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MAPPING CONFORMATIONAL CYP2E1 EPITOPES RECOGNISED BY AUTO-ANTIBODIES IN HALOTHANE HEPATITIS AND ALCOHOLIC LIVER DISEASE

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Abbreviations

ALD	alcoholic liver disease
CYP	cytochrome P450
HAL	halothane-induced hepatitis
HER	hydroxyethyl free radical
ELISA	enzyme-linked immunosorbent assay

Abstract

Auto-antibodies against cytochrome P4502E1 (CYP2E1) are associated with halothane-induced hepatitis and are also detectable in about 40% of the patients with alcoholic liver disease (ALD). Although such auto-antibodies might contribute to liver injury by targeting CYP2E1 on hepatocyte plasma membranes, their epitope specificity has not been characterized.

Preliminary experiments revealed that the recognition of recombinant human CYP2E1 by sera from halothane hepatitis and ALD patients involved conformational epitopes. Therefore a novel approach for identification of conformational epitopes was devised.

A molecular model of CYP2E1 was generated based on the CYP2C5 crystal structure and potential motifs for amino acid exchanges were selected by computer simulation in surface α helices and β sheets. Fourteen modified CYP2E1 variants produced in *E. Coli* and showing correct protein folding by spectral absorption analysis at 450 nm were investigated in immuniprecipitation experiments. Ala substitution of Lys243, Glu244, Glu248 and Lys251 as well as of Lys324, Lys342, Lys420 and Phe421 affected CYP2E1 recognition by the majority of the sera, whereas the other substitutions had minor effects. Based on the structural model these substitutions identified two distinct conformational epitopes on CYP2E1 surface corresponding to, respectively, the G-helix and an area at juxtaposition of the J' and K" helices.

Introduction

Cytochrome P450 2E1 (CYP2E1) is a haemoprotein belonging to the cytochrome P450 family that is responsible for the biotransformation of a variety of low molecular weight xenobiotics including halogenated hydrocarbons, benzene, acetaminophen and ethanol as well as for the oxidation of endogenous ketone bodies [1]. Studies by Bourdi and co-workers and Eliasson and Kenna have shown that patients suffering from halothane hepatitis develop auto-antibodies specifically targeting CYP2E1 [2,3]. Similar auto-antibodies are also detectable in pediatric anesthesiologists exposed to halogenated anesthetic gases [4]. We have reported that IgG directed towards CYP2E1 develop in chronic intragastric alcohol-fed rats and their titers correlate with the extent of hepatic injury [5]. These observations have been confirmed in humans showing that high titers of anti-CYP2E1 auto-antibodies are present in about 40% of patients with advanced alcoholic liver disease, but not in heavy drinkers without liver damage [6]. In the former the presence of anti-CYP2E1 IgG correlated with the extend of lymphocyte infiltration in liver biopsies [7], suggesting a possible role of auto-immune mechanisms in the pathogenesis of alcohol liver injury.

Anti-CYP auto-reactivity is not uncommon in liver diseases and antibodies against different CYP isoforms can be detected in the case of dihydralazine- (anti-CYP1A2) or tienilic acid- (anti-CYP2C9) induced hepatitis as well as during hypersensitivity reactions to the aromatic anti-convulsants (anti-CYP3A) or in children treated with immunosuppressive drugs (CYP3A4, CYP2C9) [8-11]. Furthermore, CYP2D6 is a target of anti-liver kidney microsome type I (LKM-1), present in type II auto-immune hepatitis and in virus C hepatitis [12]. Epitopes in CYP11A (cholesterol side-chain cleavage enzyme), CYP17 (steroid-17α hydroxylase) and CYP21A2 (steroid-21α hydroxylase) are also recognized by auto-antibodies associated with autoimmune polyendocrine syndrome and autoimmune Addison's disease [13,14]. For some of these auto-antibodies extensive epitope mapping studies have been performed in order to get a better understanding of the mechanisms leading to auto-immunity [15-21]. The data so far obtained regarding anti-CYP2D6, anti-CYP2C9 and anti-CYP3A1 auto-antibodies show that several linear and conformational epitopes are recognized in the different CYPs [15-21].

These observations along with the evidence indicating that CYP2E1 auto-antibodies target functionally active CYP2E1 present on the outer layer of hepatocyte plasma membrane [3], prompted us to investigate the epitope specificity of CYP2E1 auto-antibodies associated to halothane hepatitis or alcoholic liver disease in order to understand more about their formation and to get information of value for the development of more specific diagnostic tests.

Materials and Methods

Patients

For this study the sera of 10 patients with unexplained hepatitis following multiple halothane anesthesia and negative for markers of hepatitis viruses or evidence of exposure to hepatotoxic drugs or alcohol were used along with 12 sera from patients with severe alcoholic liver disease (ALD). Sera from 10 patients with halothane hepatitis were generously provided by Dr JG Kenna, Imperial College School of Medicine, London, UK. Their properties have been described in previously published studies [22,23]. In brief, halothane hepatitis was defined clinically as severe, otherwise unexplained, histologically confirmed hepatitis occurring within 4 weeks after halothane exposure in patients with normal preoperative liver function.

The diagnosis of ALD was based on clinical, ecographical and laboratory criteria. Liver biopsies were available for all the ALD patients and showed classical feature of micronodular cirrhosis, hepatocyte balloning degeneration with Mallory's bodies and inflammatory infiltrates. The patients with alcohol abuse were negative for serum markers for HBV and for the presence of HCV RNA. The reactivity of the sera with CYP2E1 was preliminary assessed by enzyme-linked immunosorbent assay (ELISA) using as antigen recombinant human CYP2E1 (Oxford Biochemicals Inc. Oxford, MI, USA) (300 ng/well) as previously reported [6] and was at least 3 times higher (1:100 dilution) than those of 50 control sera.

Immunoprecipitation of [^{35}S]-methionine labeled CYP2E1

[^{35}S]-methionine labeled wild-type CYP2E1 and the N-terminal and C-terminal deleted forms were produced from the respective pGEM4z plasmid cDNA (1 μg) using rabbit reticulocyte "*in vitro*" translation/transcription system TnT T7-Quick Coupled T Translation/Transcription kit (Promega Co, Madison WI) and 10 μmol/L [^{35}S]-methionine (1,000 Ci/mmol) according to the manufacture instructions. The N-terminal moiety contained amino acids 1-222 and the C-terminal part the amino acids 223-493, i.e. starting with the sequence just prior to the G-helix These constructs were kindly provided by Dr Inger Johansson. For immunoprecipitation experiments 5 μL of the transcription/ translation mixture were diluted (1:20) with RIPA buffer (50 mmol/L Tris/HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl and 1 mmol/l EDTA, pH 7.4) and pre-incubated 1 hour at 4° C with 50 μL protein A-Sepharose CL4B beads (50% w/v suspension in PBS)(Amersham Biosciences, Amersham, UK). After centrifugation at 5,000 rpm for 5 min 95 μL of the supernatant were added to 5 μL of human sera (dilution 1:20 in

RIPA buffer) and first incubated 12 h at 4°C in an orbital shaker then 2 hours at 4°C in the presence of protein A-Sepharose CL4B beads (50 µL of 50% w/v suspension in PBS). As a positive control a polyclonal rabbit anti-CYP2E1 (dilution 1:100 in RIPA buffer) was used. Immunocomplexes bound to protein A-Sepharose were recovered by centrifugation (2 min at 3,000 rpm), washed 3 times with 1.5 mL PBS and solubilized in 40 µL of SDS buffer pH 6.8 (4% w/v sodium dodecylsulphate, 0.2 mol Tris/HCl, 26% v/v glycerol). One aliquot (15 µL) was added to scintillation fluid and used for radioactivity determination. The other was boiled 5 min, centrifuged and used for SDS/PAGE electrophoresis. The recovery of radioactive CYP2E1 was calculated as (DPM sample – DPM background)/(DPM pos. Control – DPM background) x 1,000, where DPM background was the radioactivity recovered in the absence of added serum. SDS/PAGE electrophoresis was performed for 45 min at 200 V using 4% stacking and 10% resolving gels. The proteins were transferred to Hybond-C Extra nitrocellulose gel (Amersham Biosciences, Amersham, UK) and exposed to autoradiography using X-Ray films (Eastman-Kodak, Rochester, NY, USA).

CYP2E1 computer simulation

A structural model of human CYP2E1 was generated using the SWISS-MODEL automated comparative protein modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL/.html) and the template coordinates of CYP2C5 (code ExPDB 1DT6A) [24]. Graphical representation was made by the use of Rastop (version 2.0.3) by Philippe Valadon.

Site-directed mutagenesis of CYP2E1

The CYP2E1 was cloned into the expression plasmid pCWori^{+} [25] between the restriction sites *Nde*I and *Hin*dIII. Additional bases encoding 6 C-terminal histidines were added and nucleotides encoding the first 18 amino acids were removed in order to optimize the expression [26]. Mutant CYP2E1 was generated by using the QuickChange™ XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacture instructions. The forward and reverse DNA primers used for each mutations are listed in Table 1. XL-1 Blue supercompetent *E. Coli* strain was transfected with the different plasmids by heat shock and selected on LB-agar plates containing ampicillin. Single colonies were further expanded by overnight culture at 37°C in 5 mL of LB medium plus ampicillin (50 µg/mL). Plasmid DNA was isolated using QIAprep Spin Miniprep columns (QIAgen Inc., Valencia CA) and plasmid concentration was evaluated spectrophotometrically. The correct sequence of the insert was confirmed by automated DNA

sequencing with the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Prokaryote expression of mutated and wild-type CYP2E1

For the expression of wild-type and mutated CYP2E1 variants 70 μL of XL1 Blue supercompetent *E. Coli* cells were transfected with 1 μL of the respective plasmid solution as described above and colonies were selected on LB-agar plates with ampicillin. Single colonies were further grown for 16 hours at 37°C and 10 hours at 4°C on LB-medium-ampicillin. Two mL of these cultures were used to seed 198 mL of LB-agar-ampicillin medium supplemented with 1 mmol/L tiamine, 0.5 mmol/L δ-aminolevulinic acid 25 μmol/L $FeCl_3$ and incubated at 30°C under continuous shaking [23]. Bacterial growth was monitored spectrophotometrically until reaching 0.7 O.D. units before adding 0.5 μmol/L imidazole and 1 mmol/L isopropyl β-D-thiogalactoside. After further 24 hours culture at 30°C, CYP expression was monitored in whole cells by recording absorption spectrum at 450 nm in the presence of reducing agent and CO [27].

Purification of recombinant CYPs.

Bacteria were separated from the incubation medium by centrifugation (2,800 g for 12 min at 4°C) and resuspended in cold 50 mmol/L Tris-HCl buffer pH 7.4 plus 250 mmol/L sucrose and 0.25 mmol/L EDTA and 0.25 mg/mL lysozyme. After 30-60 min incubation in ice, spheroblasts were recovered by centrifugation (2,800 g for 12 min at 4°C), resuspended in 0.1 M phosphate buffer pH 7.6 containing 6 mmol/L magnesium acetate, 20% (v/v) glycerol and 25 μL/mL of protease inhibitor cocktail (Hoffmann-La Roche Ltd, Basel, Switzerland) and lysed by sonication. The bacterial lysate was centrifuged at 4°C (12,000g for 12 min) and the membrane fraction was recovered from the supernatant by centrifugation at 100,000 g for 60 min. The pellet was resuspended in 2 mL of 50 mmol/L Na-phosphate buffer pH 7.4 supplemented with 300 mmol/L NaCl, 5 mmol/L imidazole 20% (v/v) glycerol, 1% (wt/v) sodium deoxycholate 1 mmol/L phenylmethyl-sulfonylfluoride and further centrifuged at 100,000 g for 60 min. The supernatant was added to 0,5 mL of nickel-charged polypropylene-agarose (QIAgen Inc., Valencia CA). Following 1 hour incubation at 4°C under shaking the mixture was loaded to Ni-NTA-agarose columns (QIAgen Inc., Valencia CA). The columns were first washed 4 times with 4 mL of 50 mmol/L Na-phosphate buffer pH 7.4 containing 300 mmol/L NaCl, 10 mmol/L imidazole 20% (v/v) glycerol, 0.1% (wt/v) sodium deoxycholate and the CYP2E1-containing

fractions were eluted by the subsequent addition of 0.5 mL 50 mmol/L Tris-HCL buffer pH 7.4 plus 100 mmol/L NaCl, 500 mmol/L imidazole 20% (v/v) glycerol, 0.1% (wt/v) sodium deoxycholate. CYP2E1 recovery was estimated spectrophotometrically at 450 nm according to Omura and Sato [27].

Reaction of human sera with mutated CYP2E1s

The recognition of mutated and wild-type CYP2E1 by the human sera was estimated in immunoprecipitation experiments using 10 pmol of CYP2E1 solubilized in RIPA buffer and 5 μL of the different sera (1:20 dilution in RIPA buffer) as described above. Immunocomplexes bound to protein A Sepharose beads were solubilized in 40 μL of SDS buffer pH 6.8 (4% w/v sodium dodecylsulphate, 0.2 mol/L Tris/HCl, 26% v/v glycerol) and used for SDS/PAGE electrophoresis (45 min at 200 V using 4% stacking and 10% resolving gels). The proteins were then transferred to Hybond-C Extra nitrocellulose gel (Amersham Biosciences, Amersham, UK) and the membranes probed with a monoclonal mouse IgG toward the His-6-tag tail (Amersham Biosciences, Amersham, UK). The antibody binding was revealed by horseradish peroxidase conjugated anti-mouse immunoglobulins (BioRad Hercules, CA, USA) using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA, USA) and X-Ray film (Estman-Kodak, Rochester, NY, USA). The intensities of the bands obtained with the differently mutated CYP2E1s were measured by videodensitometry and expressed as percent of the intensities of the bands with wild-type CYP2E1.

Results

The specificity for CYP2E1 of the sera from 5 patients with halothane hepatitis (HAL) and 5 patients with alcoholic liver disease (ALD) having high titers of IgG against recombinant human CYP2E1 in ELISA was confirmed by immunoprecipitation experiments using [^{35}S]-methionine-labelled CYP2E1 translated "*in vitro*" using the rabbit reticulocyte system (Fig. 1). In order to discriminate between conformational and linear epitopes we prepared CYP2E1 constructs with the N-terminal (222 as) or C-terminal (271 as) moieties. As shown in Figure 1 the CYP2E1 recognition by the human sera was essentially lost using these variants, indicating that the anti-CYP2E1 auto-antibodies are directed against conformational epitopes. This finding was strengthened by the observation that human sera failed to recognize CYP2E1 in Western blots (not shown).

To characterize these conformational epitopes, we decided to study the effects of single amino acid substitutions on the antigenic capacity of the whole molecule. We focused on the C-terminal domain since previous studies have shown that the epitopes in human CYPs so far identified are in this region [15-17]. A computer simulated structure of CYP2E1 was generated using the Swiss-Model automated comparative protein modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL/.html) and the crystal structure of rabbit CYP2C5, that share 59% amino acid sequence homology with CYP2E1 [28]. As shown in Figure 2 the three-dimensional model of CYP2E1 obtained was quite similar to that of CYP2C5 with the exception of a loop between Lys277 and Tyr285 and a helix between Pro213 and Pro222. This latter region was exchanged with the corresponding sequence from CYP2C3 in order to get CYP2C5 suitable for crystallization [24]. Amino acid motifs corresponding to α helices and β sheets located on the surface of the molecule were preliminary selected using the CYP2E1 structural model and their potential antigenic capability was confirmed by computer assessment of the antigenic index according to Jameson and Wolf [29] (GCG Wisconsin Package; Accelrys Inc.). ELISA experiments using purified rat or rabbit CYP2E1 showed that human anti-CYP2E1 IgG cross-reacted with the enzyme from these species (not shown). Amino acid sequences of human CYP2E1 not in common with the rat and rabbit CYP2E1 ortologous were therefore excluded. In order to produce major changes in the configuration of the possible epitopes without disrupting the tertiary structure of the molecule we decided to insert a neutral amino acid having a low steric hinderance such as alanine in place of charged residues of lysine, arginine and glutamic acid. At the end of this process 26 different amino acid substitutions were selected. The effects of these mutations on CYP2E1 structure and on the antigenic potential of

the molecules were preliminary evaluated using the CYP2E1 simulation model described above and the GCG Wisconsin software. As a result, 20 single amino acid substitutions (Table 1) that would cause major changes in the antigenic capacity without affecting the enzyme conformation were selected.

Site-directed mutagenesis was used to introduce the selected mutations into cDNA and the modified CYP2E1s were then expressed in *E. Coli* as 6-His tagged proteins. Fourteen out of the 20 mutated CYP2E1s (see Table 1) showed spectral features at 450 nm comparable to wild-type CYP2E1, indicating that the proteins had correct configurations and were able to incorporate and retain the heme. The capacity of the human sera to recognize CYP2E1 variants with correct folding was assayed by immuno-precipitation followed by western blotting. As shown in Fig 3, about half of the substitutions caused a significant decrease in the ability of the sera to immunoprecipitate CYP2E1. Alanine substitutions of Lys272, Lys342 and Lys420 affected the antibody binding to the highest extent. The effects of Lys324, Arg374, Phe421 and Lys440 substitutions were less consistent, being evident with only 2-3 different sera (Fig. 3). Conversely, the Ala substitution of Lys234, Glu320, Arg331, Arg344 and Glu346 did not interfered with the antibody recognition or had scattered effects with single sera (Fig. 3). Although the mutations able to reduce CYP2E1 antigenicity involved amino acids located far away from each other in the J'-helix and the β-sheet between K and L helices, respectively, computer simulations revealed that the positions of Lys342, and Lys420 on the tertiary structure were rather close (about 25-30 nm) and identified an area in CYP2E1 surface compatible with the presence of a distinct conformational epitope (Fig. 4). This interpretation was supported by the observation that the substitutions of Lys324 and Phe421 (Fig 4) also decreased the antigen recognition of some sera.

The poor immuno-reactivity of 3 HAL and 1 ALD sera with CYP2E1 modified by the substitution of Glu272 in the β sheets between the G and H helices, (Fig 3) prompted us to extend the investigation to include substitutions encompassing the G helix between Lys243 and Lys251. Figure 5 shows that the combined substitution of Lys243, Glu244, Glu248 and Lys251 for Ala decreased CYP2E1 recognition in 3 out of 5 of HAL and in 4 out 5 of the ALD sera. Structural simulation revealed that the mutated amino acids were closely associated with a distinct area on the enzyme surface (Fig. 5).

These results indicate that anti-CYP2E1 auto-antibodies recognize at least two distinct conformational epitopes located in the G helix and between J' and K" helices in the C-terminal portion of the molecule, respectively. This conclusion was supported by further analysis of additional 5 HAL and 7 ALD sera revealing that these two epitopes were recognized by 4 out of the 5 HAL sera and 5 out of the 7 ALD sera (Table 2). Altogether, the epitopes identified account for the antigen specificity of 19 out of all 22 (86%) human sera investigated.

Discussion

In recent years several studies have investigated the epitope specificity of LKM-1 auto-antibodies on CYP2D6 by comparing the cross reactivity of human sera with prokaryotic expressed peptides spanning the entire protein sequence. This approach has allowed the identification of several linear epitopes with different specificity for LKM-1 associated with type II auto-immune hepatitis and chronic hepatitis C [17-21]. Furthermore, it was shown that some of these epitopes share structural homologies with protein sequences present in type 1 Herpes Simplex virus and hepatitis C virus [17,21]. The use of deletion CYP mutants has also provided important information concerning the structure of different epitopes present in CYP2C9, CYP3A1 and CYP1A2 [15-17]. However, it is well known that B-cell epitopes associated to auto-immune disease are often conformational. For instance, conformational epitopes in CYP2D6 and CYP2C9 are implicated in the auto-reactivity of, respectively, LKM-1 and LKM-2 antibodies [15,30]. According to Bourdi and co-workers [2], the recognition of recombinant human CYP2E1 by the sera of patients with either halothane hepatitis or alcoholic liver disease was completely lost when the antigen was denaturated. The same sera also had low reactivity toward truncated forms of CYP2E1 not allowing the use of deletion mutants for the identification of the epitopes. We, therefore, devised a new approach based on the analysis of the whole molecule antigenic capacity following single amino acid substitutions that modify the charge distribution on CYP2E1 surface without affecting the molecule conformation. Hereby we observed that Ala substitution of Lys324, Lys342, Lys420 and Phe421 identifies a conformational epitope formed by the juxtaposition of the J' and K" helices. The measurement of the distance between the single amino acids confirms the compatibility with an antigen-antibody binding area. The J, K and L helices have been implicated as sites of conformational epitopes recognized in CYP2C9 by LKM2 auto-antibodies associated with tienilic acid-induced auto-immune hepatitis [15], while the K helix of CYP3A4 is considered a major epitope recognized by auto-antibodies of patients with hypersensitivity reactions to aromatic anti-convulsing drugs [16]. It is interesting to note that both Lys324 and Lys342 are comprised in a sequence with good homology with $CYP2D6_{321-351}$ epitope [19], while Lys324 is conserved in the $CYP2C9_{314-322}$ epitope [15]. Lys324 is also close to the $CYP2D6_{316-327}$ sequence that according to Ma and co-workers represents a key target for auto-antibodies on CYP2D6 surface [20]. Similarly, the $CYP2D6_{410-429}$ epitope [19] has good homology with the sequence containing Lys420 and Phe421. This suggests the possibility that the J, K and L helices might represent an important antigenic area in CYPs, able to give rise to antibodies against both linear and conformational epitopes.

A further conformational epitope corresponding to Lys243, Glu244, Glu248 and Lys251 was identified in a distinct area of the G helix that is located on the opposite side of the CYP2E1 surface. This epitope is recognized by 50% of the 22 sera tested, 5 of them in combination with the epitope in J'-K" helices. The C-terminal portion CYP3A4 spanning up to Thr208 and the region between Thr208 and Ser281, that comprises the G helix, are the targets of auto-antibodies present in a sub-set of alcoholics [5]. However, it is unlikely that anti-CYP3A4 auto-antibodies might account for the recognition of CYP2E1 conformational epitopes because they are directed against linear structures and there is no sequence homology between G helices of CYP2E1 and CYP3A4.

We have recently reported [31] that the N-terminal portion of CYP2E1 and particularly the amphipatic amino acids in the B-helix are responsible for electrostatic interactions with negatively charged phospholipids that anchor CYP2E1 to the cell membranes. The orientation of CYP2E1 in relation to the membrane shows that the epitopes formed by J' and K" helices and G helix are both on the outer portion of the molecule and well accessible to antibody recognition (Fig. 6). This is consistent with previous observations showing that functionally active CYP2E1 is present on the outer layer of hepatocyte plasma membrane where it is targeted by specific antibodies [3, 32, 33]. Thus, the recognition of these conformational epitopes on the portion of CYP2E1 that face the extracellular spaces can be involved in triggering antibody-mediated hepatocyte killing.

The current hypothesis explaining the development of anti-CYP auto-reactivity postulates that structural modifications of CYP by reactive drug metabolites can induce helper T lymphocytes (Th) to recognize as "non-self", alkylated peptides. In turn, these Th cells are capable of inducing the clonal expansion and maturation of B lymphocytes recognizing the alkylate epitope as well as of quiscent autoreactive B cells with specificity towards native CYP [34, 35]. This mechanism potentially explains why drug-induced immune reactivity often involves the concomitant presence of antibodies directed against both native and drug-modified proteins [36]. In the case of hatothane hepatitis or alcoholic liver disease anti-CYP2E1 auto-antibodies are present in combination with allo-antibodies recognizing, respectively, trifluoroacetyl- and hydroxyethyl free radical- (HER) CYP2E1 adducts [37, 38]. Moreover, patients with alcoholic liver disease showing anti-HER IgG appear to have a 4-fold increased risk of developing anti-CYP2E1 auto-antibodies, than subjects without anti-HER antibodies [6]. Such risk increases up

to 23 fold in subjects that also carry a single nucleotide mutation (+49 A→G transition) in exon 1 of the immunoregulatory cytotoxic T lymphocyte associated antigen-4 (CTLA-4)[6], a T lymphocytes membrane receptor involved in the down-modulation of T cell-mediated immune responses [39,40]. The observation that anti-CYP2E1 auto-reactivity is polyclonal and involves at least two conformational epitopes is consistent with the hypothesis that autoimmune reactions directed against CYP2E1 result from an antigenic stimulation by xenobiotic-modified CYP2E1 peptides combined with an poorly controlled expansion of auto-reactive Th cell clones consequent to CTLA-4 polymorphism [6].

In conclusion the results presented demonstrate that anti-CYP2E1 auto-antibodies recognize at least two distinct conformational epitopes present in the G-helix and in an area formed by juxtaposition of J' and K" helices on the C-terminal portion of the molecule surface. Moreover, we propose the combined use of molecular modeling and site-directed mutagenesis as a novel method to investigate the epitope specificity of conformational anti-CYP auto-antibodies associated to liver diseases.

References

1. Ronis, M.J.J., K.O. Lindros, and M, Ingelman-Sundberg. 1986. The CYP2E family. In: Cytochromes P450: metabolic and toxicological aspects. (Ioannides C. Ed.) pp. 211-239, CRC Press, Boca Raton.
2. Bourdi, M., W. Chen, R.M. Peter, J.L. Martin, J.T.M. Buters, S.D. Nelson, and L.R. Pohl. 1996. Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. Chem Res Toxicol 9:1159-1166.
3. Eliasson E., and J.G. Kenna. 1996. Cytochrome P450 2E1 is a cell surface autoantigen in halothane hepatitis. Mol Pharmacol 50:573-582.
4. Njoku D.B., R.S. Greenberg, M, Bourdi, C.B. Borkowf, E.M. Dake, J.L. Martin, and L.R. Pohl. 2002. Autoantibodies associated with volatile anestetic hepatitis found is the sera of a large cohort of pediatric anestesiologists. Anesth Analgesia 94:243-249.
5. Lytton S.D., A. Hellander, Z.Q. Zhang-Gouillon, K. Stokkeland, R. Bordone, S. Aricò, E. Albano, and S.W. French. 1999. Ingelman-Sundberg, M. Autoantibodies against cytochromes P450 2E1 and P450 3A in alcoholics. Mol Pharmacol 55:223-233.
6. Vidali, M., S.F. Stewart, R. Rolla, A.K. Daly, Y. Chen, E. Mottaran, D.E.Y. Jones, Y.B. Leathart, C.P. Day, and E. Albano. 2003. Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in alcoholic liver disease. Hepatol 37:277-285.
7. Bourdi, M., D. Larrey, J. Nataf, J. Bernuau, D. Pessayre, M. Iwasaki, F.P. Guengerich, and P.H. Beaune. 1990. Anti-liver endoplasmic reticulum autoantibodies are directed against human cytochrome P450 1A2; a specific marker for dihydralazine-induced hepatitis. J Clin Invest 85:1967-1973.
8. Stewart, S.F., Jones, D.E.J., Vidali, M., Haugk, B., Burt, A.D., Albano, E. and Day, C.P. 2004. Correlation between anti-CYP2E1 titre and lymphocyte infiltration in ALD. An auto-immune disease in some patients? J Hepatol 40 (suppl.1):176.
9. Beaune, P.H., P.M. Dansette, D. Mansuy, L. Kiffel, M. Finck, C. Amar, J.P. J.P. Leroux, and J.C. Homberg. 1987. Human antiendoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome that hydroxylates the drug. Proc Natl Acad Sci USA 84:551-555.
10. Leeder, J., S.R.J. Riley, V.A. Cook, and S.P. Spielberg. 1992. Human anticytochrome P450 antibodies in aromatic anticonvulsant-induced hypersensitivity reactions. J Pharm Exp Ther 263:360-367.

11. Lytton, S.D., U. Berg, A. Nemeth, and M. Ingelman-Sundberg. 2002. Autoantibodies against cytochrome P450s in sera of children treated with immunosuppressive drugs. Clin Exp Immunol 127:293-302.
12. Manns, M.P., and P. Obermayer-Straub. 1997. Cytochromes P450 and uridine triphosphate-glucuronosyl-transferases: model autoantigens to study drug-induced, virus-induced and autoimmune liver disease. Hepatol 26:1054-1066.
13. Winqvist, O., J. Gustafsson, F. Rorsman, F.A. Karlsson, and O. Kämpe. 1993 Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addisson's disease. J Clin Invest 92:2377-2385.
14. Uibo, R., E, Aavik, P. Peterson, J. Perheentupa, S. Aranko, R. Pelkonen, and K.J. Krohn. 1994. Autoantibodies to cytochrome P450 enzymes P450ssc, P450c17 and P450c21 in autoimmune polyglandular disease type I and II and in isolated Addison's disease. J Clin Endocrinol Metabol 78:323-328.
15. Lecoeur, S., C. Andre, and P.H. Beaune. 1996. Tienilic acid-induced autoimmune hepatitis: anti-liver and anti-kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9. Mol Pharmacol 50:326-333.
16. Leeder, J.S., A. Gaedigk, X. Lu, and V.A. Cook. 1996. Epitope mapping studies with human anti-cytochrome P450 3A antibodies. Mol Pharmacol 49:234-243.
17. Belloc, C., A. Gauffre, C. André, and P.H. Beaune. 1997. Epitope mapping of human CYP1A2 in dihydralazine-induced autoimmune hepatitis. Pharmacogenetics 7:181-186.
18. Manns, M.P., K.J. Griffin, K.F. Sullivan, and E.F. Johnson. 1991. LKM-1 autoantibodies recognize a short linear sequence in P450IID6, a cytochrome P-450 monoxygenase. J Clin Invest 88:1370-1378.
19. Yamamoto, AM, D. Cresteil, J.C. Homberg, and F. Alvarez. 1993. Characterization of anti-liver-kidney microsome antibody (anti-KLM1) from hepatitis C virus-positive and negative sera. Gastroenterol 104:1762-1767.
20. Ma, Y., M.G. Thomas, M. Okamoto, D.P. Bogdamos, S. Nagl, N. Kerkar, A.R. Lopes, L. Muratori, F.B. Bianchi, G. Mieli-Vergani, and D. Vergani. 2002. Key residues of a major cytochrome P4502D6 epitope are located on the surface of the molecule. J Immunol 169:277-285.
21. Kerkar, N., K. Choudhuri, Y. Ma, A. Mahmoud, D.P. Bogdamos, L. Muratori, F.B. Bianchi, R. Williams, G. Mieli-Vergani, and D. Vergani. 2003. Cytochrome P4502D6$_{193-212}$: a new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type 1-positive liver disease. J Immunol 170:1481-1489.

22. Kenna, J.G., J. Neuberger, and R. Williams. 1988. Evidence for expression in human liver of halothane-induced neoantigens recognised by antibodies in sera from patients with halothane hepatitis. Hepatol 8:1635-1641
23. Smith, G.C.M., J.G., Kenna, D.J. Harrison, D, Tew, and C.R. Wolf. 1993. Autoantibodies to hepatic microsomal carboxylesterase in halothane hepatitis. Lancet 342:963-964
24. Williams, P.A., J. Cosme, V. Sridhar, E.F. Johnson, and D.E. McRee. 2000. Mammalian cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. Molecular Cell 5:121-131.
25. Barnes HJ. 1996. Maximizing expression of the eukaryotic cytochrome P450 in Escherichia Coli. Methods Enzymol 272:3-14.
26. Gillam, E.M., Z. Guo, and F.P. Guengerich. 1994. Expression of modified human cytochrome P450 2E1 in Escherichia Coli, purification, and spectral and catalytic properties. Arch Biochem Biophys. 312:59-66
27. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. Evidence of its hemoprotein nature. J Biol Chem 239:2370-2378.
28. Lewis, D.F.V., B.G. Lake, M.G. Bird, G.D. Loizou, M. Dickins, and P.S. Goldfarb. 2003. Homology modelling of human CYP2E1 based on the CYP2C5 crystal structure: investigation of the enzyme-substrate and enzyme-inhibitor interactions. Toxicol In Vitro 17:93-105.
29. Jameson BA, and H. Wolf. 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. Comput Appl Biosci 4:181-186.
30. Duclos-Vallée, J.C., O. Hajoui, A.M. Yamamoto, E. Jacqz-Aigrain, and F. Alvarez. 1995. Conformational epitopes on CYP2D6 are recognized by liver/kidney microsomal antibodies. Gastroenterol 108:470-476.
31. Neve, E.P.A., M. Hidestrand, and M, Ingelman-Sundberg. 2003. Identification of sequences responsible for intracellular targeting and membrane binding of rat CYP2E1 in yeast. Biochem 42:14566-14575.
32. Wu, D.F., and A.I. Cederbaum. 1992. Presence of functionally active cytochrome P-450IIE1 in plasma membrane of rat hepatocytes. Hepatol 15:515-524.
33. Clot, P., M. Parola, G. Bellomo, U. Dianzani, R. Carini, M. Tabone, S. Aricò, M. Ingelman-Sundberg, and E. Albano. 1997. Plasma membrane hydroxyethyl radical adducts cause antibody-dependent cytotoxicity in rat hepatocytes exposed to alcohol. Gastroenterol 113:265-276.
34. Griem, P., M. Wulferink, B. Sachs, J.B. Gonzalez, and E. Gleichmann. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? Immunol Today 19:133-141.

35. Van Pelt, F.N.A.M, P. Straub, and M.P. Manns. 1995. Molecular basis of drug-induced immunological liver injury. Sem Liver Dis 15: 283-300.
36. Beaune, P., D. Pessayre, P. Dansette, D. Mansuy, and M. Manns. 1994. Autoantibodies against cytochrome P450: role in human diseases. Adv Pharmacol 30:199-245.
37. Kenna, J.G., J. Neuberger, and J. Williams. 1987. Specific antibodies to halothane-induced liver antigens in halothane-associated hepatitis. Br J Anaesth 59:1268-1290.
38. Clot, P., E. Albano, E. Eliasson, M. Tabone, S. Arico, Y. Israel, C. Moncada, and M. Ingelman-Sundberg. 1996. Cytochrome P4502E1 (CYP2E1) hydroxyethyl radical adducts as the major antigenic determinants for autoantibody formation among alcoholics. Gastroenterol 111:206-216.
39. Waterhouse, P., L.E.M. Marengère, H.W. Mittrücker, and T.W. Mak. 1999. CTLA-4, a negative regulator of T-lymphocyte activation. Immunol Review 153:183-207.
40. Egen, J.G., M.S. Kuhns, and J.P. Allison. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. Nature Immunol 3:611-618.

Table 1

Mutations inserted in CYP2E1 molecule and DNA primers used for in situ mutagenesis reactions

N	Mutations	Primers
1*	K234 AAA → A234 **GC**G	CTTGCCTGGAAGCCACAGA**GC**AGTCATAAAAAATGTGGCTG CAGCCACATTTTTTATGACT**GC**TCTGTGGCTTCCAGGCAAG
2*	K243 AAA → A243 **GC**A + E244 GAG → A244 G**C**G	AATGTGGCTGAAGTA**GC**AG**C**GTATGTGTCTGAAAGGGTGAAGGAGC GCTCCTTCACCCTTTCAGACACATAC**G**CT**GC**TACTTCAGCCACATT
3*	L243 AAA → A243 **GC**A + E244 GAG → A244 G**C**G + E248 GAA → A248 G**C**A + K251 AAG → A251 **GC**G	GCAGCGTATGTGTCTG**C**AAGGGTG**GC**GGAGCACCATCAATC GATTGATGGTGCTCC**GC**CACCCTT**G**CAGACACATACGCTGC
4	E252 GAG → A252 G**C**G	GTCTGAAAGGGTGAAGG**C**GCACCATCAATCTCTGG CCAGAGATTGATGGTGC**G**CCTTCACCCTTTCAGAC
5	R263 CGG → A263 **GC**G	GACCCCAACTGTCCC**GC**GGACCTCACCGACTG CAGTCGGTGAGGTCC**GC**GGGACAGTTGGGGTC
6*	E272 GAA → A272 G**C**A	GACTGCCTGCTCGTGG**C**AATGGAGAAGGAAAAGC GCTTTTCCTTCTCCATT**G**CCACGAGCAGGCAGTC
7*	E320 GAG → A320 G**C**G	CTCATGAAATACCCTG**C**GATCGAAGAGAAGCTCC GGAGCTTCTCTTCGATC**G**CAGGGTATTTCATGAG
8*	K324 AAG → A324 **GC**G	CCCTGAGATCGAAGAG**GC**GCTCCATGAAGAAATTGACAGGG CCCTGTCAATTTCTTCATGGAGC**GC**CTCTTCGATCTCAGGG
9	H326 CAT → A326 **GC**T + E327 GAA → A327 G**C**A	CCCTGAGATCGAAGAGAAGCTC**GC**TG**C**AGAAATTGACAGGGTG CACCCTGTCAATTTCT**G**CA**GC**GAGCTTCTCTTCGATCTCAGGG
10*	R331 AGG → A331 **GC**G	GCTCCATGAAGAAATTGAC**GC**GGTGATTGGGCCAAGCCG CGGCTTGGCCCAATCACC**GC**GTCAATTTCTTCATGGAGC
11*	K342 AAA → A342 **GC**G	GCCGAATCCCTGCCATC**GC**GGATAGGCAAGAGATGC GCATCTCTTGCCTATCC**GC**GATGGCAGGGATTCGGC
12*	R344 AGG → A344 **GC**G	CCCTGCCATCAAGGAT**GC**GCAAGAGATGCCCTACATGG CCATGTAGGGCATCTCTTGC**GC**ATCCTTGATGGCAGGG
13*	E346 GAG → A346 G**C**G	CCATCAAGGATAGGCAAG**C**GATGCCCTACATGGATGC GCATCCATGTAGGGCATC**G**CTTGCCTATCCTTGATGG
14	H355 CAT → A355 **GC**T	CCTACATGGATGCTGTGGTG**GC**TGAGATTCAGCGGTTCATC GATGAACCGCTGAATCTCA**GC**CACCACAGCATCCATGTAGG
15*	R374 CGA → A374 **GC**G	CCTGCCCCATGAAGCAACC**GC**AGACACCATTTTCAGAGG CCTCTGAAAATGGTGTCT**GC**GGTTGCTTCATGGGGCAGG
16	S395 TCT → F395 T**T**T	CGTAGTGCCAACTCTGGACT**T**TGTTTTGTATGACAACC GGTTGTCATACAAAACA**A**AGTCCAGAGTTGGCACTACG
17*	K420 AAG → A420 **GC**G	CCAGAACACTTCCTGAATGAAAATGGA**GC**GTTCAAGTACAGTGAC GTCACTGTACTTGAAC**GC**TCCATTTTCATTCAGGAAGTGTTCTGG
18*	F421 TTC → A421 **GC**C	CCTGAATGAAAATGGAAAG**GC**CAAGTACAGTGACTATTTCAAGCC GGCTTGAAATAGTCACTGTACTTG**GC**CTTTCCATTTTCATTCAGG
19*	E440 GAA → A440 G**C**A	GAGTGTGTGCTGGAG**C**AGGCCTGGCTCGC GCGAGCCAGGCCT**G**CTCCAGCACACACTC
20	R444 CGC → A444 **GC**C	GCTGGAGAAGGCCTGGCT**GC**CATGGAGTTGTTTCTTTTG CAAAAGAAACAACTCCATG**GC**AGCCAGGCCTTCTCCAGC

* The CYP2E1 containing these mutations showed adsorption spectra comparable to wild-type CYP2E1 and were used in further experiments.

Table 2

Effect of selected CYP2E1 mutations that identify conformational epitopes in, respectively, the J'-K" (Lys420, Lys342) and G (Lys243, Glu244, Glu248 and Lys251) helices on the antigen recognition by additional sera from patients with halothane hepatitis (HAL) or alcoholic liver disease (ALD).

Sera | Mutations

	Lys420	Lys342	Lys243+Glu244+Glu248+Lys251
HAL6	100%	62%	**59%**
HAL7	**53%**	71%	84%
HAL8	75%	78%	78%
HAL9	72%	100%	**46%**
HAL10	**20%**	85%	100%
ALD6	81%	91%	100%
ALD7	68%	100%	86%
ALD8	83%	**54%**	**43%**
ALD9	**45%**	89%	100%
ALD10	**56%**	100%	78%
ALD11	63%	100%	**49%**
ALD12	**38%**	100%	80%

Ten pmol of wild-type or mutated CYP2E1s were reacted with 5 µL of the different human sera (1:20 dilution) and the immunocomplexes recovered by protein A Sepharose beads were estimated by western blotting using a monoclonal mouse IgG toward CYP2E1 His-6 tag tail. The intensities of the bands were measured by videodensitometry and the results expressed as percent of the recovery of modified CYP2E1 as compared to the wild-type protein included as reference in each blot. The black squares indicated a reduction in the antibody recognition of CYP2E1 ≥ to 50% and the grey squares a reduction between 40 and 50%.

Legends to figures

Figure 1: Immunoprecipitation experiments showing the recognition of [^{35}S]-methionine-labelled CYP2E1 translated "*in vitro*" using the rabbit reticulocyte system (Top panel) by 5 sera from patients with alcoholic liver disease (ALD) and 5 sera from patients with halothane-induced hepatitis (HAL). The lower panel shows the relative capacity of human sera to immunoprecipitate the N-terminal or the C-terminal moieties of [^{35}S]-CYP2E1.

Figure 2: Computer simulation of the CYP2E1 three dimensional structure generated using the crystal structure of rabbit CYP2C5 as a template and the Swiss-Model automated comparative protein modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL/.html). The CYP2E1 structure overlapping that of CYP2C5 is shown in white. The sequences of CYP2E1 differing from CYP2C5 as shown in red, such as the loop between Lys-277 and Tyr-285 and the helix between Pro-213 and Pro-222, that was modified in CYP2C5 in order to get the protein crystallized [22].

Figure 3: Effect of single amino acid substitutions on the recognition of recombinant human CYP2E1 by the sera of 5 patients with alcoholic liver disease (ALD) and 5 patients with halothane-induced hepatitis (HAL). Ten pmol of wild-type or mutated CYP2E1s were incubated with 5 μL of the different human sera (1:20 dilution) and the immunocomplexes recovered by protein A Sepharose beads were estimated by western blotting using a monoclonal mouse IgG targeting the CYP2E1 His-6-tag tail. The intensities of the bands were measured by videodensitometry and the results were expressed as percent of the recovery of modified CYP2E1 as compared to the wild-type protein included as reference in each blot. The black squares indicate a reduction in the antibody recognition ≥ 50% and the grey squares a reduction between 40 and 50%.

Figure 4: Localization of the two conformational epitopes identified by the site-directed mutagenesis experiments on the CYP2E1 three dimensional simulated structure. The top panel shows the epitope formed by the juxtaposition of the J' and K" helices (light grey) identified by the mutations of Lys324, Lys342, Lys420 and Phe421 (dark grey), while the lower panel shows the epitope in the G helix (light grey) identified by the combined substitutions of Lys243, Glu244, Glu248 and Lys251 (dark grey).

Figure 5: Effect of the combined substitutions of several amino acids located in the G helix on human CYP2E1 recognition by the sera of 5 patients with alcoholic liver disease (ALD) and 5 patients with halothane-induced hepatitis (HAL). Ten pmol of wild-type or mutated CYP2E1s were incubated with 5 μL of the different human sera (1:20 dilution) and the immunocomplexes recovered by protein A Sepharose beads were estimated by western blotting using a monoclonal mouse IgG toward CYP2E1 6-His-tag tail. The intensities of the bands were measured by videodensitometry and the results were expressed as percent of the recovery of modified CYP2E1 as compared to the wild-type protein included as reference in each blot. * indicates the substitution of Lys243 and Glu244 for Ala; ** indicates the combined substitution of Lys243, Glu244, Glu248 and Lys251 for Ala.

Figure 6: Localization of the two conformational epitopes identified in the G and J'-K" helices, respectively, in relation to CYP2E1 orientation with respect to the cell membranes proposed by Neve et al [28].

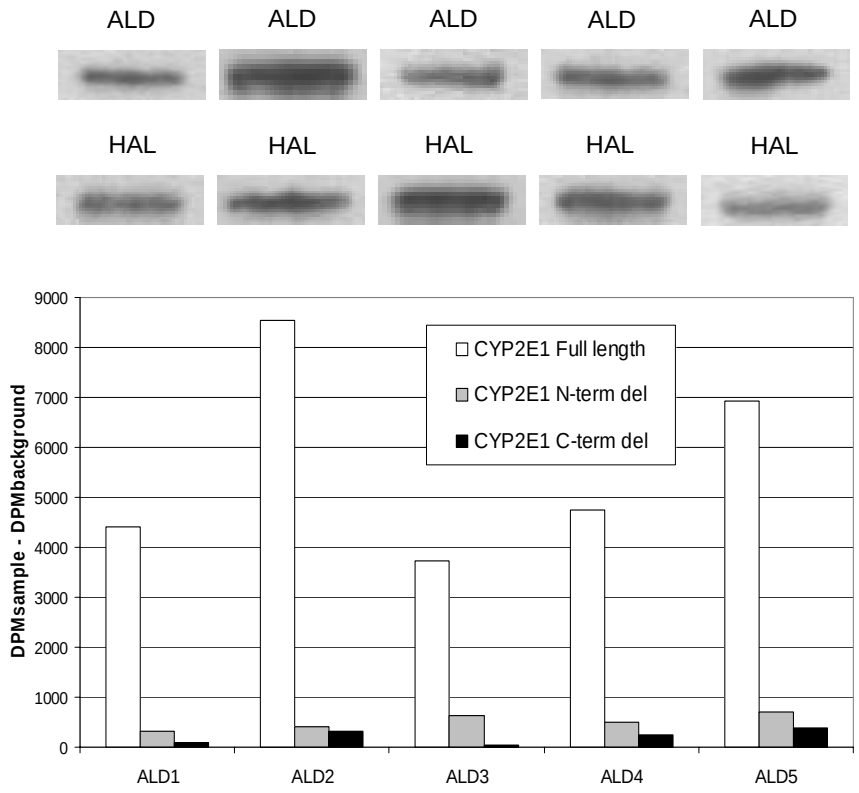
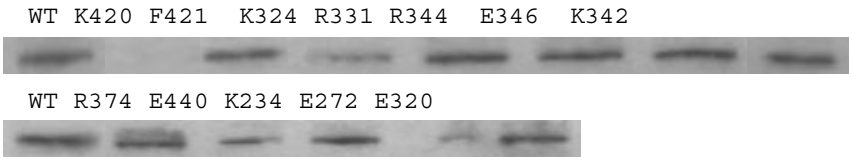

ALD
ALD
ALD
ALD
ALD
HAL
HAL
HAL
HAL
HAL
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
DPMsample - DPMbackground
CYP2E1 Full length
CYP2E1 N-term del
CYP2E1 C-term del
ALD1
ALD2
ALD3
ALD4
ALD5

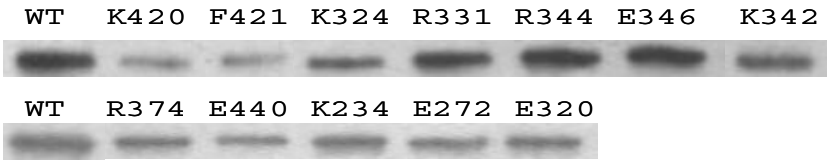
Figure 1

Figure 2

HAL5



ALD2



	Lys-420	Phe-421	Lys-324	Arg-331	Arg-344	Glu-346	Lys-342	Arg-374	Glu-440	Lys-234	Glu-272	Glu-320
HAL1	**46%**	100%	83%	96%	90%	84%	**50%**	77%	98%	80%	100%	80%
HAL2	100%	**52%**	95%	91%	79%	100%	100%	64%	100%	80%	**60%**	79%
HAL3	**55%**	96%	**53%**	100%	100%	100%	**52%**	100%	72%	80%	100%	100%
HAL4	95%	100%	73%	85%	78%	65%	**50%**	**53%**	96%	100%	**44%**	68%
HAL5	**0%**	100%	**31%**	100%	100%	100%	74%	100%	**57%**	100%	**26%**	78%
ALD1	92%	**60%**	69%	83%	70%	100%	**56%**	**44%**	**50%**	100%	92%	100%
ALD2	**23%**	**22%**	**51%**	92%	100%	99%	62%	94%	63%	100%	92%	100%
ALD3	**45%**	91%	79%	100%	96%	**42%**	**7%**	74%	71%	100%	66%	**55%**
ALD4	**48%**	100%	**26%**	91%	89%	78%	**29%**	82%	89%	100%	75%	**45%**
ALD5	**56%**	71%	62%	93%	62%	100%	67%	**59%**	80%	**43%**	**44%**	99%

Figure 3

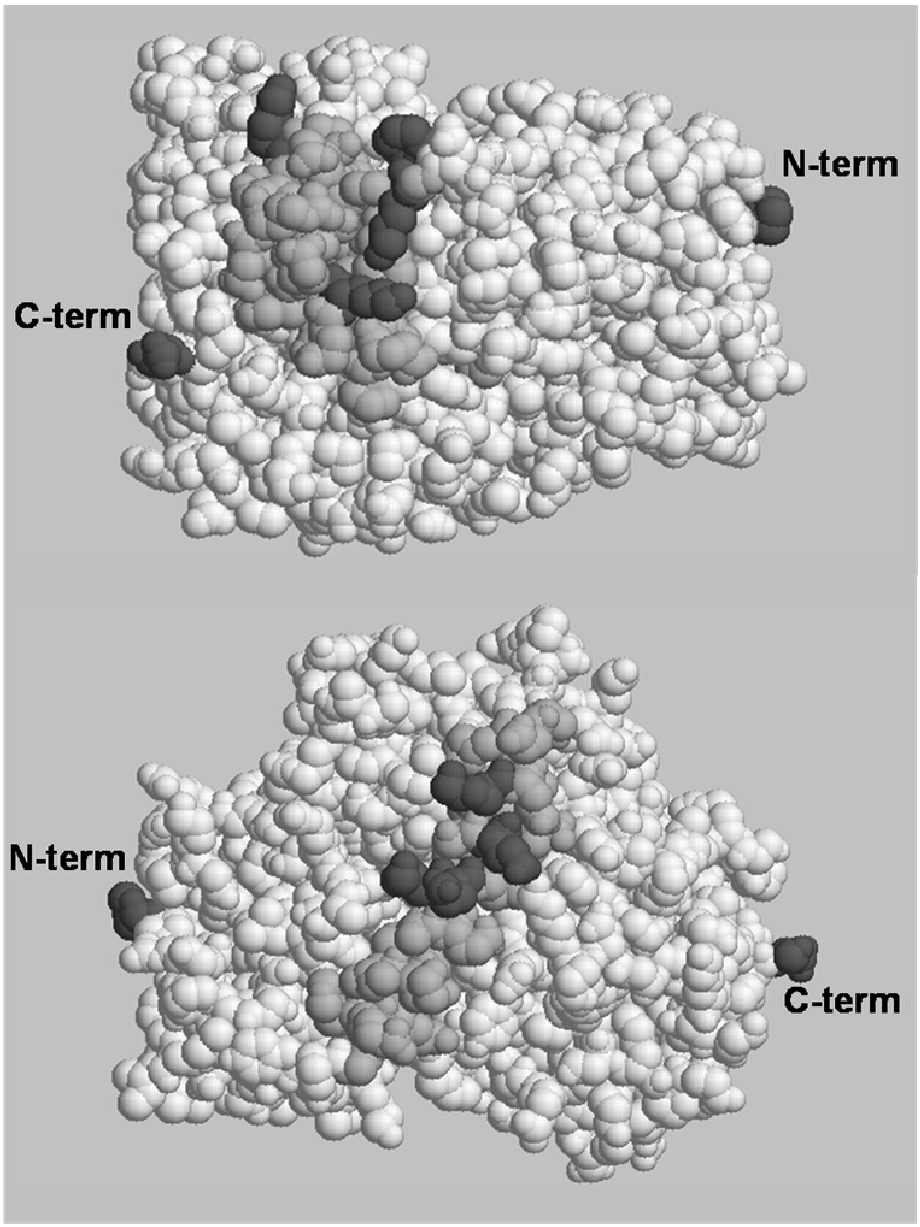
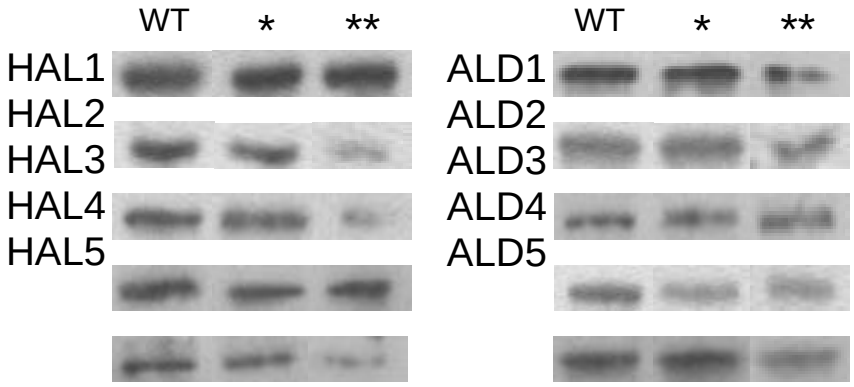


Figure 4



	WT	*	**
HAL1	100%	100%	100%
HAL2	100%	77%	19%
HAL3	100%	100%	29%
HAL4	100%	86%	86%
HAL5	100%	70%	27%

	WT	*	**
ALD1	100%	80%	46%
ALD2	100%	100%	40%
ALD3	100%	100%	100%
ALD4	100%	70%	58%
ALD5	100%	100%	51%

*Ala → Lys-243, Glu-244
**Ala → Lys-243, Glu-244, Glu-248 and Lys-251

Figure 5

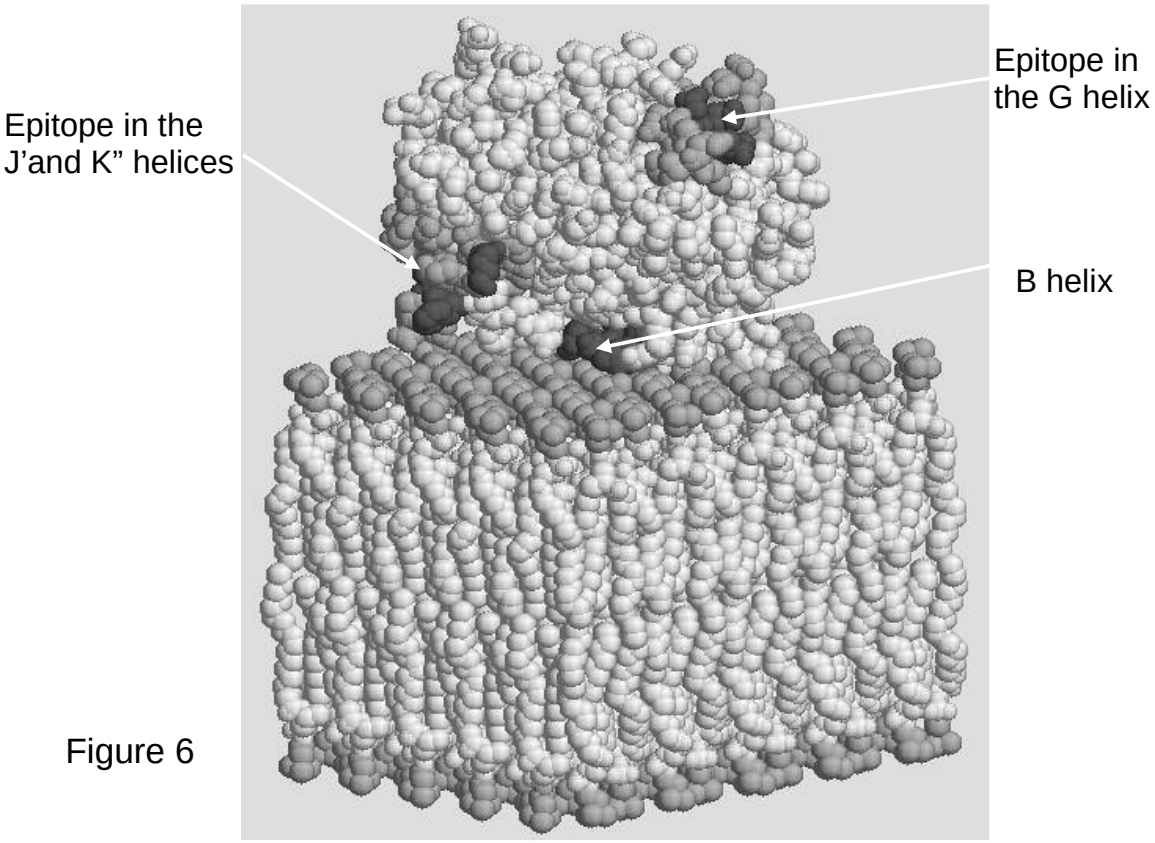


Figure 6