, we found that both KL-295 and -304 also showed the same reactivity pattern with HLA-DR1 (AEYWNSQKDLLE, β58-69 homologous region), a human class II MHC protein. From this work, we discovered that these two antibody reagents can specifically detect empty class II MHC proteins, and we used them to understand what role empty MHC may play within the immune system.

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3 This work was done in collaboration with Dr. Laura Santambrogio at the Dana Farber Cancer Institute. The initial work has been submitted for publication.
Initially, we used the antibodies to determine if empty molecules are present on the surface of the immune cells which normally express class II MHC proteins, i.e. antigen presenting cells. Using primary cultures from mice of various haplotypes and flow cytometry analysis, we found that empty molecules are only present on the surface of immature dendritic cells and speculated that they may be taking part in a unique extracellular antigen presentation pathway.

At the same time, Zarutskie et al. (1999) showed that empty HLA-DR1 undergoes a conformational change upon binding antigenic peptides. Not only does the class II molecule change in secondary structure, the heterodimer hydrodynamic radius decreases by approximately 5 Å as followed by dynamic light scattering, analytical ultracentrifugation, and gel filtration. Consistent with this observation, we found that both KL-295 and -304 react preferentially with empty DR1 (not peptide-loaded complexes) and that the trigger for the complete conformational change between empty (antibody-sensitive) and peptide-bound (antibody-resistant) DR1 is filling the large hydrophobic P1 pocket and establishing some main chain and/or pocket interactions beyond this primary pocket. This project will be addressed in a subsequent chapter.

MATERIALS AND METHODS

**Murine H2-IAα Baculovirus Expression**

DNA fragments encoding signal sequence and extracellular domains for IAα (EDDIEA...SELTET) and IAβ (GDSETL...ESARSK) subunits were amplified by PCR and cloned into pFastBac1 (Gibco BRL) to produce recombinant bacmids that were used to transfect Sf9 cells. Recombinant baculovirus clones were isolated and used to infect Sf9 cells in Sf900
medium (Gibco BRL) as described for HLA-DR1 (Stern, 1992). Flow cytometry was performed 5 days post-infection. In some experiments, 10 μM peptide added to the medium 3 days post-infection as noted.

**Human HLA-DR1 Expression**

For production of soluble DR1 in *E. coli*, the extracellular domains were expressed as insoluble inclusion bodies, isolated by denaturing ion exchange chromatography, and refolded *in vitro* with or without peptide as described (Frayser, 1999).

Refolded empty HLA-DR1 protein was recovered by immunoaffinity chromatography using a conformation-specific monoclonal antibody (LB3.1), and was transferred into PBS (136 mM NaPi, 3 mM KPi, 10 mM NaCl, 2 mM KCl, 0.02% sodium azide, pH 7.2) containing 10% glycerol for storage at 4°C. Protein concentration was measured by UV absorbance at 280 nm using ε₂₈₀ of 54375 M⁻¹cm⁻¹ for empty DR1.

Peptide-bound DR1-complexes were prepared by incubating purified empty DR1 (10⁻⁵ to 10⁻⁶ M) with at least 5-fold molar excess peptide for 3 days at 37°C in PBS with 0.02% sodium azide and a mixture of protease inhibitors. Alternately, peptide complexes were formed by refolding subunits in the presence of excess peptide with isolation by immunoaffinity as described above. The peptide-DR1 complexes were further purified by gel filtration or ion exchange to remove aggregates and unbound peptide. Peptide-DR1 complex concentrations were measured by UV absorbance at 280 nm using ε₂₈₀ of 55655 M⁻¹cm⁻¹ (Ha, Yak, Min4; one Tyr in peptide), or 54375 M⁻¹cm⁻¹ (Clip; no aromatic residues in peptide).

**Peptide Synthesis and Antibody Epitope Determination**
Epitope mapping was performed by competitive ELISA using immobilized IAβ and various concentrations of epitope analog peptides. Peptide analogs of the KL-304 epitope (Lapan, 1992) (AEYNYKQYLEQT), antigenic and control peptides (Greer, 1996) PLP[139-151] (HSLGKWLGDPDFK), PLP[35-50] (GHEALTGTEKLIETYF), and PLP[178-191] (NTWTTCSQIAFSPK), and HLA-DR1-binding peptides (Zarutskie, 1999), HA[306-318] (PKYVKQNTLKLAT), YAK(AAYAAAAAAKAAAA), Min 4 (Ac-YRAL-NH₂), and CLIP (KMRMATPLLMQALPM), were synthesized with Fmoc protection and purified by reverse-phase chromatography using standard methods.

**Antibody Enzyme-Linked Immunosorbent Assays (ELISA)**

Antibody binding specificity was measured using a sandwich ELISA. Monoclonal antibody KL295 or KL-304 was used at 10μg/mL to coat a 96-well polystyrene microtiter plate by incubation overnight at 4°C. The plate was blocked with 3% BSA in PBS plus 0.02% sodium azide and washed with PBS plus 0.05% Triton X-100 (PBST). Quadruplicate 2-fold dilutions of DR1 from 1 to 300 nM in PBST were added and allowed to bind to the plate for 30 minutes at 37°C. Then the plate was washed 5 times with PBST. For Min4 samples, 100 μM free peptide was added to the incubations to prevent dissociation. The amount of bound DR1 was detected by sequential incubations with rabbit-anti-DR polyclonal antibody, goat-anti-rabbit peroxidase conjugate, and ABTS (405 nm) as described (Stern, 1992).

**Surface Plasmon Resonance (Biacore)**

For plasmon resonance, the Fab fragment of KL-304 was prepared by papain digestion (Coligan, 1997) and was coupled to a CM5 chip (Biacore) through amine groups using N-
hydroxysuccinimide and N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide. Attempts to
couple the intact antibody through amine groups resulted in loss of activity. Samples were
applied to KL-304 and control (no antibody) flowcells at 5 µl/min in PBS with 0.005% P20
surfactant, and the control trace was subtracted from the KL-304 trace.

Primary Cell Culture

Splenic dendritic cells (DC) were derived by selective attachment and negative antibody
selection as previously described (Winzler, 1997; Pierre, 1997; Cella, 1997b) and were
maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 5% fetal
bovine serum, 2 mM glutamine, non-essential amino acids, 1 mM sodium pyruvate, and 20 mM
Hepes buffer (cDMEM). Cultures were supplemented with 10 ng/ml mouse recombinant GM-
CSF (R&D Systems) every four days. Under these conditions most cells had the phenotype of
intermediate DC, although mature DC were observed. The fraction of mature DC increased
with time in culture. Similar staining results were obtained with freshly isolated dendritic cells
isolated on bovine serum albumin gradients as described (Winzler, 1997; Pierre, 1997; Cella,
1997b). Bone marrow DC were established by ex vivo differentiation of precursor cells as
previously described (Coligan, 1997). Cultures were maintained in cDMEM supplemented
every two days with 10 ng/ml GM-CSF. B cells, T cells, and granulocytes were removed using
rat monoclonal antibodies (Pharmingen) B220/CD45R, CD90.2 (Thyl.2), and Ly6GGR-1
(RB6-8C5), in conjunction with magnetic beads coated with sheep anti-rat IgG (Dynal), upon
initial isolation from bone marrow and again immediately before staining. To obtain mature
bone marrow-derived DC, cells were collected, washed, and sub-cultured for 1-2 days in the
absence of GM-CSF. In some experiments subcultured DC were further differentiated by
treatment with 100 units/ml mouse recombinant TNFα (Pharmingen) for 1-2 days (Winzler, 1997; Pierre, 1997; Cella, 1997b). Splenic and peritoneal B cells and macrophages were obtained by adherence and negative selection protocols as described (Coligan, 1997). In some experiments (Fig. 3 D,E and Fig. 4 D,E) B cells and macrophages were treated with 15 ng/ml LPS for 48 hours to increase MHC class II expression (Coligan, 1997) to levels comparable to those of DC.

Antibodies and Flow Cytometry

Primary antibodies used in cytometry, mouse monoclonals Y3P (anti IAα) (22), 10-2.16 (anti IAβk) (Germain, 1991; Germain, 1993b), 10-3.6.2 (anti IAβk) (Herzenberg, 1978), KL-304 (anti IAβkβ 57-68) (Lapan, 1992), 11-5.2 (anti-IAβ) (Pierres, 1989), MK-S4 (anti IAα) (Kappler, 1981), hamster monoclonal (Metlay, 1990), and rat monoclonal DEC-205 (Jiang, 1995), were obtained as hybridomas (ATCC) and purified by ammonium sulfate precipitation and protein A chromatography (Greer, 1996) or used as culture supernatant. Purified rat monoclonal antibodies MRC OX3 (anti-IA) and MRC OX6 (anti-IA) (Fukomoto, 1982) were purchased from Serotech. Species and isotype-matched control monoclonal antibodies were purchased from Pharmingen. For flow cytometry, cells were incubated with saturating amounts of primary antibody for 30 minutes on ice in PBS (150 mM NaCl, 10 mM Na-phosphate, 12 mM NaN₃, pH 7.2) with 10 mg/ml BSA (staining buffer), washed, incubated with fluorescein- or phycoerythrin-conjugated (Fab')₂ secondary antibody (Jackson Immunoresearch) preabsorbed with serum, washed again, and analyzed immediately using a FACscalibur flow cytometer (Becton Dickinson). Receptor number was measured using QIFIKit calibrated beads (Dako).
In some experiments Fcγ receptor binding was blocked by pre-incubation with 1 μg of rat monoclonal antibody CD16/CD32 (Pharmingen).

**Confocal Microscopy**

Splenic DC were cultured on a coverslip for microscopy. The Fc receptor was blocked as described above. Coverslips were washed and then incubated with saturating amounts of KL-304, Y3P, or control mouse IgG₂b antibody for 1 hour at 37°C, and then washed and incubated with 1 μg FITC-conjugated donkey anti-mouse (Fab')₂ (Jackson Immunoresearch) for 30 min. at 37°C, and finally fixed with 2% paraformaldehyde. Cells were not fixed prior to staining because KL-304 can recognize denatured protein that may be formed during fixation protocols.

**Cellular Peptide Binding Assays**

For fluorescent labeling, antigenic peptide PLP[139-151] and non-binding control peptide PLP[35-50] were prepared with a 13-residue N-terminal linker, εGGGSCRRGGGGS-, where ε is amino-caproic acid, and labeled at the N-terminus using 10-fold molar excess fluorescein isocyanate (FITC) in 1:1 dimethylformamide: N-methylmorpholine before side chain deprotection. For binding assays, cells were washed, incubated with various peptide concentrations in staining buffer for 2 hr at 37°C, and washed three times, before determination of bound peptide by flow cytometry.

**T-Cell Inhibition Assays**

A T-cell hybridoma restricted by IA ε and specific for PLP[139-151] was isolated and maintained as described (Santambrogio, 1998). T-cell activation was determined by a growth
inhibition assay (Ashwell, 1987). Briefly, 1 x 10⁴ immature/intermediate DC or 2 x 10⁵ splenic B cells were pulsed with different amounts (1nM - 100 µM) of specific peptide PLP[139-151] or non-specific I-Aβ-binding control peptide PLP[178-191] for 2 hours at 37°C. Cells were washed, fixed with 1% paraformaldehyde for 5 minutes at room temperature, and then washed extensively with lysine solution to remove free paraformaldehyde (Coligan, 1997). T-cell hybridoma hPLPC.4 cells (1 x 10⁴) were cultured with the washed antigen presenting cells for 24 hours, with [³H]-thymidine (1µCi) added during the last 6-8 hours, and incorporated radioactivity was determined as described (Coligan, 1997).

Proteolysis Assays

Proteolysis was measured using highly derivatized BODIPY-casein (Molecular Probes) at 5 µg/ml in 200 mM Tris-Cl, 0.02% azide, pH 8.0, 22°C, using 485 nm excitation and 535 nm emission, for samples that correspond to 10⁶ cells. The assay relies on relief of intramolecular quenching with proteolytic fragmentation of the BODIPY-caesin (Reis, 1998; Jones, 1997). For comparison of cell types, DC, macrophages, B-, and T-cells were cultured in the same batch of DMEM + 10% fetal bovine serum. For fractionation, cells were isolated from the medium by centrifugation at 4000 x g for 10 minutes. Cells were washed in assay buffer, and the medium was further fractionated by centrifugation at 100,000 x g for 60 minutes. The high-speed pellet contained numerous 50-100 nm vesicular exosomes (Fig. 1; Zitvogel, 1998). For inhibitor analysis, splenic DC were cultured in DMEM without serum for 6 hours and the 4000 x g supernatant was assayed as above, with or without addition of protease inhibitors. Other BODIPY-labeled proteins (BSA and ovalbumin) were also purchased from molecular probes and tested in the same manner as casein.
Rhodamine peptide substrates were purchased from Molecular Probes (Leytus, 1983a, 1983b) and were tested at 100 μM concentration in 1 ml of overnight DC medium, which had

**Fig. 1.** Splenic DC Exosome Preparation. Exosomes were derived from a 100,000xg pellet of supernatant (10,000xg) from an overnight splenic DC culture. After washing with 20 mM Tris, 200 mM NaCl, pH 8 (TBS), they were spun down at the same speed and resuspended in TBS. The sample was stained with uranyl acetate and submitted for analysis by transmission electron microscopy as described (Spector, 1998). Arrows indicate examples of DC exosomes.
been precleared at 100,000 x g to remove any insoluble particles and cell fragments. The sample was excited at 498 nm and the emission monitored at 521 nm over 30 minutes. The initial rate (ΔF/s) was determined from a linear fit to the data.

RESULTS

KL-304 Antibody Biochemical Analysis

The monoclonal antibody KL-304 (Lapan, 1992) was raised to a peptide (P58-69) found as part of the antigen binding site of the murine class II MHC protein IA^β (Fig. 2A). KL-304 does not bind to native IA^β on the surface of B-cells (Lapan, 1992), but recognizes a recombinant form of IA^β produced in baculovirus-infected insect cells (Stern, 1992) (Fig. 2B). Antibody binding required expression of both IA^α and β subunits, but was abrogated by addition to the culture medium of a peptide known to bind to IA^β (Fig. 2C). Insect cells are deficient in loading endogenous peptide onto heterologously expressed class II MHC proteins (Stern, 1992), and we suspected that KL-304 might preferentially react with MHC molecules that had not acquired peptide. To test this, we used the human class II MHC molecule HLA-DR1, which can be prepared in well-characterized empty and peptide-loaded forms (Stern, 1992; Frayser, 1999). HLA-DR1 has a two-residue insertion in the epitope region relative to IA^β, but crystal structures show that the insertion forms a bulge without altering the helical register (Stern, 1994; Fremont, 1998; Scott, 1998). Peptide mapping experiments show that the KL-304 epitope is discontinuous (Fig. 2D). The key epitope residues in IA^β (YY-K-YL) are substantially conserved in HLA-DR1 (YW---K-LL), with the conserved substitutions not affecting binding (Fig. 2D). As with IA^β, KL-304 reactivity with HLA-DR1 expressed in insect
cells also required both α and β subunits, and also was abrogated by peptide binding (Fig. 2E). To establish that KL-304 reactivity was specific for empty MHC molecules and not for MHC complexes carrying peptides derived from endogenous insect cell or serum proteins, we used recombinant soluble HLA-DR1 produced in E. coli and folded in vitro in the absence of peptide (Frayser, 1999). The folded, peptide-free HLA-DR1 showed strong KL-304 reactivity as assayed by sandwich ELISA (Fig. 2F) and plasmon resonance (Fig. 2G). We tested several peptide complexes of HLA-DR1, including those of the tight binding antigenic peptide HA (K_d ~ 10^{-8} M), the polyalanine analogue YAK (K_d ~ 10^{-7} M), the weakly binding minimal tetrameric peptide MIN4 (K_d ~ 10^{-5} M), and the invariant chain fragment CLIP (K_d ~ 10^{-9} M) (Zarutskie, 1999). None of the peptide complexes bound KL-304 (Fig. 2F). Thus, KL-304 appears to be specific for the empty MHC protein, most likely by sensing a conformational change that has been reported to occur in class II MHC proteins upon peptide binding (Rabinowitz, 1998; Reich, 1997; Zarutskie, 1999).

**KL-304 Antibody Analysis of Professional Antigen Presenting Cells**

We used KL-304 to investigate expression of empty class II MHC proteins on the surface of various professional antigen presenting cells, using primary cell cultures from SJL/J (IA^k), B10.BR (IA^k/IE^k), and C3H (IA^k/IE^k) mice. Little or no empty class II MHC protein was present on the surface of splenic or peritoneal B-cells, splenic or peritoneal macrophages, or on the surface of a B-cell derived lymphoma line, as detected by KL-304 (Table I, Fig. 3). All of these cells expressed substantial amounts of class II MHC-peptide complexes as detected by the IA-specific monoclonal antibodies 10-2.16, Y3P, and 11-5.2. In marked contrast, dendritic cells isolated from spleen or derived from bone-marrow precursors expressed high levels of empty
Fig. 2. KL-304 recognizes empty class II MHC molecules. (A) The KL-304 and KL-295 epitope (shaded, lower right) on a ribbon diagram of the class II MHC peptide binding site (Stern, 1994a). (B) Flow cytometry of IA\textsuperscript{13} B-cells (B-cell lymphoma LS 102.9) or IA\textsuperscript{1} expressing insect cells (Sf9/IA\textsuperscript{1}), using control and MHC-specific antibodies as indicated at left. KL-304 preferentially stains recombinant insect cells as compared to B-cells. Y3P, MRC OX3, and other antibodies tested (10-2.16, MRC OX6, 10-3.6.2, MK-S4; not shown) preferentially stain B-cells or do not discriminate. (C) Flow cytometry using KL-304 of insect cells expressing individual IA\textsuperscript{1} subunits \( \alpha \), \( \beta \), or both, with or without antigenic peptide PLP[139-151] treatment. (D) KL-304 epitope mapping. Substitutions of the IA\textsuperscript{1} sequence shown in bold, with asterisks indicating positions of key residues. (E) As for (C) except for HLA-DR1 subunits instead of IA\textsuperscript{1}, with or without HA peptide treatment. For both IA\textsuperscript{1} and HLA-DR1, only the empty \( \alpha \beta \) complex binds KL-304. (F) Sandwich ELISA using immobilized KL-304 to detect binding to soluble empty HLA-DR1 expressed in\textit{E. coli} (open circles) and to various peptide complexes (MIN4, hatched squares; HA, solid circles; YAK, solid squares; CLIP, solid diamonds). Soluble HLA-DR1 showed no binding to heat-denatured KL-304 (x), non-specific antibody (+), or BSA (open triangle). (G) Plasmon resonance using immobilized KL-304 Fab and empty HLA-DR1 (top) or peptide-loaded HLA-DR1 (bottom).
Fig. 3. Dendritic cells express empty cell surface class II MHC molecules. (A-E) Flow cytometry of various professional antigen presenting cells using antibodies KL-304 (shaded), Y3P (unshaded), and control IgG (light shading). The relative amount of KL-304 to Y3P staining varies with developmental state for bone marrow-derived DC. (A-C) KL-304 (Y3P) staining corresponds to the following numbers of molecules per cell: DC +GMCSF, 259,000 (38,000); DC -GMCSF, 243,000 (304,000); DC -GMCSF +TNF, <3,600 (>470,000); B cells <6,000 (>470,000), macrophages 19,000 (428,000). (F,G) Flow cytometry of splenic DC either untreated (NP, shaded) or preincubated with PLP [139-151] peptide (+P, unshaded) using antibodies KL-304 (F) or Y3P (G). Control IgG staining shown in light shading. (H-J) Confocal microscopy of splenic DC stained with FITC-labeled KL-304 (H), Y3P (I), or control IgG (J).
cell surface class II MHC as detected by KL-304 (Table I, Fig. 3). Dendritic cells from BALB/c (IA<sup>d</sup>/IE<sup>d</sup>) and B6x129 F1 (IA<sup>b</sup>) mice with non-crossreactive MHC haplotypes did not react with KL-304 (not shown). The fraction of the total cell surface MHC in the empty, KL-304-reactive form varied with developmental state. For bone marrow-derived DC, which can be cultured in various developmental states depending on cytokine treatment (Coligan, 1997), higher expression levels were observed on more immature DC (Fig. 3A,B,C). Splenic DC, which comprise a mixture of intermediate developmental states, expressed an intermediate level of KL-304 reactive class II MHC molecules (Fig. 3F). After addition of antigenic peptide PLP[139-151], known to bind specifically to IA<sup>b</sup> (K<sub>d</sub> ~ 40 nM), the level of empty cell-surface MHC decreased (Fig. 3F), with a concomitant increase in level of the peptide-loaded form as detected with the complex-specific antibody Y3P (Fig. 3G). Non-binding control peptide PLP[35-50] had no effect on KL-304 or Y3P surface expression (not shown). Confocal microscopy of splenic DC confirmed the developmental regulation of empty class II MHC expression. KL-304 reactivity to splenic DC (Fig. 3H) was associated with several immature and intermediate phenotypes (Winzler, 1997; Pierre, 1997; Cella, 1997b), whereas Y3P reactivity (Fig. 3I) was mostly associated with the more mature sea urchin-like phenotype previously correlated with high levels of class II MHC surface expression (Winzler, 1997; Pierre, 1997; Cella, 1997b). Thus, both splenic and bone marrow derived dendritic cells appear to express substantial amounts of empty, cell-surface class II MHC molecules.

Cellular Peptide Binding Experiments

To investigate the functional capacity of the empty class II MHC expressed on the surface of dendritic cells, we performed peptide-binding experiments with a fluorescein-labeled version of the antigenic peptide PLP[139-151]. Cells were treated with sodium azide (12 mM) to prevent endocytosis and other energy-requiring processes, washed, and then incubated with
peptide in a two hour binding assay. Immature DC efficiently bound added antigenic peptide PLP[139-151], while control peptide PLP[35-50] did not bind (Fig. 4A). Cell surface peptide binding varied with DC developmental state, with more immature DC exhibiting more peptide binding activity (Fig. 4A,B,C). Peptide binding levels were low for B-cells (Fig. 4D) and macrophages (Fig. 4E), consistent with previous studies showing inefficient cell-surface peptide binding to B cells (Sherman, 1997; Busch, 1990), and confirming that most of the class II MHC molecules on B cells and macrophages are present as MHC-peptide complexes. Peptide binding to DC was concentration-dependent (Fig. 4F). MHC-peptide complexes formed during the two-hour pulse were able to activate an IA²-restricted, PLP[139-151]-specific, T-cell hybridoma (Santambrogio, 1998) (Fig. 4G). These results indicate that empty IA² on the surface of dendritic cells has retained its specific peptide binding function, and that captured antigens are efficiently presented to T cells.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Empty and peptide-loaded cell-surface class II MHC molecules on antigen-presenting cells</th>
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<tr>
<td>cell type / source haplotype</td>
<td>KL-304¹</td>
</tr>
<tr>
<td>B cells</td>
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</tr>
<tr>
<td>Splenic s</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Splenic k</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Peritoneal s</td>
<td>2 (5)</td>
</tr>
<tr>
<td>LS.102.9³ s/d</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Splenic s</td>
<td>8 (5)</td>
</tr>
<tr>
<td>Splenic k</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Peritoneal s</td>
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<tr>
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<td>90 (44)</td>
</tr>
<tr>
<td>Bone marrow k</td>
<td>90 (36)</td>
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¹ Percentage of cells staining positively for the indicated antibody, with mean fluorescence intensity in parentheses.
² Average values for isotype matched control antibodies.
³ B cell lymphoma.
Fig. 4. Empty cell surface class II MHC molecules on dendritic cells are functional in antigen capture and presentation to T-cells. (A-E) Peptide binding to cell surface IA^+ on bone marrow-derived DC in various developmental states (A-C), macrophages (D), or B-cell blasts (E), using 100 μM fluorescent antigenic peptide PLP[139-151] (shaded) or control peptide PLP[39-50] (unshaded), detected by flow cytometry. Fractions of cells staining positive for surface peptide binding: DC (+GMCSF) 99%, mature DC (-GMCSF) 81%, mature DC (-GMCSF, +TNF) 29%, macrophages 23%, B-cell blasts 21%. (F) Mean fluorescence as a function of peptide concentration for antigenic peptide PLP[139-151] with immature DC (+GMCSF) (filled circles), B-cell blasts (B, filled squares), and macrophages (MΦ, open triangles), or for control peptide PLP[35-50] with immature DC (P35, open circles). (G) Peptide-specific antigen presentation to an IA^+ -restricted T-cell hybridoma for antigenic peptide PLP[139-151] with immature DC (+GMCSF) (filled circles) and B cells (filled squares), or for I-A^b-binding control peptide PLP[178-191] with DC (open circle) and B cells (open square). T-cell activation was measured using a growth inhibition assay (Santambrogio, 1998).
KL-304 and Y3P Antibody Analysis of Invariant Chain Knockout Mice

The presence of abundant empty cell-surface class II MHC proteins on dendritic cells suggests that DC utilize pathways for control of class II MHC expression and intracellular trafficking that are distinct from those that have been characterized in B-cells (Watts, 1997). In B cells empty class II MHC molecules aggregate intracellularly in a functionally inactive form, and are not transported efficiently to the cell surface (Germain, 1991, 1993b). Several lines of evidence support distinct control of class II MHC expression and intracellular trafficking in DC. B-cells from mice lacking the class II-associated invariant chain chaperonin Ii exhibit defective intracellular transport of class II MHC molecules and altered expression of cell-surface MHC-peptide complexes (Fig. 5C; Rovere, 1998; Bikoff, 1993; Bikoff, 1995), whereas DC from these mice exhibit normal expression levels (Fig. 5A; Rovere, 1998). Levels of empty cell-surface I-A^k as detected by KL-304 also are normal in DC from Ii-deficient mice (Fig. 5B). H2-O (HLA-DO), another molecule that plays an important role in intracellular antigen processing in B-cells, has been reported to be absent or only weakly expressed in murine DC (Lijedahl, 1998; Karlsson, 1991). Transcriptional regulation of class II MHC molecules and associated chaperonins is different for DC relative to B cells. In many cell types including B-cells and macrophages, the transcription factors CIITA and RFX5 coordinately regulate expression of class II MHC and associated molecules, and mice lacking these factors generally do not express significant levels of class II MHC molecules (Chang, 1996; Williams, 1998; Clausen, 1998). However, class II MHC expression can be observed in DC from mice deficient for CIITA or RFX5, although at a significantly reduced level. Together, these observations support a model whereby the importance of class II-associated chaperonins in directing intracellular transport and peptide-loading activities is greatly reduced in DC as compared to B cells.
Fig. 5. Distinct antigen processing pathways in dendritic cells and other antigen presenting cells. (A-D) Expression of class II MHC molecules is impaired in B-cells, but not DC, of invariant chain-deficient mice. Flow cytometry of splenic DC (A,B) and B-cells (C,D) and using the complex specific antibody Y3P (A and C) or the empty MHC specific antibody KL-304 (B and D). In each panel cells from normal B10.BR Ii+/- mice (shaded) and transgenic knockout Ii-/- mice (open) are shown, along with a non-specific control antibody (light shading). (E, F) Dendritic cells express an extracellular proteolytic activity. (E) Proteolytic activity of splenic (Sp) DC, bone-marrow derived (Bm) DC, splenic macrophages (MΦ), or a mixture of splenic B and T cells (B,T), measured using a fluorescence assay. (F) Proteolytic activity is found in both cell-associated and secreted forms and is blocked by serine and cysteine specific inhibitors. Left, cells from a splenic DC culture were harvested by centrifugation and washed (Cells-4k). The medium (Sup-4k) was further fractionated into a high-speed supernatant (Sup-100k) and a pellet rich in exosome-like vesicles (Exo-100k). Right, medium from a six-hour serum-free culture was tested alone, after boiling for 5 min, or after addition of protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 0.6 mM), iodoacetamide (IAA, 10 mM), leupeptin (10 µM), antipain (74 µM), EDTA (5 mM), pepstatin (1 µM), α2-macroglobulin (0.2 µM). Percent activities are reported as fluorescence changes at 1 minute reaction time relative to the value for the medium alone.
Dendritic Cell Proteolytic Activity Experiments

Based on the results presented here, empty cell surface class II MHC molecules appear to function as DC antigen receptors. Similarly to previously identified DC endocytic receptors such as the mannose (Sallusto, 1995) and Fc (Sallusto, 1994) receptors and DEC-205 (Jiang, 1995), empty class II MHC molecules might serve as antigen-specific endocytic receptors, with protein fragments or even intact proteins (Castellino, 1998; Sette, 1989) binding at the cell surface, and subsequent proteolytic processing occurring after endocytosis and transport to a proteolytic compartment (Lutz, 1997). The empty MHC molecules can function in another mode, by capturing peptide antigens directly from the extracellular milieu and presenting them to T cells without further processing. Such peptides could be generated at sites of inflammation as result of cell lysis or by the proteolytic activities of macrophages and granulocytes. Dendritic cells themselves have been reported to express extracellular proteolytic activity that could contribute to antigen processing (Amoscato, 1998). We have detected such activity in splenic DC, using a generic proteolytic substrate (BODIPY-FL-labeled casein) in a fluorescent assay (Jones, 1997). Expression of extracellular proteolytic activity was much greater for splenic and bone marrow derived DC than for macrophages, B and T cells (Fig. 5E). The activity is found

![Fig. 6. Protease activity is highly specific for particular protein and peptide substrates. DC protease(s) show greater proteolytic activity with fluorescently labeled β-casein (5 μg) compared to chicken egg white ovalbumin (Ova, 25 μg) and bovine serum albumin (BSA, 25 μg). +, non-specific hydrolysis of all labeled proteins.](image-url)
in both secreted and cell-associated forms, and can be inhibited by boiling and by serine and cysteine protease inhibitors (Fig. 5F).

(A) Known Substrate Specificity |
<table>
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<tr>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P-1</th>
<th>Initial Rate (ΔF/s)</th>
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<td>Granzyme A</td>
<td>CBZ</td>
<td>Gly</td>
<td>Pro</td>
<td>Arg</td>
<td>Rhodamine-110</td>
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<tr>
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<td>CBZ</td>
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<td>Pro</td>
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<tr>
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<td>Pro</td>
<td>Arg</td>
<td>Rhodamine-110</td>
<td>89</td>
</tr>
<tr>
<td>Plasminogen Activator</td>
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<td>Leu</td>
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<td>Arg</td>
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</tr>
<tr>
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<td>CBZ</td>
<td>Pro</td>
<td>Arg</td>
<td>Rhodamine-110</td>
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<td>Pro</td>
<td>Arg</td>
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<td>38</td>
</tr>
<tr>
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<tr>
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<td>CBZ</td>
<td>Arg</td>
<td>Rhodamine-110</td>
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Fig. 7. Protease activity is highly specific for particular peptide substrates (A) DC protease(s) show high specificity for Pro-Arg peptide substrates. For each substrate, the change in fluorescence versus time (ΔF/s) is given, which is proportional to the amount of substrate hydrolyzed over time; (B) Casein shows higher level of proline content than Ova and BSA, consistent with protein specificity in Fig. 6.
In comparison to other BODIPY-FL labeled protein substrates (Ovalbumin, BSA), casein showed the highest propensity to be cleaved (Fig. 6). Casein is composed of three components, alpha, beta, and kappa caseins, which are 75%, 22%, and 3% of the total protein, respectively. To understand the proteolytic substrate specificity more clearly, a set of mono-, di-, and tri-peptide Rhodamine peptide analogs (100 μM) were tested in DC supernatant (1 ml) containing the protease and the fluorescence signal monitored over time (Fig. 7A). These substrates were test specificity in the P3, P2, and P1 substrate positions and contain rhodamine-110 at the P-I position. Upon hydrolysis between the P1 and P-I residues, rhodamine-110 is liberated and forms a highly fluorescent product. The DC protease(s) show(s) a remarkable specificity for Pro-Arg substrates (P2, P1) (Fig. 7A); although whether the protease(s) requires Arg (at P1) is unclear since all of the substrates that contain a Pro (at P2) also contain Arg (at P1). The protease(s) also prefers small residues at the P3 position, as CBZ-Ile-Pro-Arg (38 ΔF/s) shows a much lower reactivity compared to CBZ-Gly-Pro-Arg (199 ΔF/s) and CBZ-Val-Pro-Arg (109 ΔF/s). The observed substrate specificity is remarkably similar to granzyme A (Velotti, 1989; Krahenbuhl, 1988) and thrombin (Leytus, 1983b), which also show a preference for Pro-Arg sequences (Fig. 6A). Upon careful inspection of the protein substrate sequences (casein, ovalbumin, and BSA) (Fig. 7B), we observed that all the casein proteins contain a high level of proline (α, 8.5%; β, 17%, κ, 11%) compared to ovalbumin and BSA, which only contain ~4% proline. This fact is consistent with the Pro substrate preference shown above and the higher cleavage activity for casein over the other proteins.
DISCUSSION

Dendritic cells (DC) are the most potent of professional antigen presenting cells, with unique capacities to activate naive T cells and to overcome T cell non-responsiveness in vivo (Cella, 1997a; Banchereau, 1998; Mellman, 1998; Steinman, 1995). Immature DC are found in peripheral tissues where they very efficiently capture and process antigens. Upon internalization of antigen they migrate to secondary lymphoid organs and present antigen to T cells. The migration completes the DC differentiation process, and mature DC have decreased endocytic activity and increased expression of class II MHC and co-stimulatory molecules (Winzler, 1997; Pierre, 1997; Cella, 1997b). Because of the importance of DC in the de novo immune response to naive antigens and the potential of DC to serve as antigen carriers in vaccines, the mechanisms of antigen processing and presentation in DC are of considerable interest. DC are able to take up antigen through fluid-phase endocytosis (Cella, 1997a; Banchereau, 1998; Mellman, 1998; Steinman, 1995), a pathway that is prominent in macrophages but inefficient in B cells (Watts, 1997). DC also utilize receptor-mediated uptake (Cella, 1997a; Banchereau, 1998; Mellman, 1998; Steinman, 1995) for internalization of antigens, a pathway active in both B cells and macrophages (Watts, 1997). DC endocytic receptors include DEC-205 (Jiang, 1995) and mannose receptor (Sallusto, 1995) for glycosylated proteins, and immunoglobulin Fc receptors for antibody/antigen complexes (Sallusto, 1994). Both fluid-phase and receptor-mediated endocytic pathways rely on intracellular processing and MHC loading subsequent to internalization (Watts, 1997).

The extracellular proteolytic activity, together with extracellular empty cell surface class II MHC molecules that can bind peptide antigens, suggests a novel pathway for extracellular antigen processing and presentation that may function in DC in addition to well-characterized endocytic pathways. The developmental regulation of expression of the empty molecules is consistent with the role of immature DC in efficiently collecting antigen for storage and
subsequent presentation to T cells. The extracellular recognition and presentation of peptide antigens that we have observed here may be important to the sentinel function of dendritic cells, enabling them rapidly to initiate a primary immune response. Furthermore, this alternative pathway can generate and preserve antigenic peptides, which might otherwise be terminally degraded in the extremely proteolytic endosomal/lysosomal environment.

FUTURE WORK

In the future, we plan to collect large quantities of DC supernatant (~1 liter) and attempt to purify the DC protease(s) to near homogeneity. One purification strategy will be to prepare an affinity matrix (e.g. NHS Sepharose) with Gly-Pro-Arg covalently attached via the N-terminus by a long flexible 6-amino-hexanoic acid linker. Since we know that the protease binds and cleaves this substrate, the protease should bind to the prepared matrix and then elute off with a high concentration of free peptide [alternative elution buffers will include acid (glycine, pH 2.2), base (CAPS, pH 11.5), or 50% ethylene glycol]. Once purified, we will try to get initial N-terminal sequence and mass spectral data in the hopes of finding a match in the protein database. If unsuccessful, we will conduct a more thorough sequencing effort using tryptic digests and MS/MS (or Edman sequencing) techniques. To understand more clearly the substrate specificity, fragments of casein (and other BODIPY-labeled protein substrates) produced in an overnight incubation with DC supernatant will be purified and characterized using standard N-terminal sequencing (Edman sequencing) and MALDI-MS. Since we know each protein sequence, the fragment data should tell us what sites are cleaved within the protein and thus give us an idea of the overall substrate specificity. In a more directed approach, other Rhodamine peptide substrates will be synthesized (Leytus, 1983a, 1983b) to test the P1 specificity of the protease.
**V. KL-295 and KL-304: Monoclonal Antibodies that Specifically Bind Empty Class II MHC Proteins**

**B. A Conformational Change in HLA-DR1 is Dependent on Both Main Chain Interactions and P1 Pocket Occupancy**

*Introduction*

Major histocompatibility complex (MHC) proteins are heterodimeric cell surface proteins that serve as restricting elements for the cell-mediated immune system. Class II MHC proteins bind peptides produced by endosomal proteolysis and present them at the cell surface for recognition by CD4+ T-cell receptors (Germain 1994; Watts 1997). Each of the hundreds of class II allelic variants can bind a wide variety of peptides with relatively high affinity ($K_d \approx 10^{-4}$ to $10^{-6}$ M). Crystal structures determined for many different class II MHC proteins in complex with various defined peptides (Brown and al 1993; Stern, Brown et al. 1994; Ghosh, Amaya et al. 1995; Fremont, Hendrickson et al. 1996; Dessen, Lawrence et al. 1997; Murthy and Stern 1997; Fremont, Monnaie et al. 1998; Scott, Peterson et al. 1998), along with biochemical characterization of the peptide binding preferences of class II MHC proteins (Sinigaglia and Hammer 1994), have led to a good understanding of the mode of binding of high-affinity peptides. Peptides bind in an extended polyproline type II-like conformation, placing several side chains into pockets within the site that determine the peptide sequence specificity. In HLA-DR1, a common class II MHC allele, these pockets accommodate the peptide side chains at positions 1, 4, 6, and 9 (Stern, Brown et al. 1994). Many additional interactions between the peptide main chain and conserved MHC residues provide a peptide sequence-independent component to the interaction (see MHC Introduction, chapter I).

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4 This work was done in collaboration with Jennifer Zarutskie and will be published in the near future. The introduction to this chapter was taken from Zarutskie et al. (1999).
The conformations of class II MHC proteins with their bound peptides are essentially identical in the various crystal structures. However, some studies suggest that alternate conformations of the class II MHC molecule do exist under some conditions. A “floppy” species with reduced mobility in non-denaturing SDS-PAGE has been observed in vitro as an intermediate in the thermal denaturation and folding pathways for some murine class II MHC proteins (Dornmair, Rothenhausler et al. 1989; Dornmair and McConnell 1990), and also as the predominant form at low pH (Sadegh-Nasseri and Germain 1991). The “floppy” species has been observed in vivo for some class II MHC proteins produced in cells from mice lacking the class II-associated invariant chain chaperone protein (Bikoff, Huang et al. 1993; Viville, Neefjes et al. 1993). In these cells, the intracellular trafficking pathway is altered, and the class II MHC proteins do not sort into the endocytic compartments where they usually acquire peptide. Similar effects were observed for recombinant cell-surface class II MHC proteins expressed heterologously in cells that lack invariant chain (Peterson and Miller 1990; Wettstein, Boniface et al. 1991). The altered electrophoretic mobility of the “floppy” form observed in vitro or in vivo may reflect the absence of bound peptides, or the presence of a particular set of weakly-binding peptides that has been enriched by the experimental conditions.

Kinetic studies of the peptide binding reaction also have indicated alternate conformational states of class II MHC proteins. The class II peptide binding reaction exhibits rather complicated kinetic behavior with a very slow apparent forward rate constant, which has led several groups to propose a multi-step peptide binding pathway in which an initial MHC-peptide complex undergoes a unimolecular change to generate the stable complex (Sadegh-Nasseri and McConnell 1989; Sadegh-Nasseri, Joshi and Stern 1999). Although these kinetic studies cannot address directly the nature or magnitude of a conformational change, the unimolecular kinetic
step implies different conformations for the initial and final MHC-peptide complexes. For many class II MHC proteins, low pH favors peptide dissociation (Jensen 1990). Mildly acidic pH (4.5 to 6.5) decreases the SDS-stability of the protein and increases its ability to bind the fluorescent probe ANS (8-amino-1-napthalene-sulfonic acid) (Boniface, Lyons et al. 1996; Runnels, Moore et al. 1996), providing evidence for a pH-induced structural change in class II MHC proteins. Recently, direct physical comparison of empty and peptide-loaded MHC has been reported for I-E\(^k\) (Reich, Altman et al. 1997), where small changes in circular dichroism spectra observed at mildly acidic pH were interpreted as a manifestation of a peptide-dependent conformational change. In that study, the peptide-bound form exhibited a steeper thermal denaturation profile than the empty protein, suggestive of a looser or extended conformation in the empty protein, and indicating a possible similarity to the "floppy" species described above. Similar differences in CD spectra and thermal denaturation profiles have been observed for empty and peptide-loaded class I proteins (Gorga, Dong et al. 1989; Fahnestock, Tamir et al. 1992; Bouvier and Wiley 1994; Bouvier and Wiley 1998).

Taken together, these studies suggest that alternate conformations of class II MHC protein can exist when peptide binding is absent or destabilized. A direct measurement of the extent of such conformational alteration has not been reported until recently by Zarutskie et al (1999). Many previous studies which attempt to address this issue have been complicated by the presence of physical and chemical heterogeneity in the MHC protein, and by variable occupancy of the peptide binding site with endogenous and/or uncharacterized peptides. In addition, empty class II MHC proteins produced in recombinant systems have exhibited a pronounced tendency to aggregate (Stern and Wiley 1992), and the presence of these aggregates complicates physical analysis of the protein. In this study, we used homogenous, monomeric, soluble empty and
peptide-loaded HLA-DR1 complexes produced by expression in *E. coli* and folding *in vitro* (Frayser, 1999).

We have recently shown that human MHC Class II protein HLA-DR1 undergoes a conformational change when the empty form of the protein binds a peptide (Zarutskie 1999) by comparative biophysical and antibody binding analysis of the empty and peptide-loaded forms. The conversion involved a decrease of the hydrodynamic radius from ~35 to ~28 Å, an alteration in secondary structure, and an increase in the cooperativity of thermal denaturation. Together these results suggested a condensation or folding of the empty protein around the peptide. The work presented here addresses what minimal peptide-MHC interactions are needed to trigger the conformational change using peptides of various lengths and substitutions, and a DR1 mutant (G86Y). We find that the peptide length and sequence requirements for inducing the conformational change are distinct from those for tight binding, and in particular that occupancy of the P1 pocket is crucial for transition to a compact form.

**Materials and Methods**

*Empty HLA-DR1*

The extracellular portion of HLA-DR1 was produced by expression of isolated subunits in *E. coli* inclusion bodies followed by refolding *in vitro* as described (Frayser, 1999). The modified DR1G86Y β expression plasmid was constructed by ligating an AflII/BspEI DR1 gene fragment, which was derived from a longer baculovirus construct of HLA-DR1 β containing the G86Y mutation (Natarajan, 1999), into the previously described DR1 β expression plasmid (Frayser, 1999), which was cut with the same enzymes. The final DR1G86Y construct was the
same length as the previous DR1 β construct, under the T7 promoter, and contained a tyrosine, instead of glycine, at the β86 position.

As previously described by Zarutskie et al (1999), refolded empty HLA-DR1 and DR1G86Y proteins were recovered by immunoaffinity chromatography using a conformation-specific monoclonal antibody (LB3.1), and were transferred into PBS (136 mM NaPi, 3 mM KPi, 10 mM NaCl, 2 mM KCl, 0.02% sodium azide, pH 7.2) containing 10% glycerol for storage at 4°C. Protein concentration was measured by UV absorbance at 280 nm using ε₂₈₀ of 54375 M⁻¹ cm⁻¹ for empty DR1 and 55655 M⁻¹ cm⁻¹ for empty DR1G86Y.

**Peptide Synthesis and Production of Peptide-DR Complexes**

Peptides were synthesized using solid-phase Fmoc chemistry, deprotected, and purified by reverse phase chromatography using standard methods. If needed for subsequent binding assays, peptides were biotinylated on the resin prior to cleavage using a LC-LC-biotin succinimide ester derivative (Pierce), where LC corresponds to a 6-aminohexanoic acid linker. The identity of the purified peptides was confirmed by matrix-assisted laser desorption (MALDI) mass spectrometry. Peptide-bound DR1-complexes were prepared by incubating purified empty DR1 (10⁻⁵ to 10⁻⁶ M) with at least 5-fold molar excess peptide for 3 days at 37°C in PBS with 0.02% sodium azide and a mixture of protease inhibitors (Stern, 1992). Alternately, peptide complexes were formed by refolding subunits in the presence of excess peptide and purified by immunoaffinity as described above. Extent of peptide binding was routinely assayed by 10% native PAGE and by 12% SDS-PAGE with samples boiled or not in loading buffer containing 1% SDS before loading. The peptide-DR1 complexes were further purified by gel filtration or ion exchange to remove aggregates and unbound peptide. Peptide-DR1 complex concentrations
were measured by UV absorbance at 280 nm using $\varepsilon_{280}$ of 55655 M$^{-1}$cm$^{-1}$ (HA, YAK, Min4; one Tyr in peptide), or 54375 M$^{-1}$cm$^{-1}$ (HA(Y308A), no aromatic residues in peptide).

DR1$_{G86Y}$+HA(Y308A) complex concentrations were measured by UV absorbance at 280 nm using using $\varepsilon_{280}$ of 55655 M$^{-1}$cm$^{-1}$.

$K_D$ determination

To determine the Kd for each peptide in a direct binding assay, empty DR1 (0.5 nM) was mixed with a titration series of biotinylated peptide [HA or HA(Y308A)] in PBS, 0.02% azide, 0.05% Triton-X100, 0.3% BSA, and protease inhibitors. These samples were incubated at 37°C for 3 days, followed by a sandwich ELISA for bound peptide, which utilizes a complex-specific capture antibody and a Europium-streptavidin detection system. Briefly, the ELISA plate was prepared by adding LB3.1 (anti-DR antibody; 10 ng/µl, PBS, 0.02% azide) to each well of a 96 well polystyrene plate (Dynex Immulon 4). Following incubation at 37°C for 2 hours, the plate was blocked with PBS, 3% BSA and 0.02% azide at room temperature for 2 hours. Prior to loading the samples, the blocked ELISA plate was washed five times with Tris-buffered saline, 0.1% Tween-20 (TBST). After incubating the samples with the plate at 37°C for 30 minutes, the plate was washed again and streptavidin-Eu (1:1000) in Wallac DELFIA™ Assay Buffer was applied to each well. Following incubation at 37°C for 15 minutes, the plate was washed again and developed with 200 µl/well of Wallac DELFIA™ Enhancement Solution at room temperature for 5 minutes. Bound Europium was detected using a Wallac VICTOR™ 1420 Multilabel Counter (1 second count time/well; normal emission aperature).
Relative binding affinities were also determined by a competition assay using biotinylated HA (bHA) peptide (N-terminal caproyl-biotinyl succinimide ester) and a sandwich ELISA using the LB3.1 antibody and streptavidin-europium essentially as described (Jensen, 1988; see also Appendix). Peptides (10^{-10} to 10^{-4} M) were incubated with 0.5 nM bHA and 0.5 nM empty DR1 in PBS with 0.3% BSA and protease inhibitors at 37 °C for 3 days. ELISA was used to detect DR1-bHA complex formed in this reaction, and the IC_{50} values were converted to K_D using the equation, K_D = (IC_{50})/((1+[bHA])/ K_{D,bHA}). The K_D for bHA was 14 nM as determined using the direct binding assay described above, which is similar to a previously reported value for {^{125}}I-HA (Roche, 1990).

**Gel filtration**

A Superdex 200 HR gel filtration column (Pharmacia), run at 0.3 mL/min in PBS, pH 6.8, was calibrated using thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (BioRad). The molecular weights of the empty and peptide-loaded DR1 were estimated from their respective elution volumes by reference to a calibration plot (Siegel, 1966). Confidence intervals (±σ) reported in Table I reflect the standard deviation from the mean elution volume for replicate samples and uncertainty in the non-linear least squares fit to the calibration plot.

**Dynamic light scattering (DLS)**

Samples for DLS were prepared by exchange into PBS and concentration by centrifugal ultrafiltration to 1 mg/mL, and finally dilution to 0.5 and 0.3 mg/mL. Samples in the cuvette were spun at 5,000 RPM for 60 minutes immediately prior to the measurements to remove dust.
Dynamic light scattering measurements were made at 25 °C with an argon ion laser (Coherent Innova 90, 25 W, λ= 488 nm) at a scattering angle of θ=90°. Ten measurements were performed for each sample. The signal was processed by a Langley Fold model 1096 correlator, and data analysis was performed using a regularization procedure (Braginskaya, 1983), modified for the analysis of the goniodyne autocorrelation function (Thomas, 1996). The autocorrelation function was utilized to reconstruct the distribution of the scattering particles over diffusion coefficients from fluctuations in the scattering intensity of the protein solution. The values of the diffusion coefficients were corrected to 20 °C. Confidence limits for these measurements reflect the standard deviation from the mean of the replicate measurements and uncertainty in temperature. The distribution of diffusion coefficients was converted into a distribution over the particles hydrated radius $R$, using the Stokes-Einstein equation (Cantor, 1980) as described previously (Zarutskie, 1999).

**Circular dichroism spectroscopy and thermal stability**

For circular dichroism (CD) analysis, purified empty DR1 and DR1-peptide complexes (0.2 mg/mL to 0.6 mg/mL) were exchanged by dialysis into 10 mM phosphate buffer, pH 7.0, and filtered with a 0.45 μm filter. CD measurements were made at 4 °C in a 1 mm path length cell as described (Frayser, 1999). Thermal denaturations were performed as described (Frayser, 1999), monitoring the change with temperature of the CD signal at 204 nm, which is near a negative peak in the native minus denatured difference spectrum. Thermal denaturation data were fit to a seven-parameter function that describes a two-state transition (Becktel and Schellman 1987):
\[
\theta = (\theta_u + \mu_T) + \frac{(\theta_f - \theta_u) + T(m_f - \mu)}{1 + \exp \left( \frac{\Delta H}{RT} + \frac{\Delta C_p}{R} \left( \frac{Tm}{T} - 1 + \ln \frac{T}{Tm} \right) \right)}
\]

where \(\theta_u\) and \(\mu\) describe the slope and y-intercept of the unfolded state baseline, \(\theta_f\) and \(m_f\) describe the slope and y-intercept of the folded state baseline, \(Tm\) is the midpoint of the transition (where \(\Delta G = 0\)), \(\Delta C_p\) is the heat capacity change upon unfolding (assumed to be independent of temperature), and \(\Delta H\) is the enthalpy of unfolding at the \(Tm\). All \(Tm\) values were verified by first derivative analysis.

**KL-295 ELISA**

Antibody binding specificity was measured using a sandwich ELISA as described previously (Zarutskie, 1999). Monoclonal antibody KL-295 (Lapan, 1992) was used at 10\(\mu\)g/mL to coat a 96-well polystyrene microtiter plate by incubation overnight at 4\(^\circ\)C. The plate was blocked with 3% BSA in PBS plus 0.02% sodium azide and washed with PBS plus 0.05% Triton X-100 (PBST). Quadruplicate 2-fold dilutions of DRI from 1 to 300 nM in PBST were added, allowed to bind to the plate for 30 minutes at 37\(^\circ\)C, and washed 5 times with PBST. For Min4 samples, 100 \(\mu\)M free peptide was added to the incubations to prevent dissociation. The amount of bound DRI was detected by sequential incubations with rabbit-anti-DR polyclonal antibody, goat-anti-rabbit peroxidase conjugate, and ABTS (405 nm) as described (Stern, 1992).
Results

*Peptide specificity in triggering conversion to the compact conformation*

To investigate roles played by different regions of the peptide ligand in triggering the HLA-DR1 conformational change, we designed several analogues of the tight-binding peptide HA (Table 1) derived from influenza haemagglutinin (Roche, 1990; Stern, 1994a). Peptides HA(Y308A) and YAK were designed to investigate the role of interactions between peptide side chains and MHC side chain-binding pockets. They are full length analogues (13 residues) with alanine substitutions for the tyrosine at P1 (Y308A), or with alanine substitutions at all of the positions except tyrosine at P1 and the lysine at P8 lysine which was retained for solubility (YAK). These peptides have been used in an earlier study demonstrating the key importance of interactions in P1 site to the overall binding affinity (Jardetsky, 1990). Peptide Min4 is a shorter peptide designed to investigate the role of interactions between peptide main chain atoms and MHC residues and retains interactions in the P1-P4 region only with optimal side chains at every position (Hammer, 1992, 1994). Min4 is the shortest peptide known to bind to class II MHC proteins (Zarutskie, 1999). These peptides were complexed with empty HLA-DR1 refolded from subunits separately expressed in *E. coli*. For all low affinity peptides, DR1 was refolded in the absence of peptide, purified by affinity chromatography, and then incubated with peptide. After 3 days incubation, peptide-DR1 complexes were purified by gel filtration chromatography. For high affinity peptides, peptide was added during the initial refolding and then peptide-DR1 complexes were purified by immunoaffinity and gel filtration chromatographies.
Several of these peptides bind very weakly, in general agreement with the effects of substitution at various positions observed previously (Rammensee, 1997). HA(Y308A) (Jardetsky, 1990) and Min4 (Zarutskie, 1999), for example, have apparent Kd’s greater than 1 μM in competition with high affinity binding peptides [HA(306-318) or Influenza Matrix(17-31)] for DR1 binding. Many of these more weakly bound DR1 complexes [Min4, HA(Y308A)] also dissociated into free subunits in the presence of 1% SDS on SDS-PAGE (Fig. 1). Even though we get nearly quantitative peptide occupancy in the presence of excess peptide, this assay is not useful for peptide complexes where the peptide off-rates are faster than the time course of the experiment. In such a situation, one cannot distinguish whether the complex is truly SDS-sensitive or just lost its peptide during the course of running the gel. For this reason, the data derived from these peptide complexes is not useful.

On other hand, high affinity peptides do not dissociate from the class II molecule during the course of the assay and therefore, data derived from such experiments show us something relevant about a complex’s structure. As seen previously, both DR1+HA and DR1+YAK are completely SDS-stable (Zarutskie 1999), indicating that these complexes can exclude SDS and remain intact within the gel (Fig. 1). Upon binding DR1, all of the peptides (Table I) were able
to trigger the conversion from the larger empty form (~35 Å) to the smaller, compact form (Rh ~ 28-30 Å), as measured by either gel filtration (Fig. 2, Table II) and/or dynamic light scattering (Table II). Complexes of each of the peptide analogues exhibited decreased apparent molecular weight and decreased width of the elution profile relative to the empty protein (Fig. 2). In each case, the changes were very similar to those induced by the unmodified HA peptide. For most of the complexes, the decrease in hydrodynamic radius upon peptide binding was confirmed using dynamic light scattering (Table II). Thus, each of the peptide analogues were able to trigger HLA-DR1 conversion to the compact conformation. Furthermore, it also appears that the minimal peptide elements are localized to the P1-P4 area of the binding groove since Min4 binding was able to trigger the hydrodynamic conversion.

Fig. 1. SDS-PAGE analysis of DR1 and DR1<sub>G86Y</sub> peptide complexes. All of these samples were run on 12.5% SDS-PAGE under reducing conditions. Boiling (+/-) corresponds to whether (+) or not (-) the sample was boiled for 2 minutes before loading.
Table II Hydrodynamic Properties: ND, not determined; agg’d, too aggregated to conduct analysis. Note: The gel filtration data is given in apparent molecular weight, based upon a standard curve on known protein standards. Since all of the complexes are approximately the same mass, a decrease in Mwapp indicates a decrease in hydrodynamic radius. *Taken from Zarutskie et al. (1999).

<table>
<thead>
<tr>
<th>Complex</th>
<th>GF, Mwapp</th>
<th>DLS R&lt;sub&gt;H&lt;/sub&gt;, nm; 25° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1-empty</td>
<td>56.4</td>
<td>3.50 ± 0.05</td>
</tr>
<tr>
<td>DR1+HA</td>
<td>36.3</td>
<td>2.82 ± 0.12</td>
</tr>
<tr>
<td>DR1+YAK</td>
<td>~36</td>
<td>2.77 ± 0.11</td>
</tr>
<tr>
<td>DR1+Min4</td>
<td>38.4</td>
<td>3.04 ± 0.2*</td>
</tr>
<tr>
<td>DR1+HA(Y308A)</td>
<td>38.4</td>
<td>2.86 ± 0.18</td>
</tr>
<tr>
<td>DR&lt;sub&gt;G86Y&lt;/sub&gt;-empty</td>
<td>40.6</td>
<td>N.D., agg’d</td>
</tr>
<tr>
<td>DR&lt;sub&gt;G86Y&lt;/sub&gt;+HA(Y308A)</td>
<td>38.4</td>
<td>2.95 ± 0.14</td>
</tr>
</tbody>
</table>

Fig. 2. Gel filtration of DR1 and DR<sub>G86Y</sub>, including their peptide complexes.
Previously, the hydrodynamic radius change was linked to changes in accessibility of the KL-295 epitope (localized at β58-69 region, see previous chapter introduction) and to an increase in the thermal cooperativity (Zarutskie, 1999). None of the peptide-DR1 complexes were recognized by the antibody KL-295, suggesting that the epitope is inaccessible in each case (Fig. 3). As followed by CD thermal denaturation at 204 nm, all of the peptide complexes exhibited a steep denaturation profile compared to empty DR1, indicative of cooperative denaturation (Fig. 4, Table III). This is evident from the dramatic increases in both ΔH and ΔCp values upon binding any of the tested peptides (Table III). From this data, therefore, it appears that peptide binding initiated folding and/or movement in DR1 (at least within the β58-69 region), which increased intraprotein interactions within the monomer and caused an overall increase in thermal cooperativity.

Fig. 3. Reactivity with the conformation specific antibody KL-295. KL-295 Reactivity of Various Empty, Peptide Loaded, and Mutant HLA-DR1 Complexes. Sandwich ELISA using immobilized KL-295 to detect binding to soluble empty HLA-DR1 (empty circles) and HLA-DR1G86Y (empty diamonds) expressed in E. coli and to various peptide complexes [DR1+MIN4, hatched squares; DR1+HA, solid squares; DR1+HA(Y308A), diagonal squares; DR1G86Y+HA(Y308A)]. Soluble HLA-DR1 showed no binding to heat-denatured KL-295, non-specific antibody, or BSA (data not shown). DR1+YAK also showed no reactivity in this assay (data not shown).
Fig. 4. Baseline Adjusted Thermal Stability Profiles. Within each data set, the $\theta$ value at each temperature point was adjusted by subtracting the $\theta$ value (mdeg dM$^{-1}$ cm$^{-1}$) at 100°C within each data set. This baseline adjusted data allows one to clearly see the increasing cooperativity between empty DR1 and DR1+HA.

The mutant DR1$^{G86Y}$ has a compact conformation and binds a mutant P1 Ala peptide.

The results with Min4 suggested that the trigger for the hydrodynamic collapse might be localized to a small region of the peptide-binding groove. The P1 pocket is known from binding and structural work to be a likely site of important interactions (Jardetsky, 1990; Brown, 1993; Stern, 1994a). P1 is the largest and most hydrophobic of the HLA-DR1 peptide side-chain.

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pockets (Stern, 1994a), and the site of αβ subunit contacts and upper-lower domain contacts (Murthy, 1997). Most of the residues lining this site are highly conserved, but β85 and β86 are polymorphic. Examination of the structure of HLA-DR1 and H-2Kβ suggested that the substitution of HLA-DR1 at β86 with an aromatic group would allow the side chain in a common rotamer to fill the P1 pocket, exposing the terminal OH to solvent. (Natarajan 1999). Tyrosine was chosen to replace HLA-DR1 Glyβ86 to provide a relatively hydrophilic “cover” for the pocket in the form of the exposed ring hydroxyl group (Natarajan, 1999). This protein has been previously investigated in a study of its stability to SDS-induced chain dissociation (Natarajan, 1999).

The protein HLA-DR1G86Y has been previously used for study of the SDS-stability (Natarajan, 1999). For our studies, the protein was expressed in *E. coli* for comparison with the wild-type form. Results of the expression and refolding were similar to the wild-type protein, and the folded protein showed similar complex specific epitopes (LB3.1, L243) and binds peptides predicted to bind the mutant complex. HA(Y308A), for example, is the HA(306-318) peptide with the P1 tyrosine residue mutated to alanine. This peptide contains a small residue in the P1 position and was able to bind to DR1G86Y (Fig. 6), while peptides with large aromatic residues were not (Natarajan 1999), suggesting that the substitution had the desired effect. Binding constants for these peptides were low (Fig. 6), confirming observations in the wild-type protein that P1 interactions are contribute greatly to the high affinity of peptides, such as HA(306-318).

HLA-DR1G86Y exhibited the compact conformation even in its peptide-free form, as judged by gel filtration (Fig. 2, Table II). KL295 also was unable to react with the mutated protein (Fig. 3). The mutant protein also showed increased cooperativity in thermal denaturation.
experiments (Fig. 4; Table III). These results suggest that the mutant protein in its empty form has attained the compact conformation usually observed for peptide complexes with the wild-type protein.

HA(Y308A) peptide binding to DR1G86Y does not further decrease the hydrodynamic radius (Fig. 2, Table II), change the lack KL-295 reactivity (Fig. 3), or increase the degree of complex cooperativity (Fig. 4), as compared to the empty mutant form. HA(Y308A) binding to DR1G86Y, however, does increase the Tm for this complex relative to empty form, suggesting that it has other stabilizing contacts have been formed upon peptide binding (Fig. 4; Table III). As predicted, the DR1G86Y+HA(Y308A) thermodynamic parameters and Tm values are similar to those of DR1+HA (Table III), suggesting that high affinity binding (Fig. 5) is not crucial to forming a high Tm complex.

Table III  Thermal Stability

Using the raw data used in Fig. 4, the ΔH (cal/mol) and ΔCp (cal/mol*K) values were determined using a seven-parameter equation and Tm values were derived by taking the first derivative of each melt profile (see Materials and Methods). All data was rounded to two significant figures.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Peptide</th>
<th>ΔH</th>
<th>ΔCp</th>
<th>Tm</th>
</tr>
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<tbody>
<tr>
<td>DR1</td>
<td>None</td>
<td>49000</td>
<td>160</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>+HA(306-318)</td>
<td>100000</td>
<td>3800</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>+MIN4</td>
<td>96000</td>
<td>2300</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>+HA(Y308A)</td>
<td>150000</td>
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<tr>
<td>DR1G86Y</td>
<td>None</td>
<td>96000</td>
<td>3500</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>+HA(Y308A)</td>
<td>130000</td>
<td>3700</td>
<td>84</td>
</tr>
</tbody>
</table>
Both P1 occupancy and main-chain interactions are required to attain the final conformation

CD spectra show that DR1G86Y is in an intermediate conformation between empty and peptide-loaded wildtype DR1 (Fig. 6A). Thus of the KL295-resistant, compact, cooperatively melting forms, there appear to be at least two different conformers. HA(Y308A) bound to wild type DR1 also is in the intermediate conformation, like empty DR1G86Y (Fig. 6C). In light of their similar thermal melts, it is not surprising that HA(Y308A) bound to DR1G86Y is in the final conformation, like HA bound to DR1 (Fig. 6B). Since neither empty DR1G86Y and
DR1+HA(Y308A) are in the final peptide-loaded wildtype DR1 conformer, both P1 and main-chain interactions appear to be required for the complete conformational change.

Fig. 6. CD scans of experimental DR1 and DR1\textsubscript{G86Y} complexes. (A) Overlay of DR1, DR1\textsubscript{G86Y}, DR1+HA, and DR1+Min4; (B) DR1+HA and DR1\textsubscript{G86Y}+HA(Y308A) have similar CD profiles and are considered the final, compact form of DR1 [Overlay of DR1, DR1\textsubscript{G86Y}, DR1+HA, and DR1G86Y+HA(Y308A)]; (C) DR1+HA(Y308A) and DR1\textsubscript{G86Y} have similar CD scans and may be an intermediate conformer on the pathway to the compact form [Overlay of DR1, DR1+HA(Y308A), and DR1\textsubscript{G86Y}].

Discussion

Previously, Natarajan et al. (1999) expressed a mutant DR1 protein, DR1\textsubscript{G86Y}, which replaced the \(\beta86\) glycine residue with tyrosine. Their intention to was insert a large hydrophobic residue into the P1 pocket of HLA-DR1 and not perturb the structure too much from the wildtype
form. They chose the β86 position because several murine class II proteins have large hydrophobic residues at this position, e.g. IE^k has a Phe, and modeling showed that the glycine to tyrosine mutation did not cause any major movement in the structure. They subsequently went on to show that a non-boiled empty DR1^G86Y sample did not dissociate in a reduced SDS-PAGE gel, whereas empty DR1 completely dissociated under the same conditions. From this work, they hypothesized that SDS stability is a measure of the hydrophobic interaction between the bound peptide side chain and residues within the DR1 P1 pocket. If there are several hydrophobic interactions between the peptide side chain (aliphatic/aromatic) and the P1 pocket, SDS is excluded from the binding site and the subunits remain intact; whereas if the peptide side chain (small residues, e.g. alanine) doesn’t establish such interactions, SDS can infiltrate the P1 pocket and promote chain dissociation.

What their model failed to consider is the possibility that the G86Y mutation may unexpectedly cause an overall conformational change in the protein structure. If so, the structure may change such that hydrophobic residues away from the P1 pocket may no longer exposed to solvent; thus creating a more SDS-stable molecule. Since it is difficult to predict large conformational changes in protein structure, their modeling of DR1^G86Y may have only locally minimized the P1 pocket upon insertion of the tyrosine residue into the β86 position.

While the above DR1^G86Y work was being completed, Zarutskie et al. (1999) also showed that DR1 undergoes a conformational change upon peptide binding. To understand the mechanism of this conformational change, we extended the work of both projects and tested wild-type DR1 and DR1^G86Y mutant with several designed peptides using the same methods previously used by Zarutskie et al. (1999). Although the mutant does not bring about the full conformational change, we did find that many of its biophysical and immunological properties
are different from the wildtype form, suggesting that the mutation did cause a partial structural rearrangement in the protein. We also determined that the minimal requirements for the conformational HLA-DR1 change includes not only filling the P1 pocket, but also establishing peptide main chain and/or other pocket interactions within the P1-P4 region of the peptide binding groove.

These minimal elements in the conformational change may play a role in how these molecules obtain their peptides within a cell. HLA-DM is a non-polymorphic class II MHC homologue that promotes the removal of CLIP, a fragment of the invariant chain, and promotes binding of antigenic peptides within the endosomes (Avva, 1994; Roche, 1995; Sanderson, 1995; Weber, 1996; Kropshofer, 1997; Denzin, 1995). Not only does DM remove CLIP, it also edits out other endogenous peptides. Although the specific criteria for peptide removal is not well understood, Gly and Pro seem to play a destabilizing role in the DM-mediated dissociation kinetics of DR1 ligands (Raddrizzani, 1999). DM also protects empty molecules from aggregation at acidic pH and elevated temperatures. Since the fine structural details of the DM-DR interaction have not been investigated, the mechanism whereby DM elicits these activities is still a matter of debate. One possible mechanism is that DM may stabilize intermediate(s) along the pathway to stable peptide binding (Rabinowitz, 1998). This intermediate may be one of the observed intermediate conformational states, e.g. DR1+HA(Y308A) and DR1G86Y. For some peptide complexes, i.e. CLIP and some endogenous peptides, DM may promote peptide release by stabilizing this intermediate state and skewing the equilibrium towards the empty form. On the other hand, DM may also enhance the binding of other antigenic peptides by protecting this empty form in an intermediate conformational state, which may more receptive and active towards binding high affinity peptides than the empty form, i.e. without DM. If we can show
that DM promotes such a conformational change in empty DR1, then this hypothesis may indeed be at work. Future efforts will be aimed at understanding this possible mechanism.
VI. The Cytochrome P-450\textsubscript{SCC} Glycol Cleavage Reaction: Analysis of $^{18}$O and $^2$H Incorporation into Isocaproaldehyde and Evaluation of Potential Mechanisms

INTRODUCTION

Cytochromes P-450 enzymes are heme mono-oxygenases that catalyze a variety of oxidations of endogenous and exogenous compounds in bacterial and eukaryotic systems. Cytochrome P-450\textsubscript{SCC} is a mammalian form that catalyzes the conversion of cholesterol to pregnenolone in the steroid hormone biosynthetic pathway. Cholesterol is first hydroxylated at the C-22 pro-R position in a stereospecific manner to form (22R)-22-hydroxycholesterol (Fig. 1). This intermediate is then hydroxylated again at the C-20 pro-S of cholesterol to form (20R,22R)-20,22-dihydroxycholesterol. Burnstein et al. (1975) showed that these hydroxyl groups are introduced from two different molecules of oxygen. Finally, the glycol intermediate is cleaved between the hydroxyl carbons to form isocaproaldehyde and pregnenolone. The C-20 oxygen is not disturbed during this step and becomes the ketone oxygen of pregnenolone (Takemoto, 1968).

Fig. 1. P-450\textsubscript{SCC} mediated conversion of cholesterol to pregnenolone and isocaproaldehyde.
It has been firmly proposed that each of the hydroxylations occurs by a general P-450 radical rebound mechanism, whereby a hydrogen is first abstracted stereospecifically by a ferryl-oxygen heme species and then the iron bound hydroxyl adds back to the side chain radical without radical isomerization (Groves et al., 1978).

The mechanism of the glycol cleavage, however, is less well understood and has been a matter of some import. Two mechanisms have been proposed that are both within the bounds of published evidence, one based on a heterolytic cleavage of the glycol bond and another on radical chemistry (Fig. 2a). Neither have a cyclic intermediate nor do they involve hydroxylation a second time at C-22, the latter seemingly excluded by the finding that 22-tritio-glycol yields isocaproaldehyde retaining tritium at C-1 (Byon et. al., 1980). The heterolytic pathway does not incorporate any new oxygen atoms into the final products. The radical pathway, however, does potentially allow for incorporation of another oxygen atom after the formation of a carbon centered radical on either C-20 or C-22 via carbon-carbon bond scission. This radical can be quenched to give the observed products by one of five routes, which were recently reviewed by Vaz et al. (1994) for microsomal P-450 mediated alcohol oxidations (Fig. 2b). Routes C through F retain the original oxygens introduced in the formation of the (20R,22R)-20,22-dihydroxycholesterol. Route G, however, involves another rebound from the iron bound hydroxyl to generate a gem-diol, which once formed collapses to produce either isocaproaldehyde or pregnenolone, and water.
Fig. 2. (a) Two proposed mechanisms for cleavage of the diol substrate [(20R,22R)-20,22-dihydroxycholesterol] to form pregnenolone and isocaproaldehyde, one based on a heterolytic cleavage of the glycol bond (A) and another on radical chemistry (B); (b) This radical can be quenched to give the observed products by one of five routes (C, D, E, F, G).

We investigated these possible mechanisms by incubating (20R,22R)-dihydroxycholesterol in a reconstituted P-450_{scc} system under a 1:4 ¹⁸O₂:Ar environment. An excess of liver alcohol dehydrogenase and NADH was added to trap any ¹⁸O-labeled isocaproaldehyde in-situ as 4-Methyl-pentanol. In bulk media, a gem-diol (route G) with one ¹⁸O-labeled hydroxyl would
yield 50% labeled product. On the other hand, the same labeled gem-diol within the SCC active site could be encouraged to preferentially lose one hydroxyl over the other by a base-catalyzed stereospecific mechanism (Fig. 3). In such a case, the amount of $^{18}$O-incorporation could range anywhere from zero to full, depending on which hydroxyl is catalyzed to leave, the efficiency of that process, and the rate of gem-diol rearrangement in the active site. A positive incorporation result would implicate the radical centered gem-diol mechanism, $B+G$ (pathway $B$ plus route $G$), as the most likely mechanism.

Fig. 3. Stereoselective removal of a hydroxyl from a gem-diol intermediate to form an aldehyde/ketone within an enzyme active site.

The glycol intermediate was also incubated in D$_2$O and H$_2^{18}$O along with a normal air atmosphere and an excess of liver alcohol dehydrogenase and NADH. Reduction of the isocaproaldehyde prevents $\alpha$ proton exchange and gem-diol formation in the 2H and $^{18}$O water experiments, respectively. The D$_2$O experiment was done to test mechanism $B+F$, which allows for the incorporation of solvent hydrogen $\alpha$ to the carbonyl. While the H$_2^{18}$O experiment
was done to substantiate the radical and heterolytic pathways, neither of which incorporate an active site bound water.

MATERIALS AND METHODS

Materials and Suppliers: The proteins P-450SCC, adrenodoxin and NADPH-adrenodoxin oxidoreductase were isolated from bovine adrenal glands purchased at a slaughterhouse. Alcohol dehydrogenase and DEAE-cellulose were purchased from Fluka, Inc. Hydroxyapatite gel was purchased from Biorad, Inc. Glucose 6-phosphate dehydrogenase, Catalase, NADH, NADPH, cholesterol and glucose-6-phosphate were purchased from Sigma, Inc. (20R,22R)-20,22-dihydroxycholesterol was found in our lab steroid library and characterized before usage. A solid phase microextraction (SPME) kit with a polyacrylate fiber and manual fiber holder was purchased from Supelco, Inc. ¹⁸O₂ (95-98%), D₂O, and H₂¹⁸O were purchased from the Cambridge Isotope Laboratories, Inc. Isocaproaldehyde was synthesized from 4-Methylpentanol using PCC, both of which were purchased from Aldrich, Inc. All other chemicals were of the highest purity available.

Purification of Proteins: Cytochrome P-450SCC was purified to greater than 15 nmol P-450/mg protein (maximum approximately 18 nmol/mg) according to Tsubaki et al (1987). Adrenocortical mitochondria depleted of adrenodoxin and NADPH-adrenodoxin oxidoreductase were suspended in 10 mM potassium phosphate (pH 7.8) containing 20% (v/v) glycerol and 0.1 mM EDTA (Buffer A'). Sodium cholate was added to the suspension (diluted to 16 mg/ml) to a concentration of 2:1 (w/w). The solution was stirred for 45 minutes at 4 °C and then centrifuged at 20384xg for 90 minutes. The supernatant was incubated at room temperature for 30 minutes.
and then mixed with DEAE-cellulose, previously equilibrated with Buffer A’, to a concentration of 2 mg protein/ml column material. The slurry was poured into a Pharmacia XK 26 column and packed with Buffer A’. Then the column was washed with 2 column volumes of Buffer A’ at a flowrate of approximately 0.75 ml/min. Finally, the protein was eluted as a sharp band with Buffer A, Buffer A’ with 0.25% (w/v) sodium cholate and 0.1% (v/v) Emulgen 913.

Then the protein was immediately loaded onto a hydroxyapatite column, previously equilibrated with Buffer A, to a concentration of approximately 0.40 mg protein/ml column material. The column was washed successively with 8 column volumes Buffer A (10 mM KPi), 2 column volumes Buffer A (20 mM KPi), and 2 column volumes Buffer A (40 mM KPi) at a flowrate of approximately 0.33 ml/min. Finally, the protein was eluted with Buffer A (80 mM KPi) and collected.

Adrenodoxin was purified from bovine adrenocortical mitochondrial supernatant according to Takemoto et al. (1968) and stored in 0.5 M KCl and 10 mM TRIS buffer (pH 7.4). NADPH-adrenodoxin oxidoreductase was purified according to Takemoto et al. (1968) and stored in 0.5 M KCl and 10 mM Tris buffer (pH 7.4).

**Incubation under Ar:18O2 Atmosphere:** The following reconstituted P-450scc system was prepared in a 25 ml vial at 4 °C and then sealed with a septum and electrical tape to give an airtight seal: 2 μM P-450scc, 20 μM Adrenodoxin, 1 μM NADPH-Adrenodoxin oxidoreductase, 10 U/ml Alcohol Dehydrogenase, 1 U/ml Catalase, 1 U/ml Glucose-6-phosphate Dehydrogenase, 15 mM glucose-6-phosphate, 100 mM KCl, 4 mM MgCl2, 0.3% (v/v) Tween-20 and 200 μM (20R,22R)-20,22-dihydroxycholesterol in a 20 mM KPi buffer (pH 7.2). The steroid was added from a 10 mM ethanol stock solution to give a final ethanol concentration of 2% (v/v). Prior to
mixing, all protein stock solutions were concentrated to less than 0.5 ml using Amicon Centriplus concentrators to prevent destruction of P-450 during turnover. In a separate 8 ml vial, a 200 μM NADH and 100 μM NADPH solution was prepared and sealed in similar manner. The vials were removed to a room temperature environment and placed on a gas-train. Almost all oxygen (16O2) was removed by evacuating and then refilling each vial with argon at least four times over the course of approximately 20 minutes. Then the 25 ml vial was evacuated and back-filled first with 0.2 Atm 18O2 and then 0.8 Atm Ar using a manometer connected to the reaction vial. Finally, the system was activated by the addition of NADH and NADPH from a 1 ml gas-tight syringe to give a total volume of 20 ml. The vial was placed in a beaker of previously equilibrated water on a temperature controlled shaker table (24 °C) and allowed to incubate for approximately 18 hours. The reaction was terminated by the addition of 1 ml of 24% TCA to precipitate out all proteins.

Isolation and Analysis of Products Using GC/MS: Once all proteins had precipitated out, the reaction mixture was centrifuged at 12062xg for one hour. The supernatant was isolated and KCl was added to approximately 10% (w/v). Once dissolved, the solution was placed back into a sealed 25 ml vial for GC/MS analysis. The SPME fiber was conditioned and water blanks run as specified in Supelco manual. Once completed, the fiber was introduced into the high salt supernatant for approximately 30 minutes.

The GC/MS was installed with an HP-FFAP column and the following conditions were programmed prior to analysis: injector temperature, 300 °C; detector temperature, 280 °C; oven temperature program, 50 °C (0-5 minutes) with a 20 °C/min ramp (5-23 minutes) up to 240 °C (23-28 minutes); inlet purge off for the first five minutes. The MS was programmed for scan
mode (m/z = 20-200) with emv set to 2847. The SPME fiber was removed from the aqueous solution and introduced into the injector, where all analytes were desorbed onto the GC column for MS analysis. The 4-Methyl-pentanol peak was isolated on the total ion count time trace and all pertinent MS data was recorded. Baseline MS scans before the product peak were also averaged and subtracted from the 4-Methyl-pentanol MS. The polyacrylate fiber was cleaned between runs by keeping it in the injector (310 °C) for approximately 20 minutes.

**Cholesterol Control:** To determine the limit of $^{18}$O-incorporation into isocapraldehyde with this reconstituted system, cholesterol was used as the substrate under the same turnover conditions as previous. Steroid and ethanol concentrations were the same as for the (20R,22R)-20,22-dihydroxycholesterol experiment.

**4-Methyl-Pentanol Standards:** Two 4-methyl-pentanol standards were prepared as follows: A 200 μM 4-methyl-pentanol standard was prepared in a 20 mM KPi (pH 7.2) buffer with 100 mM KCl, 4 mM MgCl₂, and 0.3% (v/v) Tween-20. As a separate standard, (20R,22R)-20,22-dihydroxycholesterol was incubated in the same reconstituted system described above, except P-450<sub>scc</sub> purity was only approximately 7 nmol P-450/mg protein and normal air was used. The 4-methyl-pentanol was isolated from both the cholesterol control and 4-methyl-pentanol standards using the same SPME procedure. GC/MS analysis was also conducted in the same manner.

**D<sub>2</sub>O and H<sub>2</sub>¹⁸O Incubation Experiments with Controls:** The proteins P-450<sub>scc</sub>, adrenodoxin and NADPH-adrenodoxin oxidoreductase were each concentrated to less than 200 μl prior to mixing incubation components. All stock solutions and 5-times concentrate (5X) KPi reaction
buffer, pH 7.2 (500 mM KCl, 20 mM MgCl₂, 1.5% Tween-20 and 100 mM KPi) were made up in D₂O (99.9%) and H₂¹⁸O (10%). The incubation mixture was prepared in the same manner as above, except normal air was used.

The percentage of ¹⁸O and deuterium incorporation into isocaproaldehyde from bulk solvent was determined by incubating synthesized isocaproaldehyde (200 µM) in each isotopically enriched 1X KPi buffer with an excess of liver alcohol dehydrogenase (10 U/ml) and NADH (200 µM).

RESULTS

Mass Spectral Data: Since 4-Methyl-pentanol does not give a parent M⁺ peak, protonated formaldehyde ion (CH₂=¹⁶OH⁺, m/z 31) was the only detectable ion with the primary alcohol oxygen intact. The most abundant peak in all recorded spectra was m/z 56, a terminal aliphatic side-chain fragment [CH₂C(CH₃)₂+] of the parent compound. Mass spectrum background readings were measured by averaging a one-minute baseline prior to the 4-methyl-pentanol peak on a total ion count trace. These abundance readings were subtracted from all experimental abundances prior to determining percent m/z abundances. The percent abundance readings for each particular m/z value were recorded as percentages of the m/z 56 abundance to normalize against a region of the molecule that most likely does not undergo conversion during the enzymatic reaction.

In the ¹⁸O₂ and H₂¹⁸O experiments, the % ¹⁸O-incorporation into isocaproaldehyde from glycol turnover only was determined using Equation 1. Any positive incorporation was multiplied by 10 to account for using only 10% H₂¹⁸O. The % ²H incorporation was determined in a similar manner, except the [M(¹H)-H₂O]⁺ (m/z 84) and [M(²H)-H₂O]⁺ (m/z 85) ions were
used (Equation 2) to determine the extent of deuterium incorporation. Incorporation from bulk solvent was minimal in both H$_2^{18}$O and D$_2$O as determined in the control reduction of isocaproaldehyde with NADH and liver alcohol dehydrogenase (results not shown).

\[
\%^{18}\text{O-INC} = \left[\frac{\%^{33}\text{exp} - \%^{33}\text{std}}{\%^{31}\text{std}}\right] (\text{Equation 1})
\]

\[
\%^{2}\text{H-INC} = \left[\frac{\%^{85}\text{exp} - \%^{85}\text{std}}{\%^{84}\text{std}}\right] (\text{Equation 2})
\]

**Table I (Experimental Data)**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Atm.</th>
<th>Solvent</th>
<th>%31</th>
<th>%33</th>
<th>%84</th>
<th>%85</th>
<th>%$^{18}$O-INC</th>
<th>%$^{2}$H-INC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%) $\text{O}_2$</td>
<td>(%) $\text{v/v}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(turnover)</td>
<td>(turnover)</td>
</tr>
<tr>
<td>1. Glycol</td>
<td>Ar: $^{18}$O$_2$</td>
<td>17.2 0.456 - - 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>20%</td>
<td>H$_2^{16}$O</td>
<td>(99.76%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Glycol</td>
<td>Air</td>
<td>D$_2$O</td>
<td>- - 7.32 0 - 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>21%</td>
<td>(99.9%)</td>
<td></td>
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<td></td>
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<tr>
<td>3. Glycol</td>
<td>Air</td>
<td>H$_2^{18}$O</td>
<td>10.1 0.207 - - 0 -</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>21%</td>
<td>(10%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4. Cholesterol</td>
<td>Ar: $^{18}$O$_2$</td>
<td>4.21 13.6 - - 71.7 -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycol</td>
<td>Air</td>
<td>H$_2^{16}$O</td>
<td>18.6 0.279 5.77 0 - -</td>
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<tr>
<td>(Alcohol std. 1)</td>
<td>21%</td>
<td>(99.76%)</td>
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</tr>
<tr>
<td>Alcohol std. 2</td>
<td>-</td>
<td>H$_2^{16}$O</td>
<td>19.70 0.68 4.71 0.41 - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(99.76%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* The percent abundance readings for each particular m/z value were recorded as percentages of the m/z 56 abundance to normalize against a region of the molecule that most likely does not undergo conversion during the enzymatic reaction. Alcohol standard 2 was used for all incorporation calculations in Equations 1 and 2.
DISCUSSION AND FUTURE WORK

According to Table I, there was absolutely no detectable $^{18}$O incorporation into isocaproaldehyde from the (20R,22R)-20,22-dihydroxycholesterol (Experiment 1.), whereas there was substantial incorporation (77%) when the cholesterol was used (Experiment 4.). This result does not exclude either the heterolytic or radical pathway in Fig. 2a. Route G, however, appears unlikely because a hypothetical gem-diol would have to be held in place and not allowed to rearrange within the active site with nearly 100% efficiency to give the observed products (Fig. 3). Since $^{18}$O was not detected in pregnenolone following glycol turnover under an $^{18}$O$_2$ atmosphere, the same argument can be made for the formation of a 20,20-dihydroxypregnen-3β-ol intermediate (Takemoto, 1968).

There was also no deuterium incorporation into isocaproaldehyde from turnover of the glycol intermediate in D$_2$O, suggesting that B+F is an unlikely mechanism (Table I). Since none of the potential mechanisms show any oxygen incorporation from enzyme bound water, it is not surprising that there was no $^{18}$O incorporation in the incubation of the dihydroxycholesterol with H$_2$^{18}O (Table I).

Besides isotopic incorporation experiments, characterization of mechanism-based inhibitors can also shine some light on the mechanism. From our lab, Nagahisa et al. (1984) synthesized (5) and characterized its suicide-mediated inhibition of P-450$_{scC}$. Incubation of C-7 tritium-labeled steroid (5) with P-450$_{scC}$ in the presence of NADPH and O$_2$ produced at three identifiable steroid products, (8), (9), and pregnenolone (Fig. 4). Ethylene is also produced (0.6 mol/mol enzyme) in the turnover of steroid (5) with kinetics similar to those for inactivation. While the presence of (9) is consistent with a cationic or radical mechanism without C-20/C-22 bond cleavage (Fig.4a), the presence of pregnenolone and ethylene point toward the formation of
a carbon-centered radical or carbocation intermediate (Fig. 4b), which can react further to produce ethylene and a TMS radical (10) or cation (11). These mechanisms, however, don’t consider the possible breakdown of the glycol product (9) in Fig. 4a. If (9) is catabolized according to the C-22 radical centered pathway (see B in Fig. 2b), a C-22 carbon radical (12) would form and most likely decompose to produce acetaldehyde and either a TMS radical (10) or cation (11) (Fig. 4c). With this in mind, we propose to run the same incubation experiment of (5) again and test for the presence of acetaldehyde. In addition, product (9) will be characterized separately for mechanism-based inhibition of the enzyme. If (9) turnover follows pathway B, it should show similar or more pronounced mechanism-based kinetics and produce acetaldehyde at higher concentrations than (5). Such a result would add further credence to the carbon centered radical pathway for the conversion of (20R,22R)-20,22-dihydroxylcholesterol to pregnenolone and isocaproaldehyde.

To further test the heterolytic and radical centered pathways, we would like to have a hypersensitive mechanistic probe that could distinguish between radical and carbocation intermediates. Recently Newcomb et al. (1994) designed a cyclopropylcarbinyl probe (16) around the notion that a phenyl group would stabilize an incipient radical more strongly than a methoxy group, whereas the opposite would be true for a incipient carbocation (Fig. 5a). When a radical was initiated on the primary carbon under Barton-McCombie deoxygenation conditions, the ring opened between C-1 and C-2 to give a phenyl stabilized radical intermediate, which was trapped with tributyltin hydride (Fig. 5b). A primary carbocation probe, however, opened in the opposite manner between C-1 and C-3 to give a methoxy stabilized carbocation intermediate, which was trapped by water and reacted further to give an unsaturated aldehyde (Fig. 5c).
Fig 4. Silicon-mediated suicide inhibition of P-450_{5c}. (a) Possible mechanisms for the formation of radical or carbocation intermediates before side chain cleavage; (b) Possible mechanisms for the formation of radical or carbocation after side chain cleavage; (c) Possible mechanism for further breakdown of product 9.
Fig. 5. Cyclopropylcarbinyl probe to test for either a radical or carbocation intermediate. (a) Possible ring openings of probe based on type of initiation (radical or carbocation); (b) Trapping of radical intermediate with tributyltin hydride; (c) Trapping of carbocation intermediate with water and subsequent formation of aldehyde product.

We, therefore, propose to replace the side chain in (20R,22R)-20,22-dihydroxycholesterol with a similar mechanistic probe and incubate it in a reconstituted P-450 SCC system (Fig. 6). Unlike their probe, this analog has a hydroxyl group bound to the proposed site of radical or carbocation initiation, which could pose a problem in the carbocation ring opening mechanism. A methoxy group, however, can more effectively stabilize a carbocation than a hydroxyl group and thus potentially allow ring opening from a thermodynamic standpoint. Once the amounts of unrearranged aldehyde (17), radical rearranged aldehyde (18), and carbocation rearranged aldehyde (19) are determined, the possibility of each potential glycol cleavage mechanism will be evaluated (Table II). If (17) is the only detectable product, a C-22 carbon centered mechanism is unlikely and either a C-20 radical centered or heterolytic pathway is at work. On the other hand, a result of all three products will point towards a C-22 radical centered mechanism with an additional carbocation intermediate, B+C.
Fig. 6. Design of Cyclopropylcarbinyl analog of (20R,22R)-20,22-dihydroxycholesterol and possible products formed during enzymatic reaction. Product 17 is formed in heterolytic cleavage type mechanism (pathway A). Product 18 is formed from a radical intermediate (pathway B). Product 19 is formed from a carbocation intermediate, which could be derived from a previous radical intermediate (18) (pathway B + route C).

Table II (Predicted Outcomes of Radical Clock Turnover)

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>17</th>
<th>18</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B+C</td>
<td>?</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B+D</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B+E</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B+F</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B+G</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: +, some product predicted to formed; -, no product predicted to formed; ?, possible product formation.
All previous P-450\textsubscript{SCC} mechanism based inhibitors have centered their attention at the C-22 carbon as their site of oxidation and subsequent inhibition (Nagahisa, 1984). In comparison with other cytochrome P-450 enzymes, the chemistry of P-450\textsubscript{SCC} rational drug design is more easily tackled because one only has to exchange the cholesterol side chain with various analogs, whereas other P-450 inhibitors have changes on the steroid nucleus. P-450\textsubscript{SCC} is also the only P-450 that catalyzes a glycol cleavage reaction; thus making it a perfect target for rational design of even more selective mechanism based inhibitors. However, we must first understand the glycol cleavage mechanism before we can hope to rationally design such an inhibitor.
VII. References


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Williams, G.S. et al. *Int. Immun.* **10**, 1957 (1998);


VIII. Appendix

A. Methods Developed or Extensively Used in the Lab

1) HLA-DQ3 complex specific ELISA (p. 123)

2) DR1 Competition Peptide Binding Assay (p. 124)

3) 6-liter High Five (HF) Culture Instructions (p. 126)

4) Preparation of tBoc-protected 5-hydroxytryptophan for incorporation into peptide synthesis reaction (p. 127)

5) Preparation of Disulfide-Linked Peptide-Fab Conjugates (p. 128)

6) DQ DNA Construction and Expression (See Chapter II, III)

7) I-As Expression (See Chapter IV)

B. Archival Samples

1) Peptide Samples (p. 130)

2) E. Coli DQ DNA and Proteins Samples (p. 131)
HLA-DQ3 COMPLEX SPECIFIC ELISA

Solutions
1. PBST: PBS + 0.05% TX-100
2. Dilution solution:
   A. 0.3% BSA, 0.1% TX-100, PBS, filter and store @ 4 C
3. ABTS solution:
   A. 5 ml 10x ABTS Buffer (From Boehringer Mannheim-made and frozen at -20 in 5 ml aliquots)
   B. 45 ml ddH2O
   C. 1 ABTS tablet (Boehringer Mannheim)

Directions
1. Coat plate with anti-DQ3β monoclonal antibody IVD12 (1 mg/ml stock in PBS, 0.02% azide (PBSZ)). Dilute 1:100 in PBSZ and put 100 μl in each well of a 96-well ELISA plate. Incubate 37C 2 hrs. or 4C overnight.
2. Wash each well 3 times with PBST.
3. Block by filling each well to the top with 3% BSA in PBSZ. Incubate at least 1 hr. At this stage, plates can be stored at 4C by covering them well and wrapping them in saran wrap to prevent from drying out.
4. Put 50 ml dilution solution into every well. (It takes a while to pipet samples and standards to wells so this way wells won’t dry out as you pipet.)
5. Standards: Make up standards in dilution solution + 0.02% azide from 2 ng/μl to 0.002 ng/μl by 2 fold dilutions. Use 50 μl of each per well to make a set of standards going from 100 ng/well to 0.1 ng/well (100, 50, 25, 12.5, etc.) Be sure to include a zero (50 μl dilution solution). Put at least 2 wells of each standard.
6. Samples: Put appropriate amount of sample on. For tissue culture sup, put on 50 μl and 5 μl (add an addition 50 μl dilution solution to the 5 μl samples; final volume should be ~100 ml).
7. Incubate plate to bind the protein to the antibody: 37C 1 hr, RT 2 hr, or 4C overnight.
8. Carefully wash each well 3 times with PBST.
9. Fill each well with 100 μl of biotinylated SPVL3 (anti-DQ complex) diluted 1:1000 in dilution solution. Incubate for 1 hr at 37C, 1.5 hr at RT, or overnight at 4C.
10. Wash each well with PBST.
11. Fill each well with 100 μl streptavidin-peroxidase conjugate in diluted 1:1000 in dilution solution. Incubate 1 hr at RT.
12. Wash each well 3 times with PBST.
13. Add 200 μl ABTS solution to each well.
14. Read absorbance on ELISA reader at 405 nm.
15. Use 4-parameter curve fit on ELISA reader, fit-four in kaleidograph on the Mac, or graph paper to determine the DR concentration of the samples.
DR1 COMPETITIVE PEPTIDE BINDING ASSAY

I. Supplies and Solutions

A. TBST, tris-buffered saline, 0.1% Tween-20, pH 7.5
B. Dilution Solution, same as usual, PBST+ 0.1% BSA, pH 7
C. BLOCK, same as usual, PBSZ + 3% BSA, pH 7
D. Binding Buffer, PBST (0.1% TX100) + 0.1% BSA + PMSF + Iodoacetamide + EDTA + azide, pH 7
E. Wallac DELFIA Assay Buffer, Enhancement Soln., and Streptavidin-Eu
F. Protein-A purified LB3.1
G. PBSZ, pH 7
H. Affinity purified Sf9 or E. coli DR1, empty
I. Biotinylated HA(306-318), C18 purified in water

II. Set-up Binding Reactions

A. Binding Reaction: 1 nM DR1 and 1 nM biotinylated HA (bioHA) in binding buffer with a 8-fold log titration of competitor peptide (0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000, 10000 nM). The following is how I would do it, but you can mix up the components differently if you wish.
B. The first step is to prepare your competitor peptide titration. I usually set up a logarithmic titration series at 2 times the concentrations listed in A, i.e. 20000, 20000, 2000, 200, 20, 2, 0.2, 0.02, 0.002.
C. Then I make up a mix of 2 nM DR1 with 2 nM bioHA in binding buffer and then quickly aliquot 200 µl into a series of 8 tubes.
D. To each of the mix tubes, I add a different concentration of competitor peptide, which was prepared in B. At this point, you should have 8 tubes with 1 nM DR1, 1 nM bioHA, and one of the following concentrations of competitor peptide: 0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000, 10000 nM (total volume per tube = 400 µl).
E. Alternatively, you could do the peptide titration and binding reaction preparation in a 96 well polypropylene plate, except you will need to decrease your total binding reaction volume to 200 µl (100 µl peptide + 100 µl DR/bioHA mix)
F. Incubate the samples at 37° C for 3 days for maximal signal

C. Prepare Assay Plate: This can be done while the binding reactions are incubating. Always remember to leave blank wells (no DR) as controls.

A. Coat Immulon-4 plate with 100 µl/well of 10 ng/µl LB3.1 in PBSZ; incubate at 37° C for 1 hour
B. Wash plate 3X with TBST and then block with BLOCK; incubate O/N at 4° C or 37° C for 1 hour
C. Wash plate 3X with TBST and then apply 100 µl triplicate samples from previous titration series; so you need at least 300 µl of each sample within your titration series (make up 350 of each to be safe); incubate at 37° C for 30 minutes; go get DELFIA enhancement solution and let it warm up to RT

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D. Wash plate 5X with TBST and then apply 100 µl/well of 1:1000 streptavidin-Eu in DELFIA assay buffer; incubate at 37°C for 15 minutes

E. Wash plate 7X with TBST and then apply 200 µl/well of DELFIA enhancement solution; allow to incubate at RT for 5 minutes and then read plate using standard Europium protocol on Victor 1420 Multilabel Counter (Ask Aaron for DEMO on how to use and work up data).

IV. Below is a titration of Biotinylated-HA/DR1 complex using this assay; 0.1 nM complex gives a signal around 34000 fluorescence units; this is the maximum signal you would ever see; since you are theoretically below the Kd of HA/DR your maximal signal should be less than 34000.
6-LITER HIGH FIVE (HF) CULTURE INSTRUCTIONS

1. In 100 ml spinners grow cells to 1-2 x 10^6/ml and split 1:4 or 1:5 2-3 times a week. HF cells seem to be able to tolerate larger splits in serum-free medium than Sf9's.
2. To grow the cells in 1 liter spinners, do not split lower than 3 x 10^5/ml and don’t grow more than 600 ml in a 1 liter spinner unless you are sparging air through the culture.
3. We’ve been using Insect Xpress from Bio-Whittaker. The lab who gave us the cells use XL401 from JRH Biosciences. We have used Sf900 from Gibco-BRL but it doesn’t seem to be suitable for long term passage of cells.
4. To grow cells in 6l. spinner sparged with air I split a 600 ml culture that’s at least 1.5 x 10^6/ml to 2 liters and bubble at about .4l/min. I stir the spinner as fast as possible.
5. When cells are at least 1 x 10^6/ml I fill the spinner to 6 liters and turn air up to 1 l/min.
6. Infect with baculovirus when cells are more than 2 x 10^6/ml.

Diagram:

1. Trimmed paddles - so that they don’t hit the bubbler tube as it turns.
2. Hollow glass tube - bent so that it rests close to the side.
3. Autoclavable 2um filter - we’ve been using Gelman Acro 50 (product # 4251).
4. 1 ml disposable syringe.
5. Compression fittings with o-rings - holes were cut in the caps that came with the spinner using a hot cork borer.
6. Flowmeter - .1 - 1 liter/minute.
7. Regulator and filter if building compressed air is used.
8. Tank of breathing air or air from building or a pump.
9. Tubing connections (as few as possible) held by copper wire.

This procedure was developed and written by Mia M. Rushe.
PREPARATION of tBoc-PROTECTED 5-HYDROXYTRYPTOPHAN (tBoc-5OHW) FOR INCORPORATION INTO PEPTIDE SYNTHESIS

1. Dissolve 5-hydroxytryptophan (1 mmol, 220.23 mg) in minimal amount of 2 ml ethanol
2. Add 1.5 ml of 0.66 M sodium bicarbonate solution (1 mmol, 84.01 mg)
3. Add 0.2 g sodium chloride and then 1091 mg tBoc anhydride (5 mmol)
4. Stir for 90 minutes at room temperature
5. Cool, filter crude reaction mix through a cotton plug, and extract aqueous sample with chloroform using a separatory funnel
6. Combine organic extracts, rotovap off solvent, and dry solid under vacuum
7. Recrystallize product from ethyl acetate and then purify on silica gel chromatography using ethyl acetate as the mobile phase
8. Remove solvent by rotary evaporation and purify on C18 reverse phase using a gradient of water and acetonitrile; do not add TFA to mobile phase because it causes the removal of tBOC.
9. Characterize product by proton NMR, ninhydrin, and TLC; if product shows a positive ninhydrin, repeat C18 reverse phase to remove free amino acid.
10. Keep product in the dark at 4 C until use. The product decomposes in the presence of light.
11. Addition of the 5OHW amino acid to a peptide synthesis was done without any protecting groups on the indole ring. Pentachlorophenol (0.4 mmol/g resin) was added during the coupling of tBoc-5OHW and all subsequent couplings to minimize esterification of the 5-hydroxyl.
12. Characterize the product by reverse phase, amino acid analysis, and MALDI-MS; all should conform to expected values. An ultraviolet spectrum of the product should show a red-shifted shoulder relative to tryptophan.
During the initial stages of the KL-304/Dendritic Cell work (Chapter V), we attempted to make KL-304 Fab' fragments conjugated to an antigenic peptide that bound specifically to I-As, PLP(139-151). If the empty class II molecules acted as endocytic receptors on dendritic cells, we speculated that such Fab'-peptide conjugates might specifically bind the empty molecules and then be transported into an endocytic compartment. Upon endocytosis and processing, it is possible that the antigenic peptide could then be loaded onto another class II molecule and displayed on the cell surface. Once there, we could detect such cell-surface complexes with a specific T-cell hybridoma against I-As bound to PLP(139-151). Using a protocol routinely used in our lab to produce MHC dimers through disulfide chemistry (see below), we produced a Fab'-PLP(139-151) conjugate and tested it with dendritic cells in a T-cell hybridoma assay [Note: In this assay, the T-cell hybridoma shows inhibited growth upon TcR stimulation and therefore, the data is shown as % Inhibition of growth.]

Unfortunately, the conjugates only showed a modest increase relative to peptide alone in their ability to inhibit a T-cell hybridoma (see Figure; *, indicates that concentration not tested). As a positive control, we made conjugates of an anti-DEC205 Fab' fragment with the same peptide and found that DEC-205, a known DC endocytic receptor, was able to efficiently transport the conjugate to the proper

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The procedure below was adapted from a procedure developed by Jennifer Ogrodnick. For more information on this procedure, please consult the Stern lab plastic methods book for the procedure on “Making Self Dimers Through Free Cys.”
processing compartment for class II loading. From this data, therefore, we became uncertain as to whether empty molecules could serve as endocytic receptors. We are still conducting experiments to further address this question.

**Procedure:**
1. Produce (Fab')₂ fragments by digesting whole antibody with either pepsin or elastase as described in Harlow and Lane (1988); The amount of protease must be optimized for each antibody.
2. Purify (Fab')₂ away from whole antibody by flowing over a protein A-sepharose column and isolating the flowthrough.
3. Excess protease can be removed by concentrating and exchanging the (Fab')₂ into 50 mM Hepes, pH 8 in a Centricon-100K (Millipore).
4. Dilute the (Fab')₂ up to 1 ml and with 1 mM DTT at room temperature for 1 hour in the same centricon.
5. Isolate the Fab' fragment by spinning the centricon to dryness in a swinging bucket rotor and isolating the flowthrough. The isolated Fab' fragment should be free of protease and whole antibody.
6. To 1 mg Fab' in 1 ml of 50 mM Hepes, pH 8, add 10 molar excess FITC-X-PLP (see sequence below), which also contains a free cysteine residue.
7. Initiate oxidation by adding phenanthroline (1.3 mM final; 1/100 of a 130 mM fresh stock in ethanol) and copper sulfate (0.25 mM final concentration; 1/100 of a 25 mM stock in water).
8. Let react for 3 hours at room temperature.
9. Dialyze with a 10 K membrane against PBS to get rid of copper phenenthroline and free peptide.
10. Gel filtration purify and sterile filter using a 0.2 micron spin filter for use in cell culture.

**FITC-X-PLP:**
Fitc-ε-GGGGSČRRGGGGSHSLGKWLGHPDKF-NH₂
Note: Fitc, fluorescein isothiocyanate; ε, 6-aminocaproic acid; bold residues, outlined C, site of disulfide formation between Fab' and peptide; possible basic cleavage site, e.g. trypsin; underlined residues, PLP139-151.
# Peptides Used During Thesis Work

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### E. Coli DQ DNA and Protein Used During Thesis Work

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**Note:**

E, E. coli T7 construct (α terminates at 184 and β terminates at 190; see Chapter II);

C, full length cDNA (complete coding sequence);

N.A., not available;

Numbers in parentheses indicate slot number within clear top freezer stock box, which is labeled “Aaron’s stocks/Archival DNA, DH5a, BL21” and stored in the −80° C freezer (56-553);

Notebook column give notebook number and pages on which initial cloning and sequencing conducted;

*, indicates that some purified E. coli protein available for this allele, which is stored in 56-567 freezer in several boxes, labeled “Aaron’s Protein Stocks”.

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IX. Biographic Note / CV

Aaron K. Sato
19 Central St. #21, Somerville, MA 02143 (617)625-5814 - aksato@mit.edu

Education:
Massachusetts Institute of Technology (MIT), Cambridge, MA
PhD., Chemistry, June 1999
G.P.A. 5.0

University of Puget Sound (UPS), Tacoma, WA
B.S., Chemistry, May 1993
G.P.A. 3.89, magna cum laude

Awards: Otis Coolidge Chapmann Honors Scholar, Sprenger Chemistry Award, Sprenger Memorial Scholarship, UPS Trustee Scholarship

Research Experience:
Research Assistant (1994-present): Lab of Lawrence J. Stern, Phd. (MIT Chemistry Dept.)

Structural Studies of Major Histocompatibility Complex (MHC) Class II HLA-DQ Proteins: Implications for Autoimmunity

- Applied a wide array of molecular biology techniques to isolate recombinant MHC genes
- Produced MHC proteins using a variety of expression systems (baculovirus/insect cell expression; E. coli in vitro refolding and periplasmic expression)
- Developed extensive protein purification protocols for expressed proteins
- Synthesized peptides for in vitro peptide binding assays on an automated synthesizer and characterized peptides by reverse-phase HPLC and MALDI-TOF
- Probed protein secondary structure and stability using spectroscopic techniques (circular dichroism, dynamic light scattering, fluorescence steady-state spectroscopy)
- Developed several enzyme linked immunosorbent assays (ELISA) and radioactive immunoassays (RIA) for studying HLA-DQ protein structure and peptide binding properties
- Crystallized HLA-DQ proteins and conducted preliminary X-ray diffraction analyses
- Screened several proteins against a peptide M13 phage display library and identified positive binders

MHC Class II Protein Conformational Changes Induced by Peptide Binding

- Identified an in vitro conformational change between empty and peptide-loaded forms of HLA-DR1 and murine I-Aδ using peptide-specific monoclonal antibodies (ELISA/BIACORE)
- Mapped antibody epitopes using ELISA and synthetic peptides
- Engineered Fab′ antibody conjugates for in vitro studies on the role of this conformational change in the antigen presentation pathway of dendritic cells
- Isolated dendritic cells from murine spleen/bone marrow and analyzed them using fluorescence flow cytometry
- Discovered a novel dendritic cell protease and characterized its activity
Structural Studies of HIV Peptide-DR1 Complexes: Implications for Vaccine Development
- Developed several HLA-DR1 peptide binding assays and identified high affinity HIV-derived peptides as potential vaccine candidates
- Produced HIV peptide/DR1 complexes and conducted initial crystallization trials

Techniques/Training Not Included Above: western electroblotting, native and IEF gels, immunoprecipitation, protein and peptide iodination, radioactive DNA sequencing, nuclear magnetic resonance spectroscopy, silica gel chromatography, EBV-transformed B-cell culture (biosafety level 2 techniques), $^{35}$S metabolic cell labeling, blood-borne pathogen training

Group Responsibilities
- Maintained a UNIX computer network for the analysis of structural data
- Served as safety, waste management, and fire management officer
- Instructed others in the use of an Advanced Chemtech 357 Peptide Synthesizer
- Supervised an undergraduate researcher


Mechanistic Studies of Cytochrome P-450_{5cc} (side chain cleavage)
- Purified and reconstituted membrane proteins from bovine adrenal glands
- Probed the enzymatic mechanism with isotopically enriched oxygen and gas chromatography/mass spectroscopy (GC/MS)
- Synthesized substrate analogues for enzymatic assays

Group Responsibilities
- Served as safety and waste management officer


Phosphorescence of Spectrally Enhanced $\lambda$ Repressor
- Studied 5-hydroxytryptophan substituted $\lambda$ repressor proteins before and after binding DNA operator site using steady-state and time-resolved phosphorescence spectroscopy

Group Responsibilities
- Helped to maintain a home-built phosphorescence spectrophotometer


Synthesis of EDTA Analogues for Use in Positron Emission Tomography
- Synthesized and purified alkyl side chain analogues of ethylenediaminetriacetic acid

Teaching Experience:
Intermediate Chemical Experimentation (MIT: 1994-5): Organic chemistry lab course
Freshman Chemistry Lab (MIT: 1993): Introductory chemistry lab course for non-majors
Freshman Chemistry Lab (UPS: 1993): Introductory chemistry lab course for non-majors
Publications:


References:

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stern@mit.edu; 617-253-2849.

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lsantamb@warren.med.harvard.edu; 617-632-2258.

Kenneth Rousslang, Ph.D. Professor, UPS, Chemistry Department
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Martin E. Dorf, Ph.D. Professor, Harvard Medical School, Pathology Department
dorf@warren.med.harvard.edu; 617-432-1978.

* Both authors contributed equally to this work.