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## Molecular mechanisms and complete antigen formation in allergic contact dermatitis

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# **Molecular mechanisms and complete antigen formation in allergic contact dermatitis**

Stefan R Ahlfors



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Department of Clinical Sciences Lund, Sweden

2008

"Your mind is like a parachute, it functions best when open"

*James Dewar*

*Dedicated to my sons Johan and Jakob and in memory of Signe and Fritz. Without them this work had never been done.*

Cover: A model of the H2-A<sup>q</sup> /antigen aligned with a TCR (2pxy), the Dnp-modified lysine (center) highlighted with a space filling representation.

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# List of papers

The thesis is based on the following papers which are referred to by the roman numerals I, II, III and IV. Published papers are reproduced with permission from the publishers.

- I Ahlfors SR, Sterner O, Hansson C. Reactivity of Contact Allergenic Haptens to Amino Acid Residues in a Model Carrier Peptide, and Characterization of Formed Peptide-Hapten Adducts. *Skin Pharmacology and Applied Skin Physiology* 2003; 16: 59–68.
- II Ahlfors SR, Kristiansson MH, Lindh CH, Jönsson BAG, Hansson C. Adducts between nucleophilic amino acids and hexahydrophthalic anhydride, a structure inducing both types I and IV allergy. *Biomarkers*, 2005; 10(5): 321-335.
- III Holmdahl M, Ahlfors SR, Holmdahl R, Hansson C. Structure-Immune Response Relationships of Hapten-Modified Collagen II Peptides in a T-Cell Model of Allergic Contact Dermatitis. *Chemical Research in Toxicology* 2008, 21, 1514–1523.
- IV Ahlfors SR, Sundin A, Hansson C. Exploring the Immunologic Synapse in Allergic Contact Dermatitis by Modelling the TCR-MHC-Peptide Complex. *Manuscript*.

# Abbreviation

ACD	Allergic contact dermatitis
APC	Antigen presenting cell
BQ	1,4-Benzoquinone
tBuBQ	4- <i>t</i> Butyl-1,2-benzoquinone
CII	Collagen type II
DNFB	2,4-Dinitrofluorobenzene
Dnp	2,4-Dinitrophenyl
Fmoc	9-Fluorenylmethyloxycarbonyl
GSH	Glutathione
HHPA	<i>cis</i> -1,2-Hexahydrophthalic anhydride
HPLC	High performance liquid chromatography
MHC	Major histocompatibility complex
MS	Mass spectroscopy
PDB	Protein Data Bank
NMR	Nuclear magnetic resonance
SPPS	Solid phase peptide synthesis
TCR	T cell receptor

# Abstract

Allergic contact dermatitis (ACD) is a common T cell mediated skin disease. Small reactive organic molecules called haptens induce ACD. Haptens are not recognized by themselves, but need to bond to endogenous proteins in the skin, and processed further into complete antigens. Antigen specific T cells then recognize the resulting antigen. Although the haptens are well known, the chemical reactions of haptens inducing ACD are not well known. Neither are the chemical structures of the complete antigens associated with ACD in humans.

This study is composed of three parts. Reactivity study of strong sensitizers like 2,4-dinitrofluorobenzene (DNFB), 1,4-benzoquinone (BQ), 4-*t*-butyl-1,2-benzoquinone (tBuBQ) and *cis*-1,2-hexahydrophthalic anhydride (HHPA) under physiologic conditions with nucleophilic amino acids and peptides by analytical methods (HPLC, LC/MS/MS). The new reaction products were isolated and their structures were determined by NMR and MS. We found that most haptens reacted predominantly with cysteine and cysteine containing peptides.

We then synthesized an array of 2,4-dinitrophenyl- (Dnp) modified collagen II peptides, as complete antigens, by solid phase peptide synthesis (SPPS). In a MHC class II (H2-A<sup>q</sup>) restricted T cell model of ACD, we evaluated the responses of these antigens. The length of the amino acid side chain bonding the Dnp-group determined the T cell response. Substitution of lysine for ornithine gave a complete loss of T cell recognition.

We finally used homology modeling to construct a model of the H2-A<sup>q</sup>/antigen/TCR complex, a part of the "immunological synapse". Our new model could explain the experimental T cell responses in terms of a defined structure of the MHC/antigen, and how this structure is displayed for the TCR. This study may contribute to better diagnosis, and possibly a cure of ACD in the future.

# Populärvetenskaplig sammanfattning

I vårt moderna samhälle ökar förekomsten av allergier generellt och så även kontaktallergier, en vanlig hudsjukdom. Huden är ett av våra viktigaste organ. Det utgör bl a den yttersta skyddsbarriären mot allehanda skadliga bakterier, virus, kemikalier och andra ämnen som finns i vår omgivning.

Man delar upp allergier i olika typer där de vanligaste är snabballergi (typ I) som ger en hudreaktion inom några minuter (men även andra symptom som astma och hösnuva) och fördröjd kontaktallergi (typ IV) som ger symptom först efter 1 dygn. Kontaktallergi av denna typ orsakas av s k haptener som är små reaktiva organiska ämnen eller metallsalter vilka inte känns igen som de är av immunförsvaret. Man känner f n till mer än 4500 olika sådana kemiskt väldefinierade ämnen med sensibiliserande egenskaper. För att utlösa en allergisk reaktion krävs att haptenet binder till något kroppseget protein, som efter metabolism till mindre haptensmodificerade peptider skall kunna kännas igen som främmande, och vid upprepade kontakt med ämnet starta den allergiska reaktionen i huden, man säger att man blivit sensibiliserad. Detta leder vid nästa kontakt till symptom som eksem med inflammation, en immunologisk reaktion vilken ofta medför livslånga besvär för den som drabbas. Någon botande behandling finns inte heller utan endast symptomatisk behandling (steroider).

För att förstå varför kontaktallergi uppstår måste man känna till hur de kemiska processerna sker och hur antigenstrukturen ser ut. Det är ännu okänt hur den exakta kemiska strukturen för de bildade fullantigenen ser ut, och till vilken eller vilka aminosyror haptenen binder. Om samma haptener kan ge olika typer av allergi beroende på det sätt som kroppen exponeras för ämnet är också okänt. Vad som gör att vissa personer får en kontaktallergi medan andra inte reagerar alls på samma ämne vet man också lite om.

Vanliga haptener är elektrofila organiska ämnen som  $\alpha,\beta$ -omättade karbonylföreningar vilka är vanliga bland akrylater och kinoner. Andra vanliga haptener är epoxider, anhydrider, karbamater och olika typer av organiska halogenföreningar. Dessa ämnen förekommer i eller används vid tillverkning av en mängd olika produkter som lim, tandmaterial, plaster, gummi, konserveringsmedel, färger, läkemedel o s v men är också vanliga i växter och naturprodukter av olika slag. Dessa elektrofila haptener kan reagera med nukleofila grupper som finns i vissa naturligt förekommande aminosyror sidokedjor. Bland aminosyror så är tio-, amino-, imidazol- och hydroxigrupper sådana möjliga nukleofila grupper som representeras av cystein, lysin, arginin, histidin, tryptofan, prolin, tyrosin och serin. Inom immunologin är det allmänt accepterat att lysin kan binda haptener och vara det "handtag" som haptener kan fästa vid. Vilken betydelse som de andra nukleofila aminosyror spelar som bärare av haptener är till stor del okänt, både vad gäller de kemiska förloppen vid fullantigenbildningen och den



efterföljande immunologiska processen. Åtminstone några typ IV haptener kan reagera med andra aminosyror, t ex tiol gruppen i cystein, och ge fullantigen där haptener inte binder till lysin.

Som modellhaptener för reaktivitets studier har vi bl a använt 1,4-bensokinon (BQ), 4-*t*-butyl-1,2-bensokinon (tBuBQ), *cis*-hexahydroftalsyraanhydrid (HHPA) och 2,4-dinitrofluorobensen (DNFB) som representerar några vanliga typer av kontaktallergen. Av dessa är ftalsyraanhydrider en speciell typ av haptener som både kan ge typ I och typ IV allergi och därför speciellt intressant att studera beträffande likheter och olikheter i reaktivitet.

Vi har klarlagt de kemiska mekanismer som leder till bildandet av fullantigen från några vanliga typer av organiska kontaktallergen genom reaktivitetsstudier och kunnat bestämma den kemiska strukturen för de bildade addukterna. Vi har identifierat cystein och cysteinnehållande peptider som den dominerande bäraren av alla dessa kontaktallergen. Men för HHPA, som är ett speciellt haptener, fann vi att den bildade HHPA-Cys addukten i närvaro av lysin reagerade vidare och bildade motsvarande HHPA-lysine addukt. För att studera de krav som ett fullantigen måste uppfylla för att kännas igen av T-celler, studerade vi DNFB modifierade kollagenpeptider. Med fastfasyntes (SPPS) och Fmoc kemi har vi syntetiserat ett 30-tal olika haptenermodifierade peptider. I en välkänd mus modell, där fullantigenet presenteras för specifika T-cells hybridom av ett specifikt MHC, har T-cells svar från dessa fullantigen undersökts. Vi fann att T-cell svaret är helt beroende av längd och struktur på aminosyrans sidokedja som DNFB är bundet till. Dessutom har vi visat att andra peptidvarianter, där DNFB är utbytt mot socker eller ett annat haptener inte ger något T-cells svar alls. T-cellerna som är inblandade i ACD är med andra ord ytterst specifika både avseende vilket haptener är och strukturen på fullantigenet.

Som en avslutande del har vi undersökt vilka krav som är väsentliga för den "immunologiska synapsen" som är aktiv vid ACD. Den "immunologiska synapsen" består av tre olika delar; fullantigenet, i komplex med ett specifikt MHC och en T-cells receptor (TCR). För människor som är drabbade av ACD är det ännu inte känt vilken struktur fullantigen, MHC eller TCR har. Därför har vi med datormodellering konstruerat en modell av den immunologiska synapsen och jämfört den med andra kända strukturer. Den visade mycket god överensstämmelse med flera andra kända strukturer. Vår modell ger ökad kunskap om mekanismen vid allergisk kontaktdermatit samt ger möjligheter för framtida studier över strukturen för humana fullantigen och T-cells svar på dessa. Modellen kan även bli användbar vid framtida *in vitro* diagnostisering av allergisk kontaktdermatit.

# 1 Introduction

## 1.1 Background

Allergy is common in modern society today and so is allergic contact dermatitis (ACD, delayed hypersensitivity or type IV allergy). In the western and northern Europe as many as 20 % of the population is affected by this hypersensitivity (Thyssen *et al* 2007). ACD are induced by small reactive organic substances or metal salts called haptens. These substances are frequently found in all sorts of consumer products such as plastics, rubber products, preservatives, dyes, glues and many are used as industrial materials. Many strong haptens can also be found in plants and other natural products. Today more than 4500 chemically well-defined haptens are known with a capability of inducing this form of hypersensitivity (de Groot 2008). With the introduction of new consumer products, new haptens are continuously being discovered.

## 1.2 Allergic contact dermatitis

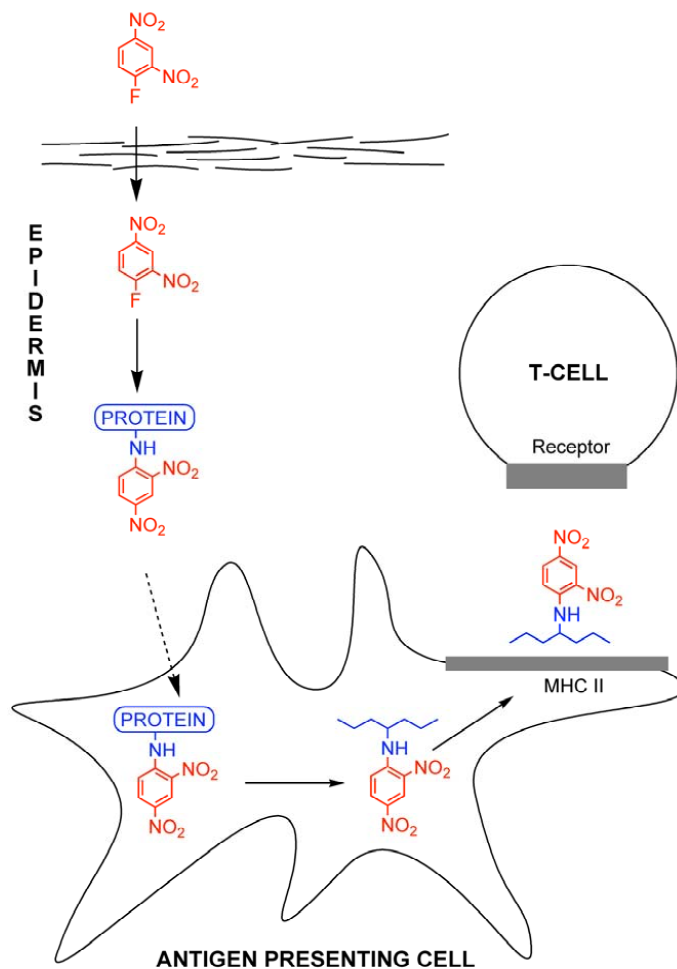
ACD or delayed hypersensitivity (type IV allergy) is usually elicited within 24 hours after exposure to the hapten, with symptoms like swelling, inflammation and blisters. ACD differ in this way from type I allergy, which is manifested within minutes after exposure to allergens like pollen proteins and latex peptides, although some small weight organic substances also can induce this type of allergy, with symptoms like asthma, rhinitis, conjunctivitis and urticaria, a condition "familiar" to many people especially at springtime.

ACD is a T cell-mediated immune reaction resulting from exposure to low-molecular-weight xenobiotics or haptens, which are able to penetrate the skin and bind to endogenous proteins or peptides (Lepoittevin 2006). These hapten-modified proteins are captured and processed by antigen-presenting cells (APC), leading to the presentation of hapten-modified peptides to T cells in a major histocompatibility complex (MHC) restricted way (for the immuno biology see Murphy *et al* 2008). At the *sensitization* T cells bearing unique T-cell receptors (TCR) recognising the antigen are formed. On subsequent skin exposure to the same antigen (*challenging*) an allergic reaction is initiated at the place of exposure. The classic contact allergic reaction involves highly specific T cells recognizing modifications of self-proteins conjugated with foreign molecules. A

similar type of antigen recognition is thought to occur in many autoimmune diseases in which self-proteins are modified and poorly tolerated. At the present time there is no curing treatment for this skin disease.



**Figure 1.1** A patient's hand severely affected by ACD.



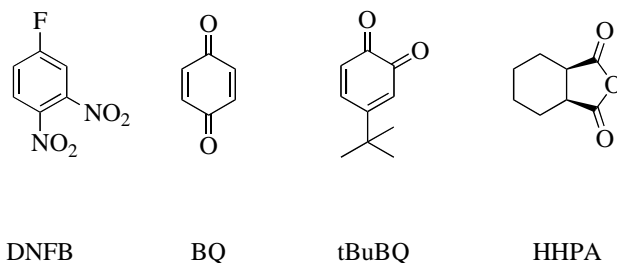
**Scheme 1.1** Illustration of the sensitization process associated with allergic contact dermatitis. First the hapten must penetrate the *stratum corneum*. The hapten then reacts with a protein nucleophile resulting in a hapten modified protein which is further processed to smaller peptides by antigen presenting cells and transported to MHC class II molecules and displayed for T cells.

## 1.3 The adaptive immune system

The immune system is a very complex protective system that normally protects us from bacteria, virus, parasites, etc. The adaptive immune system is a vital part of this defence system responsible for defence against such environmental pathogens. The process associated to ACD can be thought of as a malfunctioning adaptive immune response. The endogenously hapten modified protein are taken up and processed by antigen presenting cells (APC). The hapten modified protein is degraded into short peptides that are loaded on MHC class II molecules which are transported to the cell surface where they are presented to T cells. TCRs and co-receptor molecules bind to the MHC/peptide complex forming a large complex, the immunological synapse that activates the APC and T cell with the release of a cascade of cytokines and other immune modulators. The activated APCs are transported to local lymph nodes where they recruit other lymphocytes for a response at the site of exposure to the hapten. The activation will also stimulate the production of new antigen specific T cells. The initiation of an immune response has started. The principle of the sensitization (that also applies for the challenge) is shown in scheme 1.1.

## 1.4 The haptens

More than 4500 defined haptens are known that have the ability to induce ACD (de Groot 2008). Most of them are small (a mass of 1000 a u or less) reactive organic substances. A few of them have been known since ancient times, but the majority have been defined and characterised during the late 20th century, and their number are increasing. The pioneering work of Landsteiner and Eisen is the foundation of our knowledge regarding haptens and their conjugation to peptides and proteins and the principles put forward at that time still apply (Landsteiner and Jacobs 1936, Landsteiner and Chase 1941, Eisen *et al* 1952).



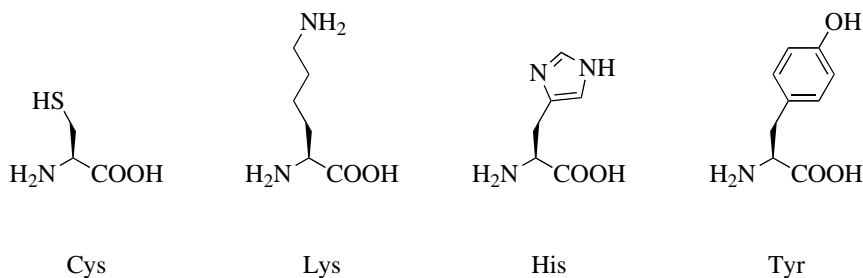
**Figure 1.2** Haptens used in this study.

## 1.5 Nucleophilic amino acids and peptides

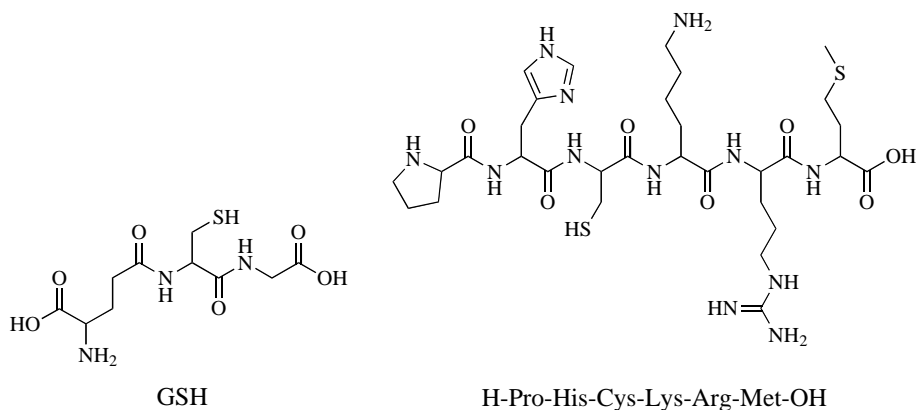
Haptens are thought to bind covalently to endogenous peptides or proteins. The target is a nucleophilic group in amino acid side chains like the  $\epsilon$ -amino group in lysine or thiol group of cysteine. Normally there are protective peptides like glutathione (GSH) present in all parts of the living organism. This tri-peptide is an essential part of the defence system that act as a scavenger of harmful substances like free radicals and conjugates to foreign toxic substances and thereby facilitates their excretion from the body. Reactive haptens can be thought to deplete the GSH available, and thus the haptens not being able to be detoxified by GSH instead it form addition products with nucleophilic groups in proteins present in the skin.

The sites for the binding are not well known and much research has been done in the past to determine the chemical reactions of haptens in model systems (Alvarez-Sánchez *et al* 2004, Hansson *et al* 1995, Kristiansson *et al* 2002, Liberato *et al* 1981, Meschkat *et al* 2001). The nature of the hapten and the mode of reaction influence the outcome of the chemical reaction when the hapten adds to a nucleophilic amino acid. However, for haptens of similar type it is reasonable to make some generalisations if the reaction and addition sites and the formed products are known for one or more of the same class of hapten.

In this study we used all nucleophilic amino acids, GSH and a hexapeptide (HProHisCysLysArgMetOH, as models of nucleophilic proteins. Also the use of this hexapeptide with the presence of essentially all amino acids with nucleophilic character in the peptide allowed direct comparison of the reaction at the different nucleophilic groups present in this peptide.



**Figure 1.3** Example of nucleophilic amino acids.



**Figure 1.4** Nucleophilic peptides used in this study.

## 1.6 Aim of the study

The aim of the study was to determine the chemical reaction mechanisms of some contact allergenic haptens under biomimetic conditions. To which amino acid do haptens bind and are there differences depending on the nature of the hapten? Determination of the addition site for the haptens will contribute to better understanding of the processes of complete antigen formation. Haptens and their reactivities towards various nucleophiles are described in paper I and II.

Furthermore we wanted to determine important characteristics of the complete antigen. In order to do this we synthesised an array of 2,4-dinitrophenyl- (Dnp) modified collagen II peptides and used them as complete antigens in a well established mouse model originally used in rheumatoid arthritis (RA) (Bäcklund *et al* 2002, Rosloniec *et al* 1998). The synthesis of the antigens used and our experimental model of ACD e. g. MHC restricted antigen presentation to CD4+ T cells and the immune response of them are described in paper III.

Also, we decided to apply computer modeling to obtain a three dimensional (3D) model of the MHC/antigen/TCR complex “the immunological synapse” and this work is described in paper IV.

Paper I and II are presented in chapter 2. Paper III is dealing with the use of the mouse model to determine immune responses from complete (ACD) antigens that is summarised in chapter 3. Generation of a homology model of the MHC/antigen complex, the “immunological synapse” is described in chapter 4.

## 2 Haptens - reactivity and processing

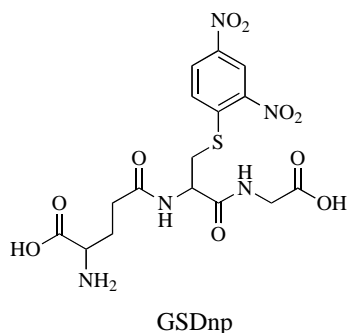
### 2.1 Background and experimental procedures

This is a summary of the reactivity studies in paper I and II. The structure of the haptens investigated and discussed in this study can be seen in figure 1.2. All of the reactivity studies were performed in buffer solutions at pH 5.4 representing the pH of the skin and at pH 7.4 representing the pH of the living cell (Öhman and Vahlquist 1994). Hapten reactions were monitored at ambient temperature by analytical high performance liquid chromatography (HPLC). We used a diode-array detector with ultraviolet/visible (UV/vis) light detection working between 190-900 nanometers. The aromatic or quinonoid haptens had characteristic UV/vis spectra and were useful for the identification of new reaction products. New products were formed for all of the investigated haptens. We performed synthesis at biomimetic conditions for all of them and isolated new products. We used nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) to determine the chemical structure for all of them. The isolated substances were used as HPLC reference and for many new substances additional properties like pH stability and red-ox behaviour was also determined.

### 2.2 Reaction of DNFB

DNFB is one of the strongest sensitizers known, and can in fact sensitize anyone with a normal immune system. It has been used as a treatment for baldness (*alopecia aerata*) and as a diagnostic tool to determine the status of the immune system prior to cancer therapy (Hansson C personal communication). DNFB is also used as a reagent in protein sequencing. This hapten was mainly used for SPPS of the complete antigens, but we observed that the addition of DNFB to thiol containing peptides such as GSH and the CIIhCys peptide resulted in the formation of thiol addition products only (figure 2.1).

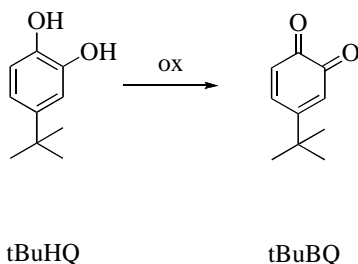




**Figure 2.1** The Dnp-modified glutathione.

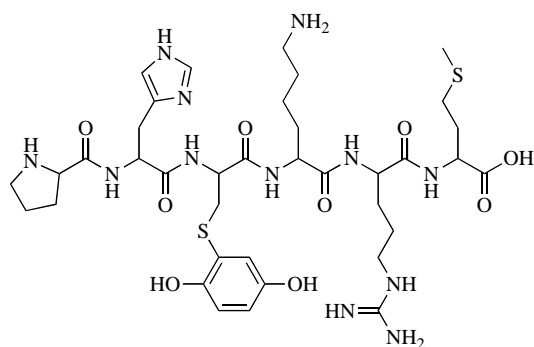
## 2.3 Reaction of Quinones

The quinones 1,4-benzoquinone (BQ) and 4-*t*-butyl-1,2-benzoquinone (tBuBQ) are representatives of electrophilic  $\alpha,\beta$ -unsaturated compounds. Quinones and other substances like caffeic acid derivatives and urushiols, can be oxidized to the corresponding quinones and have been studied over the past years (Hansson *et al* 1995, Liberato *et al* 1981).

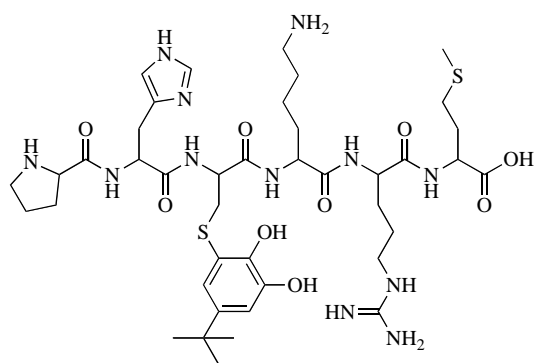


**Figure 2.2** Oxidation of catechol and derivatives forming quinonoid substances.

We found that for quinonoid haptens BQ and tBuBQ the only site of addition on the model hexapeptide was on the thiol group of cysteine. Although almost all other nucleophilic side chains of naturally occurring amino acids were present in the model peptide, no addition at any other site was registered. The BQ- and tBuBQ-adduct was shown to form red-ox pair in the presence of excess quinone the addition product could be oxidised to its quinonoid form. Also addition of ascorbic acid would lead to the quinonoid adduct being reduced to the fenolic substance. However the products were not stable and free radical reactions lead to formation of polymeric compounds. The isolated adducts of the hexapeptide can be seen in figure 2.3.



A



B

**Figure 2.3** BQ (A) and tBuBQ (B) hexapeptide addition products.

## 2.4 Reaction of HHPA

*cis*-1,2-Hexahydrophthalic anhydride (HHPA) is an interesting representative of phthalic anhydrides. They are known to induce both type I and type IV allergy (Kanerva *et al* 1997 and 1999). The reactions between HHPA and the nucleophilic amino acids and the hexapeptide at pH 5.5 and 7.4 were monitored by analytical HPLC. Adducts were observed to form in several cases (table 2.1).

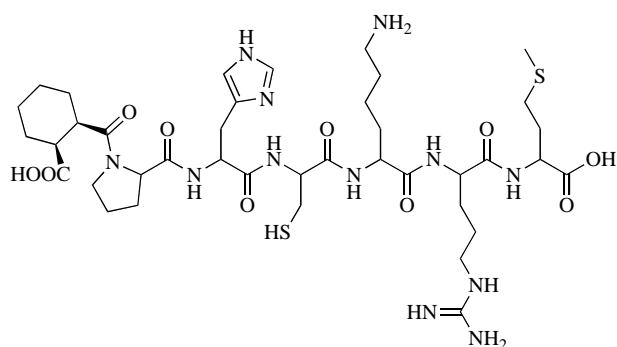
HPLC analysis showed a rapid reaction at both pH 5.5 and 7.4 between NAcCys and HHPA. The formation of the proline adduct was somewhat surprising at a low pH as 5.4. The reason for this is not obvious but could depend on the secondary nature of the amino group. The HPLC chromatogram showed the formation of double peaks and by NMR and MS analysis it was possible to

determine that this was a diastereomeric pair. The formation of diastereomers was observed for lysine and proline also. For proline we also noted a *cis-trans* isomerisation as measured by NMR. At a higher pH all of them showed a reaction except arginine, methionine and serine. However, the reaction products of cysteine, histidine, tyrosine and tryptophane were not stable at pH 7.4 (or 5.4 for cysteine). The reason is that the formed bond is weaker than for an amide and prone to hydrolysis. The formed lysine adduct was stable since the isolated product was shown to be an amide. The cysteine adduct was synthesised and identified as an thioester and was somewhat more stable than the tyrosine, histidine and tryptophane adducts which due to their unstable nature were not possible to isolate.

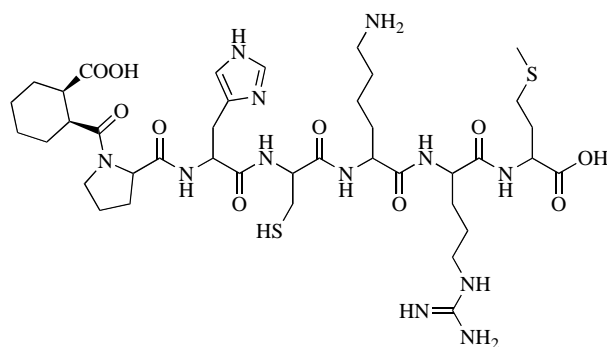
	pH 5.5	pH 7.4
NAcCys	+	+
NAcMet	-	-
NAcHis	-	+
NAcTrp	-	+
NAcSer	-	-
NAcTyr	-	+
NAcLys	-	+
NAcArg	-	-
Pro	+	+

**Table 2.1** The reactions between HHPA and all nucleophilic amino acids, as their N-acylated derivatives except Pro, at pH 5.5 and 7.4.

The model hexapeptide used in this study contained several nucleophilic residues, and HHPA was found to primarily form stable adducts with the  $\alpha$ -amino group of proline even at pH 5.5. This is somewhat surprising but the observation is in agreement with the result for the formation of proline adducts as described above. It was not possible to isolate HHPA bound to the cysteine residue in the peptide. However, such an adduct was detected by LC/MS. Furthermore, up to three HHPA bound to the peptide were detected, although the MS spectral data was not sufficient to determine the structure of the peptide with three HHPA bonded.



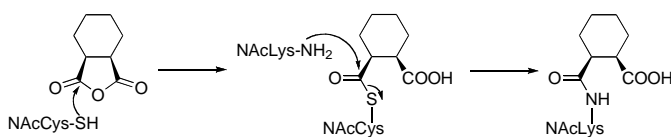
C



D

**Figure 2.4** HHPA hexapeptide proline adducts C and D, the main products formed at biomimetic conditions.

However, the main finding of this study was that HHPA bound to *N*-acetyl-L-cysteine could be transferred to the  $\epsilon$ -amino group of *N*<sup>ε</sup>-acetyl-L-lysine at pH 7.4. The reaction was fast and the formation of NAcLys-HHPA levelled out within 10-15 min.



**Figure 2.5** HHPA reaction path at pH 7.4, first is the cysteine adduct formed which reacts further transferring the HHPA to the  $\epsilon$ -amino group of lysine.

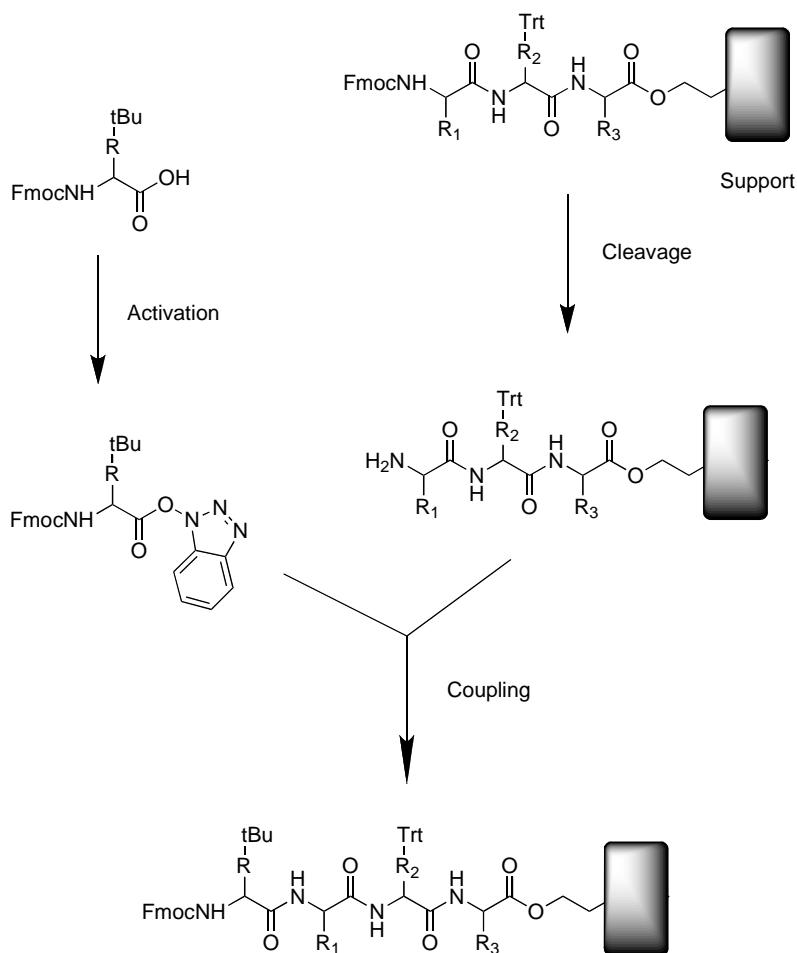
It is an important observation that the cysteine HHPA adduct formed initially could participate in a further reaction with lysine. Despite the high reactivity of HHPA, adducts to haemoglobin have been detected in exposed workers (Jönsson *et al.*

1997, Lindh and Jönsson 1998) as well as HHPA bound to tissue in the kidneys of an HHPA exposed rat (Lindh *et al.* 1999). Since HHPA is quickly hydrolysed to the corresponding acid under physiological conditions, it has been suggested that the mechanism of transport to these distant sites is mediated by a reactive intermediate.

## 3 Antigens and T cell recognition

### 3.1 Peptide synthesis

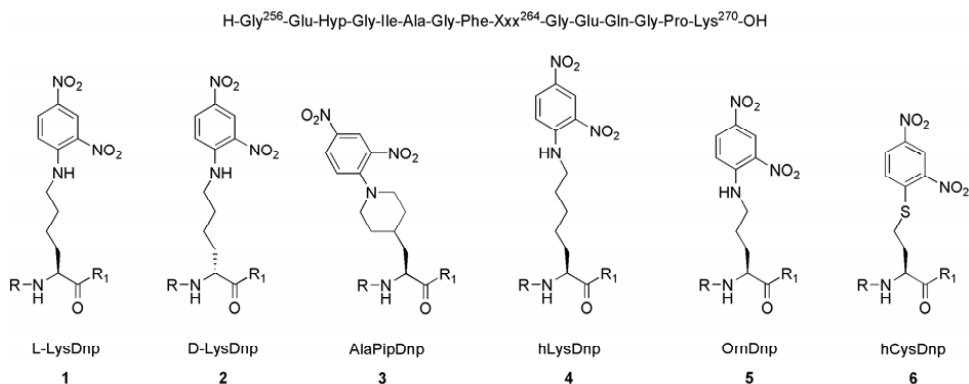
An array of more than 20 peptides were synthesised by solid phase peptide synthesis (SPPS). The principle is shown in figure 3.1 below. The procedure in SPPS is a multistep synthesis with repetitive cycles, where each step in the synthesis has a yield of > 98 % (Bodanszky 1993, Grant 1992). Synthesis is performed according to 9-fluorenylmethoxycarbonyl chemistry (Fmoc), a common protective group chemistry in SPPS (Carpino and Han 1972). Taking one of our Dnp-antigens as an example CIILysDnp a 15 amino acid long peptide, this means, for each amino acid coupling three steps, a total of 44 synthetic steps, with a final yield of 32 % which means that every step has in fact a higher yield. Also, strategy in choosing the best synthetic path is of some importance for the synthesis. If we consider CIILysDnp as an example, we have at least two options. To develop a new synthesis for FmocAlaPipDnp (since it's not a commercial substance) and use it as a building block in SPPS of this target, or as I did, use the commercial product FmocAlaPip(Boc)OH and add the Dnp-group specifically at the desired amino acid after SPPS is completed and the peptide has been cleaved and deprotected. The synthesis was under "biomimetic" conditions, since it was performed in water and the adjustment of the pH was sufficient to obtain the product (although in low yield).



**Figure 3.1** Synthetic scheme in SPPS.

## 3.2 The antigens

Many autoimmune diseases like type I diabetes, multiple sclerosis, celiac disease, pemphigus vulgaris and rheumatoid arthritis (RA), are mediated by T cell recognition of self-antigens. In the case of RA there is one well established animal model of RA, the collagen induced arthritis (CIA) that mimics the disease in humans. In CIA the antigenic peptide has been shown to be a peptide sequence from collagen type II and the immune dominant sequence has been shown to be 256-273. Thus we applied this model for ACD using the same MHC but with our Dnp-modified CII peptides prepared by SPPS in addition to the glycosylated CII peptide.



**Figure 3.2** Selection of synthetic complete antigens used in our T cell model of ACD.

Synthesis was performed with a peptide synthesiser, except peptides **3** and **6** which were modified after SPPS by the addition of DNFB in water at pH 9-10 and 5.4 respectively. It should be noted that SPPS gave the peptides in fair yields.

### 3.3 T cell specific response

We used CIIILysDnp **1** as a complete antigen with which to immunize mice and produce hybridoma cells with a T-cell receptor specific to this antigen. These T-cell hybridomas were used to investigate how small changes in the chemical structure, for example, changes in the length of the hapten-binding amino acid side chain in position 264, influenced the T-cell response.

We changed the stereochemical structure by replacing L-lysine with its D isomer and by altering the hapten-binding side chain of the amino acid, making them one- and two-carbon-shorter and one-carbon-longer structures (CIIOrnDnp, CIIhCysDnp and CIIhLysDnp respectively shown in figure 3.4). We also studied the results when the Dnp-group was bound to the amine of a less flexible piperidine ring structure (CIIAlaPipDnp). We found a high specificity and a strong response for the CIIILysDnp antigen used for sensitization in all the studied T-cell hybridomas, but only a weak response to the corresponding D-form. Only the piperidine analogue, i.e., CIIAlaPipDnp **3**, and the peptide with a one-carbon-longer Dnp-binding chain CIIhLysDnp **4**, produced stimulation similar to that of the CIIILysDnp **1** antigen in the T-cell hybridomas. We also replaced lysine with homocysteine, since most electrophilic haptens, such as DNFB, have a stronger reactivity towards thiols than towards amines. However, homocysteine is two carbons shorter than lysine and the CIIhCysDnp **6** peptide did not stimulate the T-cell hybridomas at all. By binding the Dnp to a piperidine-type ring structure, the



mobility of the bound hapten will be decreased while the length of the carbon chain is comparable to that of the lysine side chain.

T-cell response was measured by their IL-2 production when co-cultured with spleen cells as APCs. All the tested hybridomas displayed a similar strong reactivity towards the immunized peptide **1**. We found only small differences in specificity between the hybridomas tested with the Dnp-modified peptides.

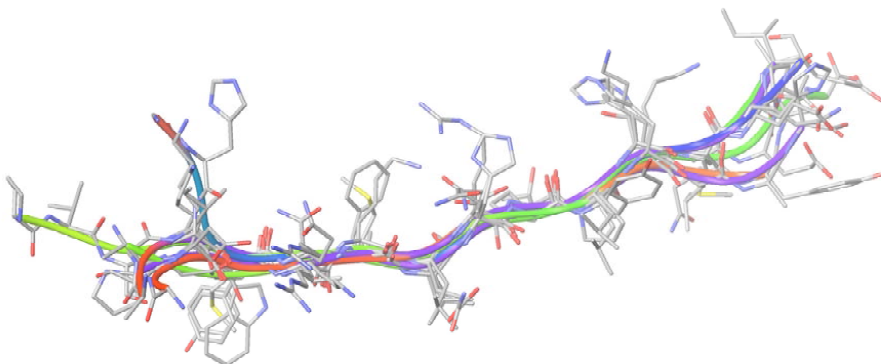
Peptide	Response	
	CIILysDnp-specific T cells	CIIfglyco-specific T cells
CIILysDnp ( <b>1</b> )	+	-
CII-D-LysDnp ( <b>2</b> )	+	-
CIIAlaPipDnp ( <b>3</b> )	+	-
CIILhLysDnp ( <b>4</b> )	+	-
CIIOrnDnp ( <b>5</b> )	-	-
CIILhCysDnp ( <b>6</b> )	-	-
CIILHylGal	-	+

**Table 3.1** T cell responses from selected antigens obtained in an H-2A<sup>d</sup> restricted mouse model of ACD.

Additionally we could not detect any cross reactivity with other antigens like the glycosylated CIIGalHyl peptide or modification with BQ instead of DNFB.

## 4 Modeling the MHC/antigen/TCR

In order to explore the structural basis for previous immune response data in our murine T cell model of ACD and to determine characteristics of the associated MHC/antigen complex, and how they can be presented to the TCR, we prepared a comparative model of H-2A<sup>q</sup> by using comparative homology modelling (Leach A R. 2001). Our model of the immunological synapse associated with ACD was built from templates obtained through BLAST searching (Altschul *et al* 1990) of the Protein Data Bank (PDB). We found 13 entries with crystal structures with >90% sequence identity or similarity for both the  $\alpha$ - and the  $\beta$ -chain. Further inspection made us decide for the crystal structure of 2IAD. Based on crystal data of the other MHC class II and their bound peptide ligands we examined the binding orientation of the peptides in these MHCs (Figure 4.1). We also compared our model with a similar comparative model associated with RA, which was published during our work (Andersson *et al* 2007). Fortunately, we also found recently deposited crystal data of several TCRs in complex with MHC/peptide complexes that were closely related to the H-2A<sup>q</sup> molecule.



**Figure 4.1** Superpositioned ligands found by BLAST search in the PDB for templates to H-2A<sup>q</sup> (CLIP, HA, HEL, E $\alpha$ 3K, MBP and OVA peptides). All of them have the backbone oriented in almost identical extended conformations.

Since the MHC/peptide complex was more or less identical we decided to simply mutate the MHC/peptide in the template (2IAD) for the H-2A<sup>q</sup>/Dnp-antigen. We prepared an array of models where all (1, 2, 3, 4, 5 or 6) of the antigens were mutated in place. For each model in complex with the antigens 1, 2, 3, 4, 5 or 6, a conformational search was performed. The generated conformations with low energy (conformations with energy up to ca. 20 kJ) were all energy-

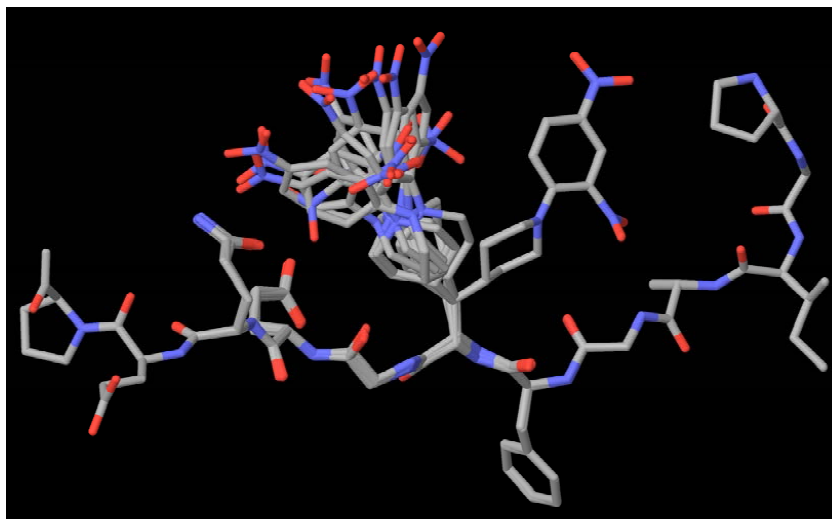
minimized and superimposed to determine preferred orientation both individually for every antigen, and then superpositioning all of the antigens.



**Figure 4.2** Final homology model with the CII<sub>LysDnp</sub> peptide.

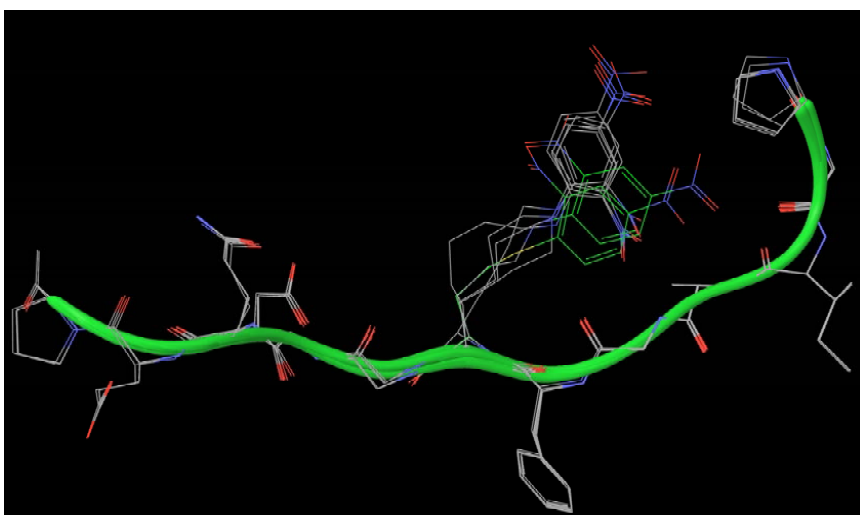
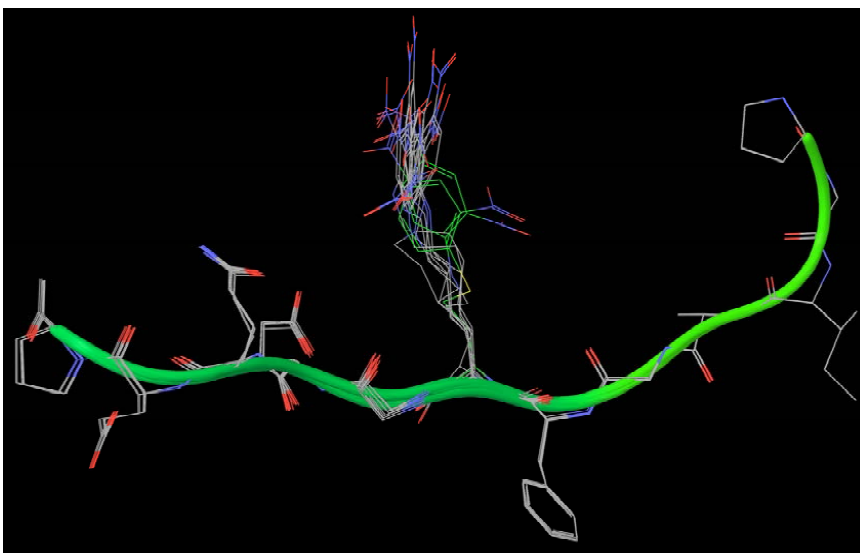
The CII<sub>AlaPipDnp</sub> (Figure 4.3) showed a preferred orientation for almost all of the conformations of low energy. It seems reasonable, therefore, to assume that this is the most likely conformation for this as well as the other T cell responsive antigens, when it is bound to the MHC molecule. This orientation was compared with conformations of the other antigens to identify similar conformations and

orientation. We observed that two of the antigens, **5** and **6**, had only high-energy conformations in this orientation of space. Consequently we assigned this orientation as the preferred conformation of all MHC/antigen complexes (Figure 4.4).

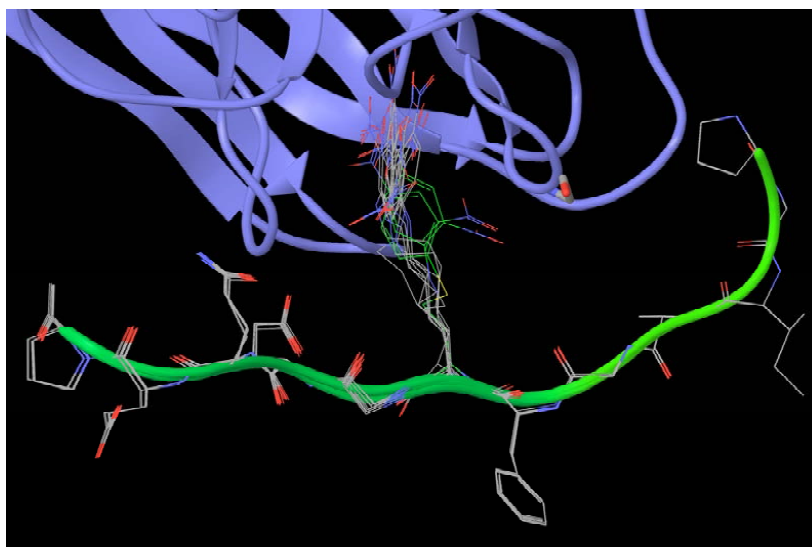


**Figure 4.3** The found low-energy conformations of CIIAIPipDnp **3**. One dominating orientation with minor differences in their position can be seen (the H-2A<sup>q</sup> comparative model not shown).

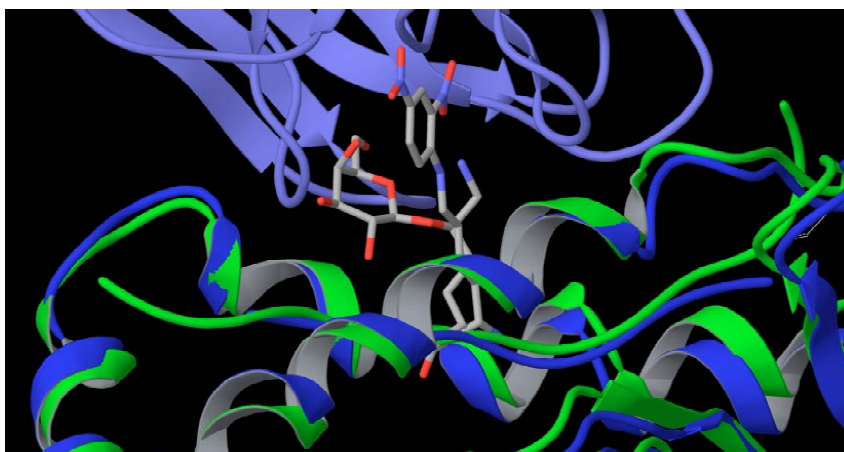
We also compared other conformations of low energy. We also considered another alternative pose for the antigens studied with a different orientation in space (bottom). However, in this orientation the total energy of the antigens are higher and the superpositioning of several TCRs indicated an electrostatic clash with backbone carbonyl groups of the TCRs CDR3 loops but also an unfavourable interaction with the CDR3 loops and the Dnp-group.



**Figure 4.4** Two possible binding poses of the antigens, the proposed (top) and one with higher energy (bottom).



**Figure 4.5** The Dnp-group is well within the CDR3 loops of 3c5z.



**Figure 4.6** Our model in green aligned with the other model published (in blue), superpositioned with 3c5z (top).

The alignment of the TCRs associated with the templates 2PXY and 3C5Z revealed that the hapten was well within the CDR3 loops in these crystal structures, a most revealing observation. Furthermore, when comparing the antigens (**1**, **2**, **3** and **4**) with the orientation of CIIHylGal in another model (Andersson *et al* 2007), this showed similarities in orientation of the galactose moiety, but the distance from the Hyl  $\alpha$ -carbon to the GalOH4 was considerably shorter than the corresponding distance from LysDnp to the *para*-nitro group (8.4

Å and 12.6 Å, respectively). This could be one reason for the lack of crossreactivity, also the Dnp and galactose groups are in different positions in space. The MHC backbone structure of the two models was, however, very similar. We think this model can be of use in further studies of how other haptens are recognised by TCRs.

## 5 Conclusions and future perspectives

Contact allergenic haptens like BQ, tBuBQ HHPA and DNFB form addition products with many nucleophilic amino acids like cysteine and thiol groups in the model peptides. However, at *biomimetic* conditions all of these haptens except HHPA preferably form stable covalent adducts with cysteine. HHPA being a special type of hapten was shown by the initial formation of a cysteine adduct, a reactive ester that reacted further acting as a "transporter" of HHPA to the  $\epsilon$ -amino group of lysine thus forming a stable adduct. To determine if this is a general pathway for HHPA and other phthalic anhydride derivatives further studies are needed.

The observation that thiols are the main target for quinones and other  $\alpha,\beta$ -unsaturated haptens implicate that the study of hapten reactivity with thiol containing endogenous proteins can be rewarding. The search and identification of hapten-modified proteins can help in targeting the true antigens associated with ACD and currently we are performing research in this field in collaboration with a group at the University of Southampton, England. Another point of view is that one can expect similarities regarding the chemical reaction mechanisms and protein targets between ACD and related types of allergies like drug associated hypersensitivity.

Targeting the actual antigens associated with ACD and other hypersensitivities will improve the possibility of better diagnosis. Also, the application of computer modeling will provide a better understanding of how and why the MHC/antigen complexes interact with TCRs associated with ACD. In the future it's possible that an effective curing treatment of ACD can be at hand.



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