STRUCTURE-FUNCTION ANALYSIS OF HUMAN CYP3A4 USING A SPECIFIC PROINHIBITORY ANTIPEPTIDE ANTIBODY

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ABSTRACT:

An anti-peptide antibody targeted against residues 253 to 269 of human CYP3A4 was produced that specifically and potently inhibited its activity in human hepatic microsomal fraction (>90%). The function of this region in P450 catalysis was investigated. Antibody binding to CYP3A4 was unable to affect the magnitude of the Type I spectrum on addition of testosterone. It also had no effect on the kinetic constant (Km) of the enzyme for testosterone, but it did cause a marked decrease in Vmax (>90%) of testosterone 6β-hydroxylation. There was no change in the ability of the antibody-bound CYP3A4 to form the steady-state level of the enzymatically or chemically reduced P450-CO complex or even the steady-state level of the dioxygen complex during testosterone metabolism, but the oxidation of NADPH by CYP3A4 in the presence of antibody was 60% that of CYP3A4 in the absence of antibody. The binding of the antibody also resulted in potent inhibition of cumene hydroperoxide-supported testosterone 6β-hydroxylase activity of human liver microsomal fraction (>90%). Our conclusion is that the loop region targeted in CYP3A4 is not involved in substrate binding, in reductase binding, in the transfer of the first or second electron from the reductase to CYP3A4, or in the binding of molecular oxygen. We speculate that antibody binding to CYP3A4 inhibits enzyme activity by destabilizing the ternary hydroperoxo complex, by interfering with the second proton transfer, and/or by interfering with the conformational changes that are suggested to be induced by substrate binding.

The superfamily of P450 monooxygenases catalyze the metabolism of numerous compounds, from the biotransformation of natural endogenous compounds such as steroids and cholesterol to the detoxification of xenogenous compounds, including drugs and environmental pollutants (Guengerich, 1996). These enzymes display broad but unique substrate specificities, with many isoforms able to metabolize the same compounds, often at different rates and at different sites (Gonzalez, 1992). The use of anti-peptide antibodies that bind specifically to a P450 enzyme and significantly inhibit its activity provides a useful approach to quantitatively determine the contribution of each P450 enzyme to the metabolism of a particular drug, in vitro, in the presence of multiple forms of P450 contained in a tissue (Boobis et al., 1996). As the active site of these enzymes is likely to be inaccessible to an antibody, such antibodies would have to be targeted against small unique regions on the surface of the enzyme of interest, regions that are involved in the catalytic function of the enzyme (Boobis et al., 1996).

A proinhibitory region on the surface of human cytochrome P450 3A4 has been identified previously using an anti-peptide antibody, targeted against residues 254 to 273 of this enzyme, which was able to significantly inhibit CYP3A4 activity (Wang and Lu, 1997). Based on epitope-mapping studies using this antibody, the inhibitory sequence of the CYP3A4 enzyme was shown to correspond to residues 261–267 with the core sequence comprising residues 262–264 (Wang et al., 1999). Based on three-dimensional models of mammalian P450 enzymes, the proinhibitory site on CYP3A4 is predicted to lie in a surface loop region between helices G and H (Lewis, 1996), and more crystallographic studies of the bacterial P450 enzymes CYP101, CYP102, and CYP108 have shown that this proinhibitory site lies some distance from the active site of the enzyme (Hasemann et al., 1995).

A number of previous studies using site-directed mutagenesis have been performed on related P450 enzymes to determine the function of the proinhibitory region in P450 catalysis (Parkinson et al., 1986; Miles et al., 1992; Shen and Strobel, 1992, 1993; Bridges et al., 1998). Although some reports have implicated that the equivalent surface loop region containing the proinhibitory site in bacterial CYP102 and rat CYP1A1 is involved in the binding of NADPH-P450 reductase and thereby plays a role in electron transfer (Miles et al., 1992; Shen and Strobel, 1992), others have suggested, based on studies with rat CYP1A1, rat CYP2B1, and rabbit CYP2B4, that the binding site for the reductase is not localized to this region of the P450 enzyme (Parkinson et al., 1986; Shen and Strobel, 1993; Bridges et al., 1998). It has even been proposed that the region possibly may play a role in substrate entry and subsequent interaction with the substrate because movement of the G-helix of bacterial CYP101 and movement of the F-G domain of CYP102 have been suggested to occur to allow

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interaction of the substrate with the P450 enzyme (Deprez et al., 1994; Chang and Loew, 1999). Consequently, the functional role of this region in the catalytic process of P450 enzymes still remains unresolved.

Therefore, to determine the mechanism by which an anti-peptide antibody binding to the proinhibitory region in human CYP3A4 inhibits activity, an anti-peptide antibody that specifically and potently inhibits human CYP3A4 activity was produced by targeting residues 253–269 of this enzyme. The ability of the antibody to affect substrate binding and electron transfer was assessed and is reported here.

**Experimental Procedures**

**Materials.** N-α-9 4-fluoroenzymethoxycarbonyl (Fmoc)2-protected amino acids linked to a Rink resin and N-α-Fmoc-protected amino acid pentafluorophenyl esters were purchased from Novabiochem (Nottingham, UK). A Li-chrospher RP18e 5-μm HPLC column (250 × 4 mm) was supplied by Merck (Darmstadt, Germany). Sulfosuccinimidyl 4-[(N-maleimidyl)acyclohexane-1-carboxylate was purchased from Pierce (Rodovre, Denmark). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (Nottingham, UK). A 1-carboxylate was purchased from Pierce (Rodovre, Denmark). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (Nottingham, UK). A Hitrap Protein G-Sepharose affinity column (gel volume of 1 ml), Excel Gel SDS 12.5% polyacrylamide gels (250 × 110 × 0.5 mm), and ExcelGel SDS polyacrylamide anode and cathode buffer strips were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane, ECL reagent, and Hyperfilm ECL were obtained from Amersham International (Little Chalfont, UK). Samples of microsomal protein from insect cells expressing individual human P450 enzymes were purchased from Gentest Corporation (Woburn, MA). All other chemicals were purchased from Sigma (Vallensbaek Strand, Denmark) or Merck (Darmstadt, Germany) and were of analytical grade or the best equivalent.

**Microsomal Fraction from Human Liver.** A mixed pool of human liver microsomal fraction (Lot no. HMM0252) from histologically normal human liver samples was obtained from the International Institute for the Advancement of Medicine (Exton, PA) and had been previously assessed for P450 activity. The specific protein content in each microsomal fraction was determined using the Pierce Protein Assay Kit (Rockford, IL). The P450 content was determined by the method described previously (Omura and Sat0, 1964).

**Microsomal Protein from Escherichia Coli Membranes Expressing Human P450 Enzymes.** The LINK consortium1, a collaboration between industry and UK-based academia, provided stocks of transformed cells with human P450 enzymes individually coexpressed with human NAPDH-P450 reductase in E. coli, which were stored as glycerol stocks at −80°C. Expression of the recombinant protein and preparation of the E. coli membranes were carried out essentially as described previously (Pritchard et al., 1998).

**Peptide Synthesis and Coupling to Carrier Protein.** The peptide VKRMKESRLEDTQKHRV, representing residues 253–269 of human CYP3A4, was synthesized on an Applied Biosystems 431A peptide synthesizer expressing CYP3A4 (0.1 nmol) and reductase, preincubated for 30 min at room temperature with varying amounts of purified IgG (1.25–5 mg IgG/mg protein) in a total volume of 115 μl. Buffers and cofactors (5 mM MgCl2 for human hepatic microsomal fraction and 5 mM ascorbic acid for microsomal protein from E. coli; 2 mM NADPH) were then added, before the reaction was started with the addition of 10 μl of 6.25 mM testosterone (final volume 250 μl). After 12.5 min at 37°C, the reaction was stopped by the addition of 1.5 ml of dithoromethane, spiked with 100 μl of 26 μM corticosterone (internal standard), vortex-mixed, and centrifuged at 1000g for 5 min. The organic layer was transferred to fresh tubes and evaporated to dryness at 35°C under a gentle stream of nitrogen. The resultant residue was then dissolved in methanol/water (50:50, v/v) and analyzed by HPLC as described previously (Reinerink et al., 1991).

In a cumene hydroperoxide-supported system, reactions were performed as described above for the NADPH and reductase-supported reaction, except that buffer and substrate were added before the reaction was started with 10 μl of 5 mM cumene hydroperoxide (final volume 250 μl).

**Other CYP-Mediated Reactions.** Ethoxyresorufin O-deethylase (CYP1A2), tolbutamide methyl-hydroxylase (CYP2C9), dextromethorphan O-demethylase (CYP2D6), and 4-nitrophenol hydroxylase (CYP2E1) activity of human hepatic microsomal fraction were measured according to the methods described previously (Burke and Mayer, 1983; Miners et al., 1988; Tansaney-akul et al., 1993; Jones et al., 1996).

**Substrate Binding Spectra.** The binding of testosterone to CYP3A4 was determined by difference spectroscopy as described previously (Schenkman et al., 1981). Microsomal fraction from E. coli expressing CYP3A4 (0.1 nmol) and reductase, preincubated for 30 min at room temperature with 1.5 mg of purified IgG, was diluted to 1 ml with 0.1 M sodium phosphate buffer (pH 7.4). The sample was divided between two 0.5-ml cuvettes, and a baseline scan with cofactors (5 mM MgCl2 for human hepatic microsomal fraction and 5 mM ascorbic acid for microsomal protein from E. coli; 2 mM NADPH) was then added, before the reaction was started with the addition of 10 μl of 6.25 mM testosterone (final volume 250 μl). After 26 min at 37°C, the reaction was stopped by the addition of 1.5 ml of dithoromethane, spiked with 100 μl of 26 μM corticosterone (internal standard), vortex-mixed, and centrifuged at 1000g for 5 min. The organic layer was transferred to fresh tubes and evaporated to dryness at 35°C under a gentle stream of nitrogen. The resultant residue was then dissolved in methanol/water (50:50, v/v) and analyzed by HPLC as described previously (Reinerink et al., 1991).

**NADPH Oxidation.** The rate of NADPH oxidation was determined spectrophotometrically at 340 nm using the method described previously and based on an extinction coefficient of NADPH of 6.26 mM−1 cm−1 (Imai, 1979; Parkinson et al., 1986). Microsomal fraction from E. coli expressing CYP3A4 (0.05 nmol) and reductase, preincubated for 30 min at room temperature with 1.5 mg of purified IgG, was diluted to 1 ml with 0.1 M sodium phosphate buffer (pH 7.4). Testosterone (250 μM) and ascorbic acid (5 mM) were then added. The sample was divided equally between two 0.5-mI cuvettes, and the
absorbance at 340 nm was measured. Reactions at 37°C were then initiated with NADPH (0.125 mM), and the absorbance was measured for 5 min at 37°C.

**Ferrous-Carbonyl P450 Complex Formation.** The formation of a steady-state ferrous-carbonyl P450 complex in human hepatic microsomal fraction (1 mg) or microsomal protein from *E. coli* coexpressing human CYP3A4 (0.025 nmol) and reductase was determined spectrophotometrically essentially as described previously (Omura and Sato, 1964). Microsomal protein and testosterone (250 μM) in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) was reduced with NADPH (0.1 mM). The sample was then divided between two 0.5-ml cuvettes, and a baseline scan was recorded between 500 and 400 nm on a Shimadzu UV-1601 spectrophotometer. Carbon monoxide was then bubbled through the sample cuvette for 1 min and spectra were recorded repetitively until a steady state was reached. At this point, a small amount of sodium dithionite was added to both cuvettes and the spectra were recorded repetitively again. The amount of the ferrous-carbonyl P450 complex formed was determined from A450–490 nm, essentially as described by Omura and Sato (1964).

**Dioxy-Ferrous P450 Complex Formation.** The formation of a steady-state dioxy-ferrous P450 complex of microsomal protein from *E. coli* coexpressing human CYP3A4 and reductase was monitored spectrophotometrically during the metabolism of testosterone as described previously (Estabrook et al., 1971; Guengerich et al., 1976). Microsomal protein (0.2 nmol) was added to 50 mM sodium phosphate buffer, pH 7.4, containing 250 μM testosterone (final volume 1 ml). The sample was divided between two 0.5-ml cuvettes. A baseline scan was recorded between 490 and 360 nm on a Shimadzu UV-1601 spectrophotometer. Reactions at 22°C were initiated by the addition of 50 nmol NADPH to the sample cuvette (final concentration of 0.1 mM) and spectra recorded repetitively between 490 and 360 nm. The amount of the dioxyferrous P450 complex formed during testosterone metabolism was determined from A412–440 nm, essentially as described previously (Guengerich et al., 1976).

**Immunoblotting.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis were carried out essentially as described previously (Edwards et al., 1988, 1998), except that microsomal protein was separated on a 12.5% (w/v) polyacrylamide gel (250 × 110 × 0.5 mm) and electro-transferred onto a nitrocellulose membrane using a Multiphor II electrophoresis system (Pharmacia, Uppsala, Sweden).

**Kinetic Analysis.** *V_\text{max}* and *K_m* values were determined from the Hill equation \( v = (V_{\text{max}}[S]^n) / (K_m^n + [S]^n) \) through nonlinear regression. The Hill coefficient (n), an indicator of the degree of cooperativity, was determined from the plot log [v / (\( V_{\text{max}} – v \))] versus log [S] through linear regression.

**Results**

An anti-peptide antibody targeted against human CYP3A4 was produced by immunizing a rabbit with a KLH conjugate of the peptide representing residues 253–269 of this enzyme (Fig. 1). In immunoblotting studies using a sample of human liver and samples of microsomal protein prepared from *E. coli* that expressed a single form of human P450 enzyme, the antibody was found to bind to a single band in a microsomal sample of human liver that comigrated with the band in a sample of microsomal fraction from *E. coli* or insect cells expressing human CYP3A4 (data not shown). The antibody did not recognize any immunoreactive protein in samples of microsomal fraction from *E. coli* expressing CYP1A2, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CY2E1 (data not shown), or any immunoreactive protein in a sample of microsomal fraction from insect cells expressing CYP3A5 (data not shown). The amount of recombinant CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP2D6 used was sufficient for detection by immunoblotting using their respective antibodies (data not shown, other forms not tested).

The effect of the antibody on enzyme activity was investigated by preincubating human hepatic microsomal fraction with the antibody before measuring testosterone 6β-hydroxylase activity. The antibody was able to progressively inhibit enzyme activity with increasing amounts of antibody (Fig. 2). When compared with preimmune IgG,
a maximum of greater than 90% inhibition of enzyme activity was achieved. Similar inhibition of testosterone 6β-hydroxylase activity of microsomal protein from *E. coli* coexpressing human CYP3A4 and reductase was also found (Fig. 2). This level of inhibition was achieved with antisera from the third bleed, where maximal titer was observed (data not shown). Inhibition was found to be specific, because the antibody had little or no effect on other P450-mediated reactions in human hepatic microsomal fraction (data not shown).

The ability of the antibody to block the binding of testosterone to CYP3A4 and, subsequently, to affect the low to high spin state transition of the enzyme (Fig. 3) was examined by differential spectroscopy. It was found that the binding of the probe substrate, testosterone (250 μM final concentration), to microsomal protein from *E. coli* coexpressing human CYP3A4 and reductase produces a Type I spectrum with an absorption peak at 390 nm and trough at 420 nm (Fig. 3). A similar result was obtained when the microsomal protein from *E. coli* expressing human CYP3A4 was preincubated with purified IgG before the addition of testosterone (Fig. 3). The antibody was also unable to affect the low to high spin state transition of CYP3A4 following binding of testosterone to microsomal protein from lymphoblastoid and insect cells expressing human CYP3A4 (data not shown). Addition of purified IgG alone to microsomal protein from transfected *E. coli* did not produce a type I difference spectrum in the absence of testosterone (data not shown).

The effect of antibody binding on the steady-state level of both the enzymatically and chemically reduced ferrous-carbonyl P450 complex was determined from the carbon monoxide difference spectrum. Preincubation of hepatic microsomal fraction with purified IgG did not change the ability of CYP3A4 to form the steady-state level of the enzymatically reduced CYP3A4-carbonyl complex when compared with the control, containing no IgG (Fig. 5). The level of the enzymatically reduced CYP3A4-carbonyl complex, in the absence and presence of antibody, was found to be 38 and 37%, respectively, of the level of the chemically reduced ferrous-carbonyl complex (Table 1). This indicates that 37 to 38% of the total P450 present in the sample

**FIG. 3.** Substrate-induced difference spectra of *E. coli* membranes expressing human CYP3A4.

Microsomal protein from *E. coli* membranes expressing human CYP3A4 (0.1 nmol) and reductase was preincubated for 30 min at room temperature with 1.5 mg purified IgG raised against residues 253–269 of human CYP3A4 in 0.1 M sodium phosphate buffer, pH 7.4. The mixture was then divided between two 0.5-ml cuvettes and a baseline scan was recorded between 500 and 360 nm. Testosterone (250 μM) was then added to the sample cuvette and difference spectra in the absence (A) and presence (B) of the antibody targeted against CYP3A4 recorded between 500 and 360 nm as described under Experimental Procedures.

**FIG. 4.** Kinetic analysis of testosterone 6β-hydroxylation of human hepatic microsomal fraction.

A, 6β-hydroxytestosterone formation rates versus substrate concentration in the absence (••••) and presence (———) of purified IgG (0.7 mg); B, testosterone 6β-hydroxylation fitted to plots of the Hill equation, log [v/(V max − v)] versus log [S], in the absence (••••) and presence (———) of purified IgG (0.7 mg). The value of n was determined to be 1.83 and 2.11 in the absence and presence of the antibody, respectively. Linear regression was used to fit the both sets of data.

antibody had little effect on reducing the inhibition seen (data not shown). Furthermore, addition of NADH together with NADPH had little effect on enzyme activity (data not shown).

The effect of antibody binding on the steady-state level of both the enzymatically and chemically reduced ferrous-carbonyl P450 complex was determined from the carbon monoxide difference spectrum. Preincubation of hepatic microsomal fraction with purified IgG did not change the ability of CYP3A4 to form the steady-state level of the enzymatically reduced CYP3A4-carbonyl complex when compared with the control, containing no IgG (Fig. 5). The level of the enzymatically reduced CYP3A4-carbonyl complex, in the absence and presence of antibody, was found to be 38 and 37%, respectively, of the level of the chemically reduced ferrous-carbonyl complex (Table 1). This indicates that 37 to 38% of the total P450 present in the sample...
Microsomal protein from human liver (1 mg) was preincubated for 30 min at room temperature with 0.7 mg of purified IgG raised against residues 253–269 of human CYP3A4. Buffer, testosterone (250 μM) and NADPH were then added (total volume of 1 ml) and the mixture was divided between two 0.5-ml cuvettes. A baseline scan was recorded as described under Experimental Procedures. Carbon monoxide difference spectrum of human hepatic microsomal fraction.

The ability of cumene hydroperoxide to support testosterone 6β-hydroxylation (Fig. 6). The formation of this complex during catalysis was not affected by the presence of the antibody, because A 440 – 412 nm = 0.002 in the absence as well as in the presence of antibody (Fig. 6).

The ability of cumene hydroperoxide to support testosterone 6β-hydroxylation, in the absence and presence of the antibody, was determined and compared with the NADPH and reductase-supported metabolism of testosterone. Cumene hydroperoxide-supported testosterone 6β-hydroxylase activity of human liver microsomal fraction was progressively inhibited with increasing amounts of antibody, as found with the NADPH and reductase-supported system (data not shown). Maximum inhibition of greater than 90% was achieved with the antibody, similar to that found with the NADPH and reductase-supported system (Table 3).

TABLE 1

<table>
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<th>Protein</th>
<th>Ferrous-Carbonyl P450 Complex</th>
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<tr>
<td></td>
<td>Enzymatically reduced</td>
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<td>Human liver microsomes</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>E. coli</td>
<td>–</td>
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<tr>
<td></td>
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</table>

The reactions were stopped by the addition of 50 μl of 3 M HCl and 6β-hydroxytestosterone was quantified as described in Experimental Procedures. Values shown represent the mean of duplicate determinations, after the value of NADPH oxidation in the absence of testosterone had been subtracted from each value.

**Fig. 5.** Carbon monoxide difference spectrum of human hepatic microsomal fraction.

**Fig. 6.** Formation of an oxy-ferrous P450 complex during the metabolism of testosterone.
Effect of an antipeptide antibody targeted against human CYP3A4 on cumene hydroperoxide-supported testosterone 6β-hydroxylation

Microsomal protein from human liver (75 μg; calculated to correspond to 10 pmol CYP3A4, when CYP3A4 is considered to comprise 30% of total P450 in the sample), microsomal protein from E. coli expressing human CYP3A4 (10 pmol) and NADPH-P450 reductase (+ Red), or microsomal protein from E. coli expressing only human CYP3A4 (10 pmol) (−Red) was preincubated for 30 min at room temperature with 0.7 mg of purified IgG. The reaction was then started by the addition of 250 μM testosterone and 2 mM NADPH or 200 μM of cumene hydroperoxide and incubated for 12.5 min at 37°C. Reactions were terminated by the addition of 1.5 ml of dichloromethane and the amount of 6β-hydroxysterterone produced was determined as described in Experimental Procedures. Values represent the mean of duplicate determinations.

### Protein
- Human liver microsomes
- E. coli (+ Red)
- E. coli (− Red)

### System
- Cumene
- Hydroperoxide
- NADPH

### Testosterone 6β-Hydroxylation
- Control
- Antibody

### Relative Activity (% of control)
- Control
- Antibody

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<th>IgG</th>
<th>Testosterone 6β-Hydroxylation</th>
<th>Relative Activity (%)</th>
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N.D., not determined.

### Notes
- By interfering in the transfer of the second proton, and/or by retarding the movement of the F-G domain, which, based on structural studies of bacterial CYP102, has been suggested to occur to further optimize the electrostatic interactions between the substrate and the enzyme (Chang and Loew, 1999).

- The production of anti-peptide antibodies that inhibit enzyme activity requires the targeting of an appropriate region of the P450 enzyme of interest that interferes with the catalytic process. The region chosen in this study is predicted from models of the three-dimensional structure of P450 to be a loop region between the G- and H-helix on the surface of the enzyme (Lewis, 1996). It has been shown previously that this region contains a proinhibitory site, with the core sequence corresponding to residues 262–264 (Wang et al., 1999).

- The antibody produced in this study, which was raised against a peptide corresponding to residues 253–269 of CYP3A4, inhibited testosterone 6β-hydroxylase activity of human hepatic microsomal fraction and microsomal fraction from E. coli expressing CYP3A4 by greater than 90%. Similarly, Wang and Lu (1997) found that an antibody raised against a peptide corresponding to residues 254–273 of CYP3A4 significantly inhibited (>90%) the 6β-hydroxylation of testosterone of human hepatic microsomal fraction.

- Although antibody binding caused a substantial decrease in Vmax in this study, it did not alter the apparent Km value of the enzyme for testosterone, indicating that the mechanism of inhibition of enzyme activity by the antibody does not involve a decrease in the affinity of CYP3A4 for testosterone. The results of the substrate binding study support this notion. Binding of testosterone to CYP3A4 was shown to produce a typical type I difference spectrum with a maximum at 390 nm and a minimum at 420 nm (Schenkman et al., 1981). In the presence of the antibody, the ability of testosterone to induce a low to high spin transition with CYP3A4 was not affected, demonstrating that the antibody is not interfering with the binding of substrate to the P450 enzyme (Fig. 7). In the absence of testosterone, addition of the antibody (enough to cause inhibition of enzyme activity) to microsomal protein from E. coli expressing CYP3A4 did not produce a type I difference spectrum, indicating that the antibody does not bind to a substrate-binding site in CYP3A4. Indeed, based on the location of the six known substrate recognition sites in the CYP2 family (Gotoh, 1992; Lewis, 1996), the region targeted by the antibody in CYP3A4 does not form part of any of these substrate recognition sites.

- From work performed with bacterial CYP102 and NADPH-P450 reductase, the low to high transition elicited by the binding of substrate induces the elevation of the reduction potential of the P450 heme iron, thereby allowing the transfer of one electron from NADPH via the reductase to the P450 enzyme (Fig. 7) (Munro et al., 1999; Sverruokova et al., 1999). To determine whether antibody binding blocked this reduction step in CYP3A4, the ability of CYP3A4 preincubated with the antibody to form the steady-state level of the enzymatically reduced P450–CO complex was investigated. The percentage of the enzymatically reduced CO complex formed by CYP3A4 relative to the chemically reduced CO complex in the presence of the antibody was virtually identical when compared with the amount formed by a human liver microsomal preparation containing no antibody, indicating that the active site of the protein has not been modified. The result also suggests that the transfer of the first electron to oxidized CYP3A4 was not blocked by antibody binding, thereby indicating indirectly that the binding site for NADPH-P450 reductase is not localized to this region of the P450 enzyme.

- Support for this came from the observation that the amount of the steady-state level of the dioxy-ferrous P450 complex (440-nm chromophore) formed during testosterone metabolism was not reduced following antibody binding. The formation of this complex is the next intermediate step following the reduction of the P450 heme iron by NADPH and reductase (Fig. 7) (Ortiz de Montellano, 1999), and is the last step that has been directly observed under physiological conditions (Estabrook et al., 1971; Guengerich et al., 1976). Because the formation of the dioxy-ferrous P450 complex during catalysis is also dependent on the binding of oxygen, in addition to the presence of substrate and the use of NADPH as the electron donor (Estabrook et al., 1971; Guengerich et al., 1976; Ortiz de Montellano, 1999), the results described above also demonstrate that antibody binding does not block the binding and activation of molecular oxygen.

- Organic hydroperoxides such as cumene hydroperoxide have been shown previously to support the metabolism of substrates in the absence of NADPH, reductase, and molecular oxygen (Nordblom et al., 1976). In this study, cumene hydroperoxide was found to support the catalytic activity of testosterone 6β-hydroxylation in human hepatic microsomal fraction, although not as efficiently as NADPH and reductase-supported activity. This finding was supported by a number of previous studies using human hepatic microsomal fraction and microsomal protein from cells expressing human CYP3A4 (Brian et al., 1990; Kim and Kim, 1998). The binding of the antibody to CYP3A4 was shown to inhibit the cumene hydroperoxide-supported testosterone 6β-hydroxylation activity of human hepatic microsomal fraction as well as microsomal protein from E. coli expressing CYP3A4 by greater than 90%. This level of inhibition is virtually identical with that found in the NADPH and reductase-supported system. Support of testosterone hydroxylation in the cumene hydroperoxide system is through a peroxide-dependent or ‘peroxide shunt’ pathway (Nordblom et al., 1976). The proposed mechanism involves the binding of cumene hydroperoxide and substrate to P450 enzymes at the substrate binding site (Nordblom et al., 1976; Cvrek and Strobel, 1998) and the formation of a reactive ferric ternary hydroperoxo complex through peroxide reduction by P450 enzymes (Fig. 7) (Nordblom et al., 1976; Blake and Coon, 1980; Benson et al., 1997). Protonation and subsequent loss of water or cumenol from this complex provides the reactive oxygen that will subsequently attack the substrate. Because the ternary hydroperoxo complex is also produced...
by two-electron reduction of the P450 enzyme and binding of molecular oxygen (Nordblom et al., 1976), it appears that antibody binding inhibits enzyme activity by possibly blocking a step after the first proton transfer (Fig. 7).

The rate of NADPH oxidation in a sample of microsomal protein from *E. coli* expressing CYP3A4 was decreased by 40% in the presence of antibody, but the decrease did not correlate with the observed inhibition of testosterone 6\β-hydroxylation of the same microsomal sample (>75%). It has been shown previously that P450 enzymes catalyze the formation of water and hydrogen peroxide in addition to products from NADPH, oxygen, and substrate (Nordblom and Coon, 1977). However, because uncoupled reduction of oxygen to hydrogen peroxide occurs before the formation of the ternary hydroperoxo complex, it is distinctly possible that the decrease in enzyme activity could be due to an increase in the uncoupled reduction of oxygen to water as a result of a decrease in the stability of the ternary hydroperoxo complex. This, however, has yet to be determined.

Molecular dynamic simulations (MDSs) of CYP102 have shown that the binding of the substrate palmitoleic acid to CYP102 may induce conformational changes in the enzyme (Chang and Loew, 1999). The entrance of the substrate access channel of CYP102 was shown to close down in the presence of this substrate during a 500-ps MDS at room temperature. Furthermore, comparison of the X-ray structure of the substrate-bound CYP102 with the substrate-free form revealed that the F-G domain appears to undergo a large-scale rotation around the H-helical axis on substrate binding, as the structure of the substrate-bound CYP102 does not superimpose well with the substrate-free form (Chang and Loew, 1999). However, as the rotation was not detected during the MDS in the time frame of 500 ps, it was suggested that this could occur at a later stage. As the antibody produced in this study was targeted, based on sequence alignment with CYP102 against the loop region between the G- and H-helices, it is distinctly possible that antibody binding to CYP3A4 is inhibiting enzyme activity by retarding or blocking the large scale rotational movement of the F-G domain that may possibly be occurring in CYP3A4 following substrate binding. These possibilities need to be investigated in future studies.

In conclusion, the inhibition of CYP3A4 activity by an anti-peptide antibody is not the result of interference with substrate binding, electron transfer, or binding of molecular oxygen to the enzyme, but is due to either a decreased stability of the ternary hydroperoxo complex leading to uncoupling, interference with the second proton transfer, and/or interference with the substrate-induced conformational changes that are postulated to occur in P450 enzymes.

**References**


Edwards RJ, Singleton AM, Sesarick D, Boobis AR and Davies DS (1988) Antibodies to a...
synthetic peptide that reacts specifically with a common surface region on two hydrocarbon-inducible isoenzymes of cytochrome P-450 in the rat. Biochem Pharmacol 37:3735–3741.


