EXPERIMENTAL STUDY

Analysis of the interaction between human steroid 21-hydroxylase and various monoclonal antibodies using comparative structural modelling

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Abstract

Objective: To study the interaction between human steroid 21-hydroxylase (21-OH) and monoclonal antibodies (MAbs) to 21-OH directed to 3 different epitopes recognised by 21-OH autoantibodies characteristic of autoimmune Addison's disease.

Design: Build comparative structural models of 21-OH, 21-OH MAbs and complexes of 21-OH–21-OH MAbs and study the effects of 21-OH MAbs on 21-OH enzyme activity. Then, analyse the relationship between sites important for binding of 21-OH MAbs and 21-OH autoantibodies and sites important for 21-OH enzyme activity.

Methods: Variable (V) regions of 21-OH MAbs (M21-OH1, M21-OH3, M21-OH5) were sequenced and models of the MAbs built using structures of antibodies in the database as templates. A comparative model of 21-OH was built using the crystal structure of rabbit cytochrome p450 2c5/3LVdH as template. 21-OH enzyme activity was measured in terms of conversion of [³H]progesterone to deoxycorticosterone and the effect of purified MAb IgGs on 21-OH enzyme activity was assessed.

Results: M21-OH1, M21-OH3 and control MAb had no effect on 21-OH enzyme activity with 88.8%±24% (*n* = 6), 86.7%±7.6% (*n* = 6) and 86.5%±10.6% (*n* = 6) of activity remaining in the presence of the respective IgGs. This was consistent with the epitopes for M21-OH1 and M21-OH3 being located distant from 21-OH enzyme active sites in our 21-OH model. The epitope for M21-OH5 which inhibited 21-OH enzyme activity (48.5±8.3% activity remaining; *P* < 0.001 compared with control MAb IgG) was found close to the redox protein binding site in our 21-OH model.

Conclusions: A comparative model of 21-OH has been produced. Analysis of experimental data in the context of the model suggests that M21-OH5 inhibits 21-OH enzyme activity through interference with redox protein binding.

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Introduction

Autoantibodies to steroid 21-hydroxylase (21-OH) are characteristic of autoimmune Addison's disease (1). 21-OH autoantibodies react with conformational epitopes located predominantly in the central and the C-terminal parts of 21-OH (amino acids (aa) 280–495) and have the ability to inhibit 21-OH enzyme activity *in vitro* (2–5). Some of the mouse monoclonal antibodies (MAbs) to 21-OH inhibit binding of 21-OH autoantibodies to 21-OH and the epitopes recognised by these MAbs involve three 21-OH aa regions: aa 391–405 (epitope region (ER) 1), aa 406–411 (ER2) and aa 335–339 (ER3) (6). Previous studies have indicated that ER2 and ER3 are parts of two distinct major epitopes recognised by 21-OH autoantibodies while ER1 is part of a minor epitope (6).

In order to gain a better insight into the interaction between 21-OH MAbs and 21-OH, we have constructed structural models of 21-OH, 21-OH MAbs and the MAbs in complex with 21-OH. The effect of 21-OH MAbs on 21-OH enzyme activity has also been studied. The relationship between the sites on the 21-OH molecule recognised by 21-OH MAbs and the sites recognised by 21-OH autoantibodies was then analysed.

Materials and methods

21-OH MAb preparation and characterisation

21-OH MAbs (M21-OH1, -3 and -5) were obtained as described previously (6) from BALB/C mice immunised with highly purified recombinant human 21-OH-GST fusion proteins. IgGs were isolated from the culture

supernatants using chromatography on ProsepA (Millipore UK Ltd, Watford, Herts, UK) according to the manufacturer's instructions, dialysed against phosphate buffered saline (PBS; 8.1 mmol/l Na_2HPO_4 , 1.5 mmol/l KH_2PO_4 , 2.7 mmol/l KCl, 137 mmol/l NaCl, pH 7.3) and stored in aliquots at -70°C (6).

Total RNA was prepared from 1×10^7 hybridoma cells using the acid phenol guanidine method (7). RT-PCR reactions were performed using reagents from Invitrogen Ltd (Paisley, Strathclyde, UK). Sense and antisense primers including additional restriction endonuclease site sequences to facilitate cloning of PCR products were synthesised according to Kettleborough *et al.* (8). The complete panel of heavy chain (HC) and light chain (LC) gene primers were used in RT-PCR and the products cloned into pUC18 and sequenced as described before (9). Variable (V) region sequences were compared with available sequences of mouse immunoglobulin genes using Ig blast (<http://www.ncbi.nlm.nih.gov/igblast/>).

A recombinant Fab of M21-OH5 was produced in *Escherichia coli* using a previously described method (10). The HC and the LC RT-PCR products were cloned into ImmunoZap H/L vector (Stratagene Europe, Amsterdam, The Netherlands) under the *lacZ* promoter and the cloning confirmed by sequencing. M21-OH5 Fab was expressed in HB2151 cells (Amersham Biosciences) using conditions as described previously (10). M21-OH5 Fab preparations contained in the bacterial cell extract in PBS with 1% Triton X-100 and 1 mmol/l phenylmethylsulphonyl fluoride (PMSF) were analysed for 21-OH binding activity as described before (6).

The characteristics of the 21-OH MABs studied, i.e. the isotype, the LC type, 21-OH binding affinity and the epitopes recognised are summarised in Table 1. The effects of MAB F(ab')₂ on binding of 21-OH autoantibodies to ^{125}I -21-OH was tested in an immunoprecipitation assay as described before (6, 11) using reagents from RSR Ltd (Cardiff, UK) (Table 1). In addition, the effects of the MABs on 21-OH enzyme activity were studied by incubating MAB IgGs with the 21-OH preparations (12). In these experiments, microsomes prepared from 21-OH expressing yeast (0.5 μg protein) were incubated with IgG preparations (100 μg in 50 μl PBS) at 0°C for 16 h. 21-OH enzyme activity in microsomal preparations was then measured in terms of conversion of [^3H]progesterone to deoxycorticosterone as described before (12, 13).

Control experiments using IgGs obtained from a 21-OH antibody (Ab)-positive patient with autoimmune Addison's disease and from healthy blood donors were also carried out, as described previously (12). MAB (IgG preparation) reactive with human acetylcholine receptor (4B7-2C4; RSR Ltd) was used as a negative control.

Modelling of 21-OH and 21-OH MABs

The amino acid (aa) sequence of human steroid 21-OH (14) was taken from the SWISSPROT/TrEMBL data base (<http://www.ebi.ac.uk/swissprot/>). A search for conserved residues with homologous cytochrome p450 proteins was carried out by BLAST (15). The crystallographic structure of rabbit cytochrome p450 2c5/3LVdH (2c5/3) which has 29.2% amino acid sequence identity with human steroid 21-OH was used as a homologue for the structural modelling (16–18). The homologue's structure lacks a trans-membrane helix (aa 3–21) that had been deleted to increase the solubility of the protein for crystallisation purposes (17). Several steps were followed in order to obtain the preliminary alignment. First, an alignment between rabbit cytochrome p450 2c5/3 and 21-OH with the program FUGUE (19) was carried out. Then, an alignment between 21-OH and its protein family using a BLAST search followed by an alignment between the P450 family from the HOMSTRAD (20, 21) database and the previously produced 21-OH family alignment was carried out. A combination of these alignments allowed us to obtain a final alignment. The program SCORE (22) produced the core of the protein. The program CODA (23) was used to obtain the loops not present in the SCORE output. A final energy-geometry minimisation was done by Tripos force field (24). The model was verified with PROCHECK (25), VERIFY3D (26) and JOY (27) and the alignment modified as required. This process was repeated three times. Three loops of the structure were modified using the SYBYL loop database (24) and a final minimisation was carried out. The trans-membrane helix of 21-OH (aa 3–21) was modelled using the N-terminal fusion peptide of HIV-1 gp41 (28) as a homologue. The haem group taken from rabbit cytochrome p450 2c5/3 was incorporated using the program MNYFIT (29).

21-OH MAB V region sequences were used to search for the most suitable templates from the HOMSTRAD

Table 1 Characteristics of mouse MABs to 21-OH.

21-OH MAB	Isotype	Affinity constants	Epitope region	Inhibition of ^{125}I -21-OH binding to 21-OH AAB*
M21-OH1	IgG2a/ κ	7.0×10^8 l/mol	391–405 aa	0–50% ($n = 25$)
M21-OH3	IgG1/ κ	2.6×10^8 l/mol	406–411 aa	27%–75% ($n = 25$)
M21-OH5	IgG1/ κ	2.3×10^7 l/mol	335–339 aa	35%–92% ($n = 25$)

*The range of inhibitions obtained with 25 different 21-OH autoantibody (AAB) positive sera is shown. See reference (6) for details.

(20, 21) and BLAST (15) databases. Alignments were obtained using FUGUE (19). The MAb models were built using MODELLER (30) and RAPPER (31, 32) and validated with PROCHECK (25) and VERIFY3D (26). HBPLUS was used for hydrogen bond definition (33). Program HERA was used for topology calculation and secondary structure representation (34). The interface solvent accessible surface area (ASA) was calculated using the Richmond algorithm (35) based on the Lee and Richards definition (36). The contact residues were defined as the residues that possess an ASA that decreased by more than $1\text{\AA}^2$ on complexation (37). GRASP (38) was used to study the electrostatic potential surfaces of 21-OH and the combining regions of MAb variable regions.

Protein–protein docking was carried out using Global Range Molecular Matching (GRAMM) methodology (39). The graphical program RASMOL (40) was used for visualisation of protein–protein complexes and the program MNYFIT (29) was used for superimposing the co-ordinates of peptide chains.

Results

21-OH MAb characteristics

As shown in Fig. 1, the 21-OH MAb M21-OH5 but not M21-OH1 or M21-OH3 had an effect on 21-OH enzyme activity relative to the effect of a control MAb IgG. Briefly, in the presence of $100\text{ }\mu\text{g}$ IgGs from M21-OH1, M21-OH3 or M21-OH5 the remaining 21-OH enzyme activity was $88.8\% \pm 24\%$ (mean $\pm$ s.d.; $n = 5$), $86.7\% \pm 7.6\%$ (mean $\pm$ s.d.; $n = 6$) and $48.5\% \pm 8.3\%$ (mean $\pm$ s.d.; $n = 6$) respectively. This can be compared with $86.5\% \pm 10.6\%$ (mean $\pm$ s.d.; $n = 6$) of the remaining 21-OH enzyme activity in the presence of $100\text{ }\mu\text{g}$ control MAb IgG. The difference in 21-OH enzyme

Figure 1 Effect of different IgG preparations on 21-OH enzyme activity. M21-OH1, M21-OH3 and M21-OH5 are mouse monoclonal antibodies to 21-OH. Control Mab, mouse monoclonal antibody to the acetylcholine receptor. Results are shown as means $\pm$ s.d. of enzyme activity after incubation with test IgG ($100\text{ }\mu\text{g}$); see text for details.

activity remaining in the presence of M21-OH5 IgG compared with control MAb IgG was statistically significant ($P < 0.001$; Student's *t*-test) but this was not so in the presence of M21-OH1 ($P = 0.88$) and M21-OH3 ($P = 0.98$). In the same series of experiments, incubation of 21-OH with $100\text{ }\mu\text{g}$ IgG from a 21-OH autoantibody-positive patient reduced 21-OH enzyme activity to 15% of the level observed after incubation with $100\text{ }\mu\text{g}$ healthy blood donor IgG.

The V regions of the HC and LC of each 21-OH MAb were analysed as shown in Tables 2 and 3. M21-OH1 HC genes were derived from the J558.47 germline, JH genes were from JH3 and the D gene could not be assigned. The LC genes were from cr1 and JK2 germ-lines. M21-OH3 used VH7183.9, DSP2.6, JH3 (the HC genes) and 21-12 and JK2 (the LC genes) germ-lines. M21-OH5 used VHGa155.1, DSP2.6 and JH3 germ-lines for the HC and 8-30 and JK2 germ-lines for the LC. The homology of the V region genes to their respective germ-lines as well as an analysis of replacement/silent (R/S) mutation ratio and amino acid substitution within the framework and complementarity determining regions (CDRs) are shown in Table 2. The aa sequences of HC and LC CDRs (41) of the three 21-OH MAbs are shown in Table 3.

Extracts from *E. coli* transformed with M21-OH5 HC and LC sequences obtained from RT-PCR carried out on hybridoma RNA showed 21-OH binding activity. In particular, 9.1% binding of ^{125}I -labelled 21-OH was observed with Fab preparations contained in undiluted bacterial extract, 7.8% with Fab preparations diluted 1:10 and 4.5% with Fab preparations diluted 1:100 (Fig. 2). Hybridoma-derived M21-OH5 IgG at concentrations of $1\text{ }\mu\text{g/ml}$, $0.1\text{ }\mu\text{g/ml}$ and $0.01\text{ }\mu\text{g/ml}$ showed 12.1%, 7.0% and 4.2% binding of ^{125}I -21-OH respectively (Fig. 2). Control recombinant Fab with the thyrotrophin (TSH) receptor antibody activity prepared using the same method as M21-OH5 Fab (10) showed approximately 3% binding of ^{125}I -labelled 21-OH at different dilutions. In addition, undiluted and diluted 1:3 preparations of recombinant M21-OH5 Fab inhibited binding of M21-OH5 IgG to ^{125}I -21-OH by 64% and 49% respectively. Control Fab preparations at similar dilutions showed approximately 20% inhibition of M21-OH5 IgG binding to 21-OH (data not shown).

21-OH modelling

The validation parameters of the 21-OH model after incorporation of the transmembrane helix and the haem group are shown in Table 4. The overall fold of 21-OH can be divided into two domains, a helix-rich domain that contains the haem group and a β -sheet domain consisting mainly of antiparallel β strands which is involved mostly in interaction with the membrane (Fig. 3A). A core structure, conserved within the P450 cytochrome family, which is formed by a

Table 2 Characteristics of variable region genes of 21-OH MABs.

Antibody	Chain	Germline	Homology at oligonucleotide level	V(D)J R/S mutations ratio in framework and CDRs	No. of mutations in framework (replacement)	No. of mutations in CDRs (replacement)
M21-OH1	HC	J558.47	96%			
		D?	0%	3.3	8 (6)	5 (4)
		JH3	100%			
M21-OH1	LC	cr1	95%	2.5	5 (3)	9 (7)
		JK2	100%			
M21-OH3	HC	VH7183.9	93%			
		DSP2.6	92%	4.0	6 (3)	14 (13)
		JH3	90%			
M21-OH3	LC	21-12	97%	6*	3 (3)	3 (3)
		JK2	100%			
M21-OH5	HC	VHGa155.1	97%			
		DSP2.6	67%	7*	3 (3)	4 (4)
		JH3	100%			
M21-OH5	LC	8-30	95%	8	5 (4)	4 (4)
		JK2	97%			

*All mutations were replacement mutations.

Germline sequences were assigned using Ig blast, CDRs were assigned by the method of Kabat *et al.* (41).

four-helix bundle (D, E, I, L; Fig. 3B), two helices (J, K; Fig. 3B), two sets of β -sheets and a coil called 'meander' (K'-L loop; Fig. 3B) is present around the haem group (42). The haem-binding loop ('meander') contains the most characteristic pattern for the cytochrome P450 family (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly) (PROSITE database PS0086 and the Functional Protein Sequence Pattern Database (FPSPD); <http://www-cryst.bioc.cam.ac.uk/~fpspd/>; FPSPD06206) (43, 44) and is located before helix L with Cys 428 that serves as fifth ligand to the haem iron. The absolutely conserved motif (Glu-X-X-Arg) (FPSPD06209) in helix K is needed to stabilise the core structure.

The central part of helix I contains the P450 signature: Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser (FPSPD06208) that corresponds to the proton transfer groove of the haem group (42).

21-OH has a triangular prism shape with an edge length approximately double the thickness. The entrance to the active site of 21-OH is present on one of the flat surfaces of the prism, referred to as the distal surface (17) whereas the binding site for the redox partner protein is present on the opposite flat surface of the prism, referred to as the proximal surface (Fig. 4). The epitopes ER1 and ER2 are positioned on the 21-OH surface distant from the

Table 3 CDRs of 21-OH MABs assigned by the method of Kabat *et al.* (41).

Antibody	Chain	CDR sequences
M21-OH1	HC	CDR1 = SYVMH CDR2 = YINPFNDTLYNEKFKG CDR3 = VT
M21-OH1	LC	CDR1 = SSSQSLVRTSGNTYLD CDR2 = KVSNRFP CDR3 = FQGSHVPYT
M21-OH3	HC	CDR1 = TFTMS CDR2 = AIKGYGSSTFYTDSVKG CDR3 = DGYDGGFAY
M21-OH3	LC	CDR1 = RASKSVSTSGYSYMH CDR2 = LVSNLES CDR3 = QHIREAYT
M21-OH5	HC	CDR1 = RYWMS CDR2 = EINPDSSTINYTPSLKD CDR3 = PGDYGYDWFAY
M21-OH5	LC	CDR1 = KSSQSLLYRSNQKNYLA CDR2 = WASTRES CDR3 = QQYYDFPYT

Figure 2 Binding of M21-OH5 IgG or recombinant M21-OH5 Fab to ^{125}I -labelled 21-OH in an immunoprecipitation assay (6). Open circles denote M21-OH5 IgG, crosses denote recombinant (r) M21-OH5 Fab tested undiluted, diluted 1:10 and diluted 1:100 in the assay buffer (6). The horizontal dashed line indicates the mean binding of control recombinant Fab with the TSH receptor binding activity (10).

Table 4 Validation parameters obtained for the models of 21-OH and 3 MABs.

	21-OH	M21-OH1	M21-OH3	M21-OH5
Ramachandran plot				
aa in favoured regions	73.4%	93.2%	89.2%	93.3%
aa in allowed regions	26.7%	6.2%	10.6%	6.7%
aa in disallowed regions	0.0%	0.5%	0.3%	0.0%
PROCHECK				
Overall G factor	-0.03	-0.11	-0.10	-0.08
Bad contacts	6	6	8	10
Number of off graph geometrical values	2	0	1	0
VERIFY3D				
Top value	+0.75	+0.75	+0.81	+0.76
Lower value	-0.45	+0.14	+0.14	+0.11
Number of residues with negative value	23	0	0	0

substrate binding site and the redox partner protein binding site (Fig. 4). ER3 is placed away from the positions of ER1 and ER2 on the surface of 21-OH but close to the binding site of the redox partner protein (Fig. 4).

The active site of 21-OH is characterised by residues able to bind the haem group through hydrogen bonds or salt bridges - Lys120, His365, Arg426 (main chain interaction) and the fifth iron ligand Cys428 (Fig. 5). The haem group is positioned in three points by hydrogen bonds and salt bridges: the iron atom that interacts with Cys428, one carboxylic group of the haem group that interacts with Lys120 and the other carboxylic group that interacts with Arg426 and His365. Two different channels which allow substrate access to the active site of P450 enzymes have been defined (18, 45–47). Channel 1 is formed by a loop (BC) and 2 helices (G and I) (18, 47). In our model, residues Arg91, Pro92, Leu109, Gly110, Trp116 (from the BC loop), Arg366 (from strand 4 of sheet 1) and Arg426 (before helix L) define the open end of channel 1 at the bottom of the structure of 21-OH and close to the membrane (Figs. 3B and 4A). Channel 2 (on the distal flat surface) is a long hydrophobic channel lined with mostly non-aromatic hydrophobic residues (18, 45–47) (Fig. 4A). This corresponds to helix A', the FG loop and residues from the turn formed by sheet 4 (a long loop at the C-terminus) which form the channel 2 entrance (18, 45–47). In our model, residues Lys199 (from the FG loop), Glu294, Thr295 (from the helix I), Leu471 and Lys472 (from the turn of sheet 4) form the entrance of channel 2 (Figs. 3B and 4A).

The electrostatic potential of the proximal surface of the model of 21-OH is shown in Fig. 6A and there is a highly positively charged groove in the centre of the surface consistent with a binding site for the highly negatively charged flavin mononucleotide (FMN)-binding domain of the NADPH-cytochrome p450 oxidoreductase (CPR) (see Discussion and (48)).

Figure 3 (A) Cartoon representation of the 21-OH model. Helices are in red and are marked αA to αL with the transmembrane helix as αTM , beta strands are in green with the beta-sheets marked $\beta 1$ to $\beta 4$. In the haem group the carbon atoms are in black, oxygen atoms in red, nitrogen in dark blue and the iron in light blue. (B) Schematic representation of the secondary structure of the model of 21-OH based on the program HERA (34). Alpha helices are labelled αA to αL , 3_{10} helices are labelled αK , beta-sheets are labelled $\beta 1$ to $\beta 4$ and each β -strand is numbered with each β -sheet. The haem group position is shown as well as the N- and C-termini.

Modelling of 21-OH MABs

The antibody structure with identification number 1yec (Protein Data Bank; <http://www.rcsb.org/pdb/>) (49) was chosen for building the framework of M21-OH1 Fab, 1hyx (50) was chosen for the LC variable region and 1i8m (51) for the HC variable region. Antibody structure 1hi6 (52) was used for modelling of M21-OH1 HC CDR3, which is only two residues long (Table 3). Model validation values are shown in Table 4. The surface of the antigen binding site has five basic patches and two acidic patches with high electrostatic potential (Fig. 6B). The basic patches are centred on Lys50 and Arg54

Figure 4 Space-fill representation of the model of 21-OH from three different points of view (the distal surface, the edge surface and the proximal surface). The transmembrane helix is at the bottom. The epitope ER1 (aa 391-405) is in magenta, the epitope ER2 (aa 406-411) is in green and the epitope ER3 (335-339) is in cyan. The entrance of channel 2 (aa 199, 294, 295, 471 and 472) is in red with the haem group shown in white. The entrance of channel 1 is in maroon and placed at the bottom of the structure (aa 91, 92, 109, 110, 116, 366 and 426). The redox-partner protein binds in a groove (blue) centred on Cys 428 (grey). (A) The distal flat surface of the 21-OH molecule. (B) The edge surface of the 21-OH molecule. (C) The proximal flat surface of the 21-OH molecule.

(from LC CDR2) and on Lys58, Lys62 and Lys64 (from HC CDR2). The acidic patches are centred on residues Asp55 and Asp56 from HC CDR2. The surface of the antigen binding site of M21-OH1 presents a deep cavity in its centre flanked by aromatic residues: Tyr32 (from LC CDR1), Tyr36 and Tyr49 (from LC), Phe55 (from LH CDR2), Phe89 and Tyr96 (from LC CDR3), Phe98 (from LC), Tyr32

(from HC CDR1), Tyr50 and Phe53 (from HC CDR2) and Trp103 (from HC).

The structure of 1hyx antibody (50) was chosen for building of the M21-OH3 Fab framework while the structures of 1bln (53) and 32c2 (54) antibodies were chosen for building the variable HC and variable LC respectively (see Table 4 for the model validation values). The surface of the antibody combining site has four basic and five acidic patches with high electrostatic potential (Fig. 6C). The basic patches are centred on Lys27 (from LC CDR1), Arg92 (LC CDR3), Lys52 (from HC CDR2) and Lys64 (HC CDR2). The acidic patches are centred on Glu55 (from LC CDR2), Glu93 (LC CDR3), Asp61 (HC CDR2), Asp95 (HC CDR3) and Asp98 (HC CDR3). There are ten aromatic residues homogeneously distributed in the combining site. The surface of the combining site is highly irregular (Fig. 6C).

The structure of 1sbs antibody (55) was chosen for building the framework of the M21-OH5 Fab and its LC variable region, while 2fbj antibody (56) structure was used for building the HC variable region except for HC CDR3. M21-OH5 HC CDR3 was modelled on the structure of 1hil antibody (57) (see Table 4 for the model validation values). The surface of the antigen binding site has six basic and seven acidic patches with high electrostatic potential (Fig. 6D). The basic patches are centred on Lys24, Arg27E and Lys30 (from LC CDR1), Arg54 (LC CDR2), Arg31 (HC CDR1) and Lys64 (HC CDR2). The acidic patches are centred on Glu55 (from LC CDR2), Asp93 (LC CDR3), Glu50, Asp53, Asp65 (from HC CDR2), and Asp97 and Asp100I (from HC CDR3). An aromatic core is formed by LC tyrosines 27D, 32, 91 and 96. The surface of the combining site is highly irregular and presents a highly acidic patch in its centre (Fig. 6D).

Figure 5 Representation of the active site of 21-OH. Helices are shown in red, β -strands in green and loops in yellow. Protein side chains (brown bonds) and the haem group (blue bonds) are illustrated. Hydrogen bonds are represented as dashed lines. One letter code for amino acids is used (K, Lys; H, His; R, Arg; C, Cys).

Figure 6 Electrostatic potential of the proximal surface of the model of 21-OH with (A) epitope regions ER1 to ER3 marked, and (B-D) the surface of the antigen binding site of (B) M21-OH1, (C) M21-OH3 and (D) M21-OH5. L indicates amino acid from the light chain, H indicates amino acid from the heavy chain. The circled area in (D) contains four tyrosine residues as indicated in the box. The acidic patches are shown in red; the basic patches are shown in blue.

Analysis of the complexes between 21-OH and 21-OH MABs

Complexes between M21-OH1 and 21-OH obtained in protein-protein docking experiments were studied consecutively in the order produced by the program GRAMM, and the complex which showed the best interaction between the 21-OH epitope ER1 (aa 391-405) and M21-OH1 was selected. In the complex, 39 residues from 21-OH interact with 43 residues from M21-OH1. Nine residues from LC CDR1 of M21-OH1, seven from LC CDR2, five from LC CDR3, four from HC CDR1 and seven from HC CDR2 are involved in the interaction. Phe53 from HC CDR2 and Thr27E from LC CDR1 show strong interactions of 6.8% and 6.0% of the change in interface (Δ ASA) respectively. Five 21-OH sequence segments are involved in the interface in the complex with M21-OH1. The fifth segment includes aa 389-399 and contributes to 24.6% of the interface Δ ASA. Arg75 and Gln53 from 21-OH are involved in strong interactions with M21-OH1 (10.7% and 9.6% of the interface Δ ASA respectively). In the complex, the total ASA buried in the interface of 21-OH is 1479 Å² and 1486 Å² for M21-OH1. There are 66.7% and 72.1% polar residues from 21-OH and M21-OH1 respectively and ten hydrogen bonds in the complex interface, two of these hydrogen bonds are in the region corresponding to the ER1.

In the case of M21-OH3, the complex with the best interaction between M21-OH3 variable region and the 21-OH epitope ER2 (aa 406-411) was chosen. In the complex, 31 residues from 21-OH interact with 32 residues from M21-OH3 (5 are from LC CDR1, 6 from LC CDR2, 1 from LC CDR3, 3 from HC CDR1, 8

from HC CDR2 and 5 from HC CDR3). Tyr53 from HC CDR2 and Tyr97 from HC CDR3 of M21-OH3 produce strong interactions of 11.6% and 11.5% of the interface Δ ASA respectively. In 21-OH, the interface is formed by four sequence segments involving different residues between aa 316 and 435. The third segment (aa 395 to 417) includes the whole sequence of ER2 (aa 406-411) which accounts for 14.3% of the interface Δ ASA. In the complex between 21-OH and M21-OH3, the total ASA of 21-OH buried in the interface is 1217 Å² and of M21-OH3 interface is 1143 Å². There are 68.8% and 74.2% polar residues from 21-OH and M21-OH3 respectively, and 26 hydrogen bonds and one salt bridge in the complex interface. Six out of twenty-six hydrogen bonds in the complex interface are present in the region corresponding to the ER2.

In the case of M21-OH5, the complex with the best interaction with the epitope ER3 (aa 335-339) was selected (Fig. 7). In the complex, 17 residues from 21-OH interact with 16 residues from M21-OH5. Six residues from LC CDR1 of M21-OH5, one from LC CDR2, two from LC CDR3, one from HC CDR1 and five from HC CDR3 are involved in the interaction. Tyr98 from HC CDR3 and Arg27E from LC CDR1 show strong interactions of 17.0% and 15.1% of the interface Δ ASA respectively. In 21-OH, the interface is formed by two sequence segments. The first segment includes residues aa 336-339 (almost the entire ER3; aa 335-339) which contribute to 25.7% of the interface Δ ASA. Arg341 and Lys337 of 21-OH are involved in strong interactions (20.3% and 16.6% of the interface Δ ASA respectively). The total ASA buried in the

Figure 7 Structural model of the 21-OH-M21-OH5 Fab complex. Antibody heavy chain is shown in blue, light chain in green, 21-OH in red. The haem group (blue bonds) is illustrated. The epitope ER3 (aa 335-339) is shown in yellow. The N- and C-termini of 21-OH are marked as N and C respectively and the variable regions of M21-OH5: L1 = LC CDR1, L2 = LC CDR2, L3 = LC CDR3, H1 = HC CDR1, H2 = HC CDR2 and H3 = HC CDR3 are indicated (see text for details).

interface of 21-OH is $652\text{\AA}^2$ while for M21-OH5 it is $723\text{\AA}^2$. There are 64.7% and 93.8% polar residues from 21-OH and M21-OH5 respectively and ten hydrogen bonds and one salt bridge in the complex interface. Three out of ten hydrogen bonds found in the complex interface are produced by the ER3.

Discussion

We have built a comparative model of the structure of 21-OH based on the structure of rabbit cytochrome p450 2c5/3 (16–18). The validation parameters for our model (Table 4) were consistent with previously described criteria for optimum positions of residues in the structure (25–27) but VERIFY 3D showed an area with negative values between aa 205–225. This aa region 205–225 of 21-OH is involved in membrane binding and corresponds to the region where several mutations had been introduced into the sequence of the rabbit P450 2c5/3 in order to avoid aggregation and to facilitate crystallisation (17). The native sequence between aa 205 and 225, which is not associated with the membrane in our model resulted in negative values in VERIFY 3D analysis (26).

The template (the structure of rabbit P450 2c5/3) we used had its transmembrane helix (aa 3 to 21) absent (the corresponding part of the sequence was removed from the protein to avoid aggregation during crystallisation) (17). Consequently, the N-terminal peptide of HIV-1 gp41 (28) was chosen in order to obtain the structure of the transmembrane helix of 21-OH in our model and this peptide adopts a transmembrane helix structure with a hydrophobicity profile similar to that of the helical sequence of the transmembrane section of 21-OH.

A typical P450 enzyme structure contains four β -sheets ($\beta 1$ – $\beta 4$) and approximately 13 α -helices (42). Some structures of members of the P450 enzyme family also contain the $\beta 5$ β -sheet (42). The more variable structural elements are helices A, B, B', F, G, H, K', β -sheets $\beta 3$, $\beta 4$, and the loops (42). In our model of 21-OH, all helices belonging to the structurally conserved core of the P450 family (i.e. helices D, E, I, L, J and K) are present (Fig. 3B) (42). Also present are the two conserved β -sheets $\beta 1$ and $\beta 2$ as well as the coil 'meander' (loop between helices K' and L) which is highly conserved in the structure and is located on the proximal face (Fig. 3B) (42). The only structural difference between 21-OH and the conserved

P450 structural core is that 21-OH β 1 has only four strands instead of five (Fig. 3B).

The non-conserved or variable regions of P450 enzymes together with some well-conserved regions are usually associated with substrate binding and redox partner binding (58, 59). The variable regions associated with substrate binding are helices A, B, B', F and G and their adjacent loops and include the substrate recognition sites (SRS) identified by Gotoh (60) in the CYP2 family. Loops B-B' and B'-C line the active site (SRS-1), and helices F and G and the loop between them form part of the access channel 2 and ceiling of the active site (SRS-2 and SRS-3). The loop at the end of β 4 protrudes into the active site (SRS-6), as does the region at the N-terminus of the fourth strand of β 1 (SRS-5), which in 21-OH contains His365 that binds one of the carboxylic groups of the haem group (42). Finally, the highly conserved central portion of helix I (SRS-4) contributes to formation of the active site.

NADPH-cytochrome P450 oxidoreductase (CPR) is a flavoprotein composed of four domains (48). The flavin mononucleotide (FMN)-binding domain interacts with the proximal surface of 21-OH and it has been shown that the binding site of the FMN-binding domain is highly negatively charged (48). Analysis of the crystal structure of the BM3-FMN complex (61) shows that the FMN residues from the α_1 -helix and the outer FMN-binding loop interacts with the C- and L-helices and the peptide that precedes the haem-binding loop (the 'meander') of the P450. These interaction areas in P450 BM3 correspond to a highly positively charged groove in our 21-OH model (Fig. 6A) consistent with this groove being the binding site for the highly negatively charged FMN-binding domain (48).

A decade ago, Lin *et al.* (62) described models of human steroid 17- α and 21-hydroxylase based on the structure of bacterial P450cam from *Pseudomonas putida*. There are substantial differences between the soluble bacterial P450cam and membrane-bound mammalian P450 enzymes of the endoplasmic reticulum (63). The Lin *et al.* model was built by amino acid substitution of the backbone, and the side chains were positioned manually. In contrast, to build our model, we have used the program SCORE (22) for modelling the core of the protein and CODA (23) for modelling the loop regions not present in the SCORE output. Auchus and Miller have constructed a model of human P450c17 (17 α -hydroxylase/17,20-lyase) (64) using the bacterial enzyme P450BMP as a template. The model scored well for side chain parameters, bad contacts and distortion of atom geometries, but less well for main chain parameters; 73.4% of residues were placed in the favoured regions of the Ramachandran plot (the same value was obtained for our model). However, in the Auchus and Miller model some undisclosed residues were present in disallowed regions of the Ramachandran plot. In contrast, in our model of 21-OH none of the residues are present in

disallowed regions of the Ramachandran plot. The overall G-factor of the Auchus and Miller model is -0.41 (64) compared with -0.03 in our model; overall G-factor scores should be above -0.5 and values below -1.0 may need investigation (25). In the P450c17 model proposed by Auchus and Miller (64), Cys442 (Cys428 in 21-OH) acts as a ligand of the haem group and Arg440 (Arg426 in 21-OH) forms a hydrogen bond with one of the carboxylic groups of the haem group. The inability of the P450c17 mutant His373Leu to bind haem was explained on the basis that in the Auchus and Miller model His373 (His365 in 21-OH) did not interact with the haem group and the mutation creates a global alteration of the structure. In our model of 21-OH, however, His365 produces a hydrogen bond with one of the carboxylic groups of the haem group which cannot be formed after His365Leu mutation.

Lewis and Lee-Robichaud have modelled one representative member of the P450 families, CYP11, CYP17, CYP19 and CYP21, based on the structure of bacterial P450 CYP102 (65). Only two residues interacting with the haem group in addition to Cys428 were identified in their 21-OH model.

More recently, Mornet and Gibrat have published a 3D model of human P450c21 (66). The haem group in the Mornet and Gibrat model is positioned by the same amino acids as in our model. The effect of some 21-OH aa mutations on enzyme activity explained by analysis of the Mornet and Gibrat model (66) can be explained in a similar way using our model. However, the effect of Glu380Asp mutation (loss of enzyme activity) was not clearly explained by Mornet and Gibrat except that Glu380 could form a salt bridge with Arg16, Lys23 or Arg25 (66). In our model, these three residues are positioned too far away from Glu380 to form a salt bridge in a favourable conformation. Analysis of our model suggests that the area containing Glu380 is present in the surface of an area that is close to the entrance of channel 1. The oxygen of Glu380 is 5.6 Å away from the nitrogen in Arg366 which is present at the entrance of substrate access channel 1 resulting in charge compensation. In the case of mutation of Glu380 to aspartic acid which has a shorter side chain than glutamic acid, the distance between the charged atoms at positions 380 and 366 becomes longer. Consequently, the charges in this area are not well compensated in the mutant and 21-OH may bind too tightly to the substrate explaining the loss of enzyme activity.

The structure of rabbit cytochrome P450-2c5/3 (16–18) was the best template available for the comparative modelling of human 21-OH when we began our study. After all modelling processes were finished, the crystal structure of human cytochrome P450-2c9 (2c9) (67) became available. However, the amino acid sequence identity between 21-OH and 2c9 is 27.0%, slightly lower compared with the 29.2% identity

between 21-OH and rabbit 2c5/3. Also, the spatial coordinates of human cytochrome P450-2c8 (2c8) (25.2% aa sequence identity with 21-OH) (68) have become available while this manuscript was in preparation. Most of the secondary structure elements obtained for our model of 21-OH are present in the crystal structure of 2c9 (67). Two 3_{10} helices present in 2c9 in the K'L-loop are not present in 21-OH, and a beta-hairpin ($\beta 2$ in Fig. 3B) between strands 4 and 3 of sheet 1 in 21-OH is not present in 2c9. The spatial coordinates were not available for 11 residues from the FG-loop in the rabbit 2c5/3 structure used as a template; however in our model of 21-OH there are two helices for the equivalent residues. Two helices are also present in the corresponding region of the structure of human 2c9, which suggests that our model of 21-OH is consistent with the structure obtained very recently for 2c9 (67). Moreover, the rabbit 2c5/3 structure lacks a β -hairpin ($\beta 4$ in Fig. 3B) between strands 1 and 2 of sheet 3 while our model, as well as the structure of human 2c9, present this β -hairpin (17, 67). The overall structure of our model of 21-OH compares well with the crystal structure of 2c9; 412 alpha carbons out of 461 from the backbones of each structure can be superimposed with less than 3.5 Å deviation and this superimposition presents a root-mean-squared difference (RMSD) of 1.190 Å. There is only one 3_{10} helix in the K'L loop in the 2c8 structure (68) which is not present in the corresponding area of our 21-OH structure. Also, a β -hairpin ($\beta 2$ in Fig. 3B) between strands 4 and 3 of sheet 1 which is found in our model is not present in the 2c8 structure (68). However, the two helices placed in the FG loop of 21-OH are also present in the crystal structure of 2c8. 2c8 lacks the β -hairpin ($\beta 4$ in Fig. 3B) between strands 1 and 2 of sheet $\beta 3$ and this is in contrast to the structure of 2c9 (67). There is a good agreement between the overall structure of our model of 21-OH and the crystal structure of 2c8 (68); 416 alpha carbons out of 463 from the backbones of each structure can be superimposed with less than 3.5 Å deviation and this superimposition presents an RMSD of 1.167 Å.

The experimentally determined epitope regions (ER1-3) reactive with 21-OH MAbs (6) were identified on our structural model of 21-OH. All three were found to be present on the surface of 21-OH which is consistent with the ability of M21-OH1, M21-OH3 and M21-OH5 to bind to the amino acids within these ERs (6) (Fig. 4). The HC and the LC V genes of M21-OH1, M21-OH3 and M21-OH5 were derived from different germline genes (Table 2). M21-OH3 and M21-OH5 had the D genes derived from the same germline (DSP2.6) whereas the D genes for M21-OH1 could not be assigned. Genes from JH3, JH4, JK1 and JK2 were used by our MAbs. Two MAbs showed high R/S mutation ratio within the V regions which is consistent with an antigen driven maturation (69). Analysis of the sequence of M21-OH3 revealed the absence of cysteine

at position 23 of the LC. This residue is highly conserved in antibodies and is normally involved in an intra-molecular disulphide bond with the Cys residue at position 88 of the LC (70). It has been reported that recombinant antibodies could not be produced in *E. coli* when the cysteines 23 or/and 88 had been replaced in an Fv fragment (70). However, examples of functional monoclonal antibodies lacking Cys residues important for disulphide bond formation have also been described (71, 72).

The sequences of our MAbs were then used to build their respective comparative models. The models of these MAbs had the standard IgG Fab structures. The topography of the antigen binding site of each of the MAbs was studied and showed variations in the formation of the surface and in the distribution of negatively and positively charged amino acids. The antigen binding sites of our MAbs contained a relatively high proportion of aromatic residues which is characteristic of the combining site of antibodies (73, 74).

The 21-OH MAb models were used in protein-protein docking experiments with our model of 21-OH. The complexes which showed the best fit between the MAbs and their respective epitope regions on 21-OH were selected (see Results). Analysis of these complexes revealed that 21-OH MAbs in addition to their respective epitope regions also interacted with various amino acid segments on the surface of 21-OH. However, the residues involved in the interaction within the segment containing the epitope region sequence contributed a large proportion of the total interface ASA (24.6% in the case of M21-OH1, 14.3% in the case of M21-OH3 and 25.7% in the case of M21-OH5). The amino acids within the MAb antigen binding sites that showed strong interactions with 21-OH were also identified. These amino acids could be good candidates for mutation in order to modify binding of MAb to 21-OH in future studies.

As shown in Fig. 4, M21-OH1 that binds to ER1 and M21-OH3 that binds to ER2 would be unlikely to have an effect on 21-OH enzyme activity as their respective binding sites are located too far away from the two active sites (i.e. the substrate binding site and the redox partner protein binding site). Our experiments showed that M21-OH1 and M21-OH3 had no effect on 21-OH enzyme activity *in vitro* (Fig. 1) and this is consistent with positions of ER1 and ER2 in our model relative to the location of 21-OH active sites. In contrast, MAbs that bind to ER3 (for example M21-OH5) might be expected to have an effect on 21-OH enzyme activity as ER3 is in relative proximity to the redox partner protein binding site (Fig. 4). Our experiments showed that M21-OH5 has the ability to inhibit 21-OH enzyme activity *in vitro* (Fig. 1) and analysis of our model of 21-OH indicates that this may occur by M21-OH5 binding interfering with the binding of the redox partner.

As described before, patient serum 21-OH autoantibodies effectively inhibit 21-OH enzyme activity *in*

vitro (12) and a recent study has shown that they inhibit the fast phase electron transfer from the NADPH-p450 reductase (CPR) to 21-OH (75). The most likely mechanism of this effect is by interference in the interaction between the CPR and 21-OH, suggesting that the binding sites for 21-OH autoantibodies are in the proximity of the CPR binding site. The binding of 21-OH autoantibodies in a panel of patient sera was inhibited (81%–93% inhibition) by a mixture of Fab or F(ab')₂ preparations of our MABs (6, 75). Furthermore, M21-OH5 (which had the strongest inhibiting effect on 21-OH autoantibodies binding) (6) also showed inhibition of 21-OH enzyme activity and this was probably through a similar mechanism (i.e. interference in binding of redox protein to 21-OH) (75).

The 21-OH MAB models used in our studies were built using structures of antibodies with homologous sequences available in the database. In order to verify that the MAB sequence obtained by RT-PCR was correct, we expressed recombinant M21-OH5 Fab in *E. coli*. Recombinant M21-OH5 Fab bound ¹²⁵I-labelled 21-OH (Fig. 2) and inhibited binding of M21-OH5 IgG (derived from hybridoma cells) to 21-OH. The results of these experiments confirmed that the M21-OH5 sequence used for comparative modelling was that of an active 21-OH MAB.

In addition to the MABs described in this study, we have found that a further 21-OH MAB, MAB 1E5 (reactive with an epitope within 21-OH aa 1–142) was able to inhibit 21-OH enzyme activity by about 80% in our *in vitro* experiments (data not shown). In addition, another MAB (11C6 reactive with an epitope within aa 165–280) also inhibited 21-OH enzyme activity by about 40% (data not shown). We have not carried out detailed analysis of the interactions between these MABs and 21-OH using their respective comparative models but we looked at the positions of the epitopes for the 2 MABs on our model of 21-OH. The aa region 1–142 extends between the entrance to channel 1 and partially overlaps the CPR binding site, and the aa region 165–280 is situated in the vicinity of and partially overlaps the entrance to channel 2. Consequently, the effects of 21-OH MABs 1E5 and 11C6 on 21-OH enzyme activity are consistent with the location of their epitopes indicated in our model.

Overall, we have analysed the interaction between 21-OH and 21-OH MABs using comparative models of 21-OH and the MABs. Analysis of the locations of the MAB epitopes on our 21-OH model provided an explanation for the ability of M21-OH5 but not of M21-OH1 and M21-OH3 to inhibit 21-OH enzyme activity. There was also evidence for similarities in the mechanism of inhibition of 21-OH enzyme activity by M21-OH5 and by patient serum 21-OH autoantibodies. The results of these analyses and the models themselves provide useful tools for further studies on defining 21-OH autoantibody in more detail. Eventually, analysis of the crystal structure of 21-OH and 21-OH

antibody complexes should be able to confirm the accuracy of our model.

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