Identification of a functionally conserved surface region of rat cytochromes *P450IA*

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A region of rat cytochrome *P450IA1* at residues 294–301 (Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn), equivalent to a pro-inhibitory region of cytochrome *P450IA2*, was identified by sequence alignment. Anti-peptide antibodies were successfully raised when the peptide was coupled through either its *N*- or its *C*-terminus to carrier protein, but no antibodies were produced against the so-called multiple peptide antigen, which consisted of eight copies of the peptide attached through its *C*-terminus to a synthetic base. Both of the anti-peptide antibodies bound specifically to cytochrome *P450IA1* in the rat, as shown by e.l.i.s.a. and immunoblotting. They inhibited microsomal aryl hydrocarbon hydroxylase activity and the mutagenic activation of 2-acetylaminofluorene (these reactions are catalysed by cytochrome *P450IA1*), but not high-affinity phenacetin *O*-de-ethylation activity, which is catalysed by cytochrome *P450IA2*. However, there were differences in the properties of the two antisera in their binding to cytochromes *P450IA1* in species other than the rat, their relative binding to the multiple peptide antigen, the yield of antibody following affinity purification using peptide coupled through its *N*-terminus to CNBr-activated Sepharose, and the binding of the purified preparations to *N*- and *C*-terminal-coupled peptide conjugates. These observations indicated that the antibodies were directed to the region of the peptide opposite to the end which was coupled to the carrier protein. Nevertheless, both of the antibody preparations bound equally well to the target cytochrome *P450*, thus indicating that, in the native protein, the whole of the peptide region is exposed on the surface of cytochrome *P450IA1* and is available for binding by the antibodies. The role of this region appears to be the same in both cytochromes *P450IA1* and *P450IA2*, despite the difference in its primary structure in the two cytochromes *P450*.

INTRODUCTION

Cytochromes *P450* comprise a superfamily of isoenzymes which catalyse the metabolism of a vast number of compounds of both exogenous and endogenous origin. Cytochromes *P450* play a key role in the elimination of many drugs and toxins, in the activation of some compounds to toxins or carcinogens, and in the metabolism of many steroids, vitamins, prostaglandins and leukotrienes. Many of the isoenzymes involved in these reactions have been characterized following their purification from microsomal or mitochondrial preparations (Åström & DePierre, 1986; Guengerich, 1987). More recently, however, the identification of different isoenzymes of cytochrome *P450* has been achieved through the isolation and sequencing of the corresponding DNA or RNA sequences (Gonzalez, 1989). Analysis of the derived amino acid sequences has been used to produce a systematic nomenclature based on sequence similarity (Nebert *et al.*, 1987, 1989).

The sequence information already accumulated can be used to target anti-peptide antibodies towards specific cytochromes *P450* (Frey *et al.*, 1985; Edwards *et al.*, 1988, 1990; Oesch *et al.*, 1989; Myers *et al.*, 1990). Such antibodies have several advantages over polyclonal and monoclonal antibodies raised against purified cytochromes *P450*. Firstly, the purification of isoenzymes of cytochrome *P450* for immunization is unnecessary. This is particularly important when studying isoenzymes which are present in low abundance, especially human cytochromes *P450*. Secondly, the specificity of the antibody may be predicted from the primary structure of related cytochromes *P450*. Thirdly, the site of antibody binding on the target protein is known. In

addition, once a functionally important region has been identified on one isoenzyme through the production of inhibitory anti-peptide antibodies, then, if the structures of the isoenzymes are conserved, it should be possible to produce antibodies with a similar effect on other isoenzymes by directing anti-peptide antibodies to the equivalent region on those cytochromes *P450*.

We have previously raised an anti-peptide antibody specifically directed towards rat cytochrome *P450IA2* which inhibited the activity of this isoenzyme (Edwards *et al.*, 1990). We now report the production of a specific antibody directed against rat cytochrome *P450IA1* by targeting antibodies to the region analogous in sequence position to the binding site of the anti-peptide antibody in cytochrome *P450IA2*. This antibody was used to investigate the role of this region of cytochrome *P450IA1*.

EXPERIMENTAL

Materials

N- α -9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Millipore (London, U.K.), except for t-butylthiocysteine, which was from Novabiochem AG (Läufelfingen, Switzerland). CNBr-activated Sepharose was obtained from Pharmacia (Milton Keynes, U.K.). All SDS/PAGE reagents were from National Diagnostics (Aylesbury, Bucks., U.K.), except for pre-stained M_r standards, which were from Sigma (Poole, Dorset, U.K.). Keyhole-limpet haemocyanin (KLH) was from Novabiochem (Nottingham, Notts., U.K.). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billinghurst, W. Sussex, U.K.), and conjugates of immunoglobulins with horseradish peroxidase were from ICN Biomedicals (High

Abbreviations used: Fmoc, *N*- α -9-fluorenylmethoxycarbonyl; KLH, keyhole-limpet haemocyanin; 2-AAF, 2-acetylaminofluorene; PBS, phosphate-buffered saline; MAP, multiple antigen peptide; MC, 3-methylcholanthrene; AHH, aryl hydrocarbon hydroxylase; POD, phenacetin *O*-de-ethylase.

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Table 1. Aligned sequences of the synthetic peptide and cytochromes P450 in the P450IA subfamily from several species

The amino acid sequences, except for that of rabbit cytochrome P450IA2, are predicted from their cDNA sequences and are numbered from the *N*-terminal methionine.

Species	Isoenzyme	Sequence	Residues	Reference
Synthetic peptide		Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn		
Rat	P450IA1	-Cys-Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn-	293-301	Sogawa <i>et al.</i> (1984)
Mouse		-Cys-Gln-Asp-Arg-Lys-Leu-Asp-Glu-Asn-	290-298	Kimura <i>et al.</i> (1984)
Rabbit		-Tyr-Arg-Val-Asp-Arg-Leu-Asp-Glu-Asn-	293-301	Kagawa <i>et al.</i> (1987)
Human		-Cys-Gln-Glu-Lys-Gln-Leu-Asp-Glu-Asn-	289-297	Jaiswal <i>et al.</i> (1985)
Trout		-Cys-Glu-Asp-Arg-Lys-Leu-Asp-Glu-Asn-	294-302	Heilmann <i>et al.</i> (1988)
Rat	P450IA2	-Ser-Glu-----Asn-Tyr-Lys-Asp-Asn-	290-296	Sogawa <i>et al.</i> (1985)
Mouse		-Ser-Glu-----Asn-Tyr-Lys-Asp-Asn-	290-296	Kimura <i>et al.</i> (1984)
Rabbit		-Ser-Glu----Lys-Asn-Ser-Lys-Ala-Asn-	290-297	Ozols (1986)
Human		-Ser-Lys-----Lys-Gly-Pro-Arg-Ala-Ser-	291-298	Quattrochi <i>et al.</i> (1986)

Wycombe, Bucks., U.K.). Acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn, Scotland, U.K.). Benzo[*a*]pyrene, 2-acetylaminofluorene (2-AAF) and Bromocresol Purple were from Aldrich (Gillingham, Dorset, U.K.). Dimethyl sulphoxide (> 99.5%) was obtained from Fluka (Glossop, Derbyshire, U.K.). Pregnenolone 16 α -carbonitrile and clofibrate were generously provided by G. D. Searle (Skokie, IL, U.S.A.) and ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.) respectively. Sodium phenobarbitone was obtained from Macarthy's (Romford, Essex, U.K.). Tissue culture plates were purchased from Becton Dickinson (Cowley, Oxon., U.K.), and sterile 0.45 μ m filters were from Flow Laboratories (Rickmansworth, Herts., U.K.). Nutrient broth no. 2 was obtained from Oxoid (Basingstoke, Hampshire, U.K.). *Salmonella typhimurium*, strain TA98, was generously provided by Professor Bruce Ames (University of California, Berkeley, CA, U.S.A.) and was maintained in accordance with published recommendations (Maron & Ames, 1983). All other chemicals were purchased from Sigma (Poole, Dorset, U.K.) or BDH (Dagenham, Essex, U.K.) and were of analytical grade or the best equivalent.

Peptide synthesis and coupling to carrier protein

The peptide sequence chosen for synthesis (Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn) is present in rat cytochrome P450IA1, and consists of residues 294-301. This is equivalent to the region in rat cytochrome P450IA2 which has been shown to be a target for inhibitory anti-peptide antibodies (Edwards *et al.*, 1990), and was identified by alignment of rat cytochromes P450IA (Edwards *et al.*, 1989a) (Table 1). Peptide synthesis was performed manually by a solid-phase method using a polyamide support and Fmoc amino acids as described previously (Edwards *et al.*, 1990). The peptide was > 80% pure, as determined by h.p.l.c. using a C₁₈ μ Bondapak column (Millipore, Harrow, U.K.), with a gradient of 0-50% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid over 20 min at a flow rate of 1 ml/min, with constant monitoring of the eluent at 210 nm. The peptide had the correct composition by amino acid analysis, and the correct *M_r* (*M*+H⁺: *m/z* = 1045) as determined by fast atom bombardment mass spectrometry (University of London Intercollegiate Research Service, Mass Spectrometry, Department of Pharmaceutical Chemistry, The School of Pharmacy, London, U.K.).

To enable conjugation of the peptide to protein in a specific orientation, the peptide was also synthesized with a cysteine residue at either the *N*-terminus or the *C*-terminus. The *N*-terminal amine of the peptide which contained cysteine at its *C*-terminus was acetylated by treatment with a 10-fold molar excess

of acetic anhydride and an equimolar quantity of dimethyl aminopyridine dissolved in dimethyl formamide. This was performed to ensure strict equivalence in structure between this peptide, when coupled to carrier protein, and the multiple antigen peptide (MAP; see below). The two cysteinyl peptides were coupled individually to carrier protein. KLH was derivatized with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Liu *et al.*, 1979) and then added to each of the peptide preparations at 2.3 mg/ μ mol of cysteinyl peptide, as determined by the free thiol content of the peptides (Ellman, 1959). The pH of the mixtures was adjusted to 7.3; the mixtures were stirred for 2 h and then dialysed against phosphate-buffered saline (PBS: 1.5 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄, 2.7 mM-KCl, 137 mM-NaCl, pH 7.5). The degree of conjugation was assessed by quantitative amino acid analysis and was 0.2 μ mol/mg of KLH for both peptides. A 'null' conjugate, in which cysteine was substituted for peptide, was produced in the same way.

MAP synthesis

Recently it has been suggested that peptide-specific antisera can conveniently be produced by replacing the peptide-protein conjugate conventionally used for immunization with a multimeric peptide antigen which is synthesized on a core of lysine residues (Tam, 1989). The peptide itself is coupled to the core through its *C*-terminus. Since the MAP does not contain any carrier protein, only anti-peptide antibodies should be produced. The MAP was synthesized essentially as described by Posnett *et al.* (1988) and Tam (1988), except that Fmoc amino acids were used throughout. Glycine was first coupled to the resin; this was then followed by three cycles of addition of Fmoc-Lys(Fmoc), thus increasing the number of peptide anchor points (free amines) to eight. This was followed by three cycles of addition of Fmoc-glycine. A sample of the MAP was deprotected and cleaved from the resin at this stage, and is referred to as the core of the MAP. Synthesis of the MAP was continued by coupling, as above, the amino acids comprising the peptide sequence. Upon completion of the synthesis, the *N*-terminal amine was acetylated prior to deprotection and cleavage, as described above, and was then purified as described by Posnett *et al.* (1988). Amino acid analysis of the core of the MAP and of the completed MAP yielded the expected amino acid composition.

Immunization

Male New Zealand White rabbits (3 kg, from Froxfield Farms Ltd., Petersfield, Hants., U.K.) were immunized with either peptide conjugated to KLH or the MAP. Each injection was administered in Freund's adjuvant in a total volume of 1 ml and

contained either 0.2 mg of peptide conjugate or 1.0 mg of the MAP. The initial injection was administered subcutaneously in Freund's complete adjuvant. After 14 days, a second subcutaneous injection was given using incomplete adjuvant. After 28 days an intraperitoneal injection was given in Freund's incomplete adjuvant and blood samples were taken from a marginal ear vein 7 days later. Pre-immune blood samples were collected from the rabbits prior to treatment.

Affinity purification of anti-peptide antibodies

The non-cysteinylation peptide was coupled to CNBr-activated Sepharose 4B (Axén *et al.*, 1967). The incorporation of the peptide, determined by amino acid analysis, was 1.13 $\mu\text{mol/ml}$ of Sepharose. Coupling was through the amine group at the *N*-terminus of the peptide. Anti-peptide antibodies were purified from antiserum by affinity chromatography using the peptide-Sepharose as described previously (Edwards *et al.*, 1989b).

Other anti-peptide antibodies

The production and characterization of anti-peptide antibodies directed to regions common to both cytochromes P450IA1 and P450IA2, at residues 174–182 and 171–179 respectively and at residues 356–363 and 350–357 respectively, as well as of an anti-peptide antibody specific to cytochrome P450IA2 at residues 290–296, have all been described previously (Edwards *et al.*, 1988, 1990, 1991).

Immunological analyses

The method for e.l.i.s.a. has been described previously (Edwards *et al.*, 1988). The microtitre plates were coated with 100 μl of microsomal fraction (100 $\mu\text{g/ml}$), purified cytochrome P450 (1 $\mu\text{g/ml}$), peptide conjugate (5 $\mu\text{g/ml}$), unconjugated peptide (2 $\mu\text{g/ml}$) or MAP (2 $\mu\text{g/ml}$) diluted in PBS.

Immunoblot analysis was carried out using the method described by Towbin *et al.* (1979) incorporating the modifications described previously (Edwards *et al.*, 1988).

Animals, and their treatment with inducing compounds

Male Wistar rats (200–250 g) and juvenile rats (100–150 g) were obtained from Harlan Olac Ltd. (Bicester, Oxon., U.K.), and male New Zealand White rabbits (3 kg) were from Frowfield Farms Ltd. Male Balb/c mice (6–10 weeks) and guinea pigs (250–300 g) were from A. Tuck & Son, Dunkin-Hartley Ltd. (Battledridge, Essex, U.K.), and male Golden Syrian hamsters (90–110 g) were supplied by Belgrave Trading Ltd. (Lambourn, Berks., U.K.). Groups of animals were left untreated or were administered inducing compounds 3-methylcholanthrene (MC), sodium phenobarbitone, pregnenolone 16 α -carbonitrile, acetone or clofibrate, as described by Rich *et al.* (1989). Animals were killed humanely in accordance with approved Home Office procedures appropriate to the species, the livers were rapidly removed and microsomal fractions were prepared as described previously (Boobis *et al.*, 1980).

Preparation and characterization of human hepatic microsomal fractions

Human liver samples were obtained from the human tissue bank at the Royal Postgraduate Medical School (Boobis *et al.*, 1980). Local Ethics Committee approval and Coroner's permission were obtained for their use in these studies. The preparation of the microsomal fraction was as described previously (Boobis *et al.*, 1980). The characterization of the human hepatic microsomal fractions has been reported previously (Sesardic *et al.*, 1990a).

Purification of rat cytochromes P450IA1 and P450IA2

The purification of cytochromes P450IA1 and P450IA2 from the hepatic microsomal fraction of rats treated with MC has been described previously (Edwards *et al.*, 1988). The isoenzyme preparations were 85–90 % pure, as determined by SDS/PAGE, and each was free from contamination by the other form, as judged by both e.l.i.s.a. and immunoblotting using a monoclonal antibody which binds to both isoenzymes (Edwards *et al.*, 1988).

Measurement of cytochrome P450-dependent mono-oxygenase activities

Aryl hydrocarbon hydroxylase (AHH) activity. This activity was determined using the method of Atlas *et al.* (1977), except that the hepatic microsomal fraction (5 μg of protein) from MC-treated rats was mixed with antiserum for 5 min at 37 °C prior to addition to the reaction mixture.

Phenacetin O-de-ethylase (POD) activity. POD activity of hepatic microsomal fractions from MC-treated rats was determined by the method of Murray & Boobis (1986), using a final phenacetin concentration of 4 μM . Hepatic microsomal fraction (10 μg of protein) was incubated for 30 min at 4 °C with various amounts of antiserum in a total volume of 0.2 ml. Buffer and cofactors were then added, and the mixtures were warmed to 37 °C before initiation of the reaction by addition of substrate.

Mutagenicity assay. Bacterial mutagenicity was used as an end point to detect the formation of short-lived reactive intermediates produced from 2-AAF using a modification of the fluctuation assay as described previously (Edwards *et al.*, 1990). The assay was performed using the hepatic microsomal fraction from MC-treated rats which had been incubated with antiserum for 5 min at 37 °C prior to addition to the reaction mixture.

Fig. 1. Relative binding of antisera raised against the cytochrome P450IA1 peptide Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn

Microtitre plates were coated with the MAP, and series of dilutions of antisera raised against the peptide were added and antibody binding compared. Antisera were obtained from rabbits immunized with the peptide coupled to KLH through its *N*-terminus (△), the peptide coupled to KLH through its *C*-terminus (○), the MAP (●) or the MAP followed by the peptide coupled to KLH through its *C*-terminus (○). Antibody binding was determined as described in the text. When unconjugated peptide was used to coat the microtitre plates, negligible antibody binding was observed for all antisera (■). Each point is the mean of two determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

RESULTS

The region of rat cytochrome *P450IA1* chosen for investigation was selected on the basis of alignment of the isoenzymes in the IA subfamily to identify the region equivalent to residues 290–296 of cytochrome *P450IA2* (Table 1). The region strictly equivalent to that in rat cytochrome *P450IA1* comprises residues 293–301. However, as the residue at position 293 is a cysteine, and this amino acid is required for linking the peptide to the carrier protein, the sequence selected for study comprised residues 294–301 of cytochrome *P450IA1*. The peptide sequence is unique to cytochrome *P450IA1* amongst other cytochrome *P450* sequences, and indeed amongst all of the protein sequences currently listed in the Swissprot database (Kahn & Cameron, 1990), as determined by the use of the program FASTA (Pearson & Lipman, 1988).

Three immunogens were produced and tested for their ability to produce antibodies against the peptide sequence corresponding to residues 294–301 of rat cytochrome *P450IA1*. Two of the immunogens were composed of the peptide coupled to KLH through a cysteine residue introduced, during peptide synthesis, at either the *N*-terminus or the *C*-terminus of the peptide. Thus

Fig. 2. Relative binding of affinity-purified anti-peptide antibodies

Microtitre plates were coated with peptide coupled through its *N*-terminus to KLH (○), peptide coupled through its *C*-terminus to KLH (●) or cysteine coupled to KLH (▲). A series of dilutions of affinity-purified anti-peptide antibody raised against the peptide coupled through either its *N*-terminus (a) or *C*-terminus (b) was added, and antibody binding was determined as described in the text. Each point is the mean of two determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

Fig. 3. Binding of anti-peptide antibodies to purified cytochromes *P450IA1* and *P450IA2*

Microtitre plates were coated with cytochrome *P450IA1* (●, ▲), cytochrome *P450IA2* (○, △) or bovine serum albumin (◆). A series of dilutions of affinity-purified anti-peptide antibody raised against the peptide coupled through either its *N*-terminus (●, ○) or *C*-terminus (▲, △) was added, and antibody binding was determined as described in the text. Each point is the mean of two determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

Fig. 4. Specific binding of anti-peptide antibodies to cytochromes *P450* in hepatic microsomal fractions from several species

Immunoblots of 5 pmol of cytochrome *P450IA1* (lane 1), 5 pmol of cytochrome *P450IA2* (lane 2) and 25 μg portions of hepatic microsomes from untreated rat (lane 3), MC-treated rat (lane 4), MC-treated mouse (lane 5), MC-treated rabbit (lane 6), human (lane 7), MC-treated hamster (lane 8) and MC-treated guinea pig (lane 9). The immunoblots were developed with antibody raised against the peptide coupled through either its *N*-terminus (a) or *C*-terminus (b) to KLH.

the peptide was coupled to carrier protein in one of two defined orientations. The third immunogen constructed was the MAP. The orientation of the peptide in the MAP was the same as that of the peptide coupled to the carrier protein through its *C*-terminus. Rabbits were immunized with each of these three preparations and the resultant antisera were tested by e.i.s.a. for the production of anti-peptide antibodies (Fig. 1). Microtitre plate wells were coated with either the MAP or unconjugated peptide. Serum from the rabbits immunized with peptide conjugated to KLH bound to the MAP, although it was evident that the antiserum from the rabbit immunized with the peptide coupled through its *C*-terminus bound more strongly than antiserum from the rabbit immunized with the peptide coupled through its *N*-terminus. In contrast, of three rabbits immunized

Fig. 5. Relative binding of affinity-purified anti-peptide antibodies to hepatic microsomes from several species treated or not with MC

Microtitre plates were coated with hepatic microsomal preparations from rat (○), rabbit (●), mouse (▲), guinea pig (◆) or hamster (▼) which had either been treated with MC (a and c) or were untreated (b and d). Human samples (■) had either a high (a and c) or a low (b and d) microsomal POD activity. A series of dilutions of affinity-purified anti-peptide antibody raised against the peptide coupled through either its *N*-terminus (a and b) or *C*-terminus (c and d) was added and antibody binding was determined as described in the text. Where no curve is shown for a species, then binding of the antibody to that preparation was negligible and equivalent to the curve shown that has the lowest absorbance measurements for each concentration of antibody applied to the microtitre plate. Each point is the mean of two determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

with the MAP, none produced any detectable antibodies, as determined by e.l.i.s.a. (Fig. 1). However, subsequent immunization of one of these rabbits with peptide coupled through its *C*-terminus to KLH elicited antiserum which displayed strong binding to the MAP (Fig. 1). None of the antisera bound to the core of the MAP (results not shown). In addition, none of the antisera bound to microtitre wells coated with unconjugated peptide (Fig. 1).

Anti-peptide antibodies were purified from serum samples by affinity chromatography using peptide coupled through its *N*-terminus to CNBr-activated Sepharose. From 5 ml of serum obtained from rabbits immunized with peptide conjugates coupled through their *N*-terminus or *C*-terminus, 4.0 mg or 0.3 mg of antibody was produced respectively. The success of the purification of the anti-peptide antibodies and demonstration of their specific binding to the peptide was shown by e.l.i.s.a. through a comparison of the binding of the purified antibody preparations to the peptide-KLH conjugates and the null conjugate (Fig. 2). The antibody preparations obtained from rabbits immunized with the *N*-terminal-coupled peptide showed a clear increase in binding to the peptide-KLH conjugates compared with the null conjugate, thus demonstrating the specificity of the purified antibody for the peptide. In addition, this antibody preparation bound more strongly to the *N*-terminal-coupled peptide than to the *C*-terminal-coupled peptide (Fig. 2a). The purification of anti-peptide antibody from the rabbit immunized

with the *C*-terminal-coupled peptide yielded less antibody. Nevertheless, there was an increase in binding to the peptide-KLH conjugates compared with the null conjugate (Fig. 2b). This antibody preparation bound equally to the *N*-terminal- and *C*-terminal-coupled peptides (Fig. 2b).

Both of the purified antibody preparations bound strongly to purified rat cytochrome P450IA1, and neither bound to purified rat cytochrome P450IA2 in e.l.i.s.a. (Fig. 3). This specificity was also seen in immunoblots performed using both purified cytochrome P450 preparations and a hepatic microsomal preparation obtained from rats treated with MC. Whereas strong binding to cytochrome P450IA1 was observed, there was no binding to cytochrome P450IA2 (Fig. 4). This specificity is complementary to that of an anti-peptide antibody raised against the corresponding residues 290–296 of cytochrome P450IA2, which binds specifically to cytochrome P450IA2 (Edwards *et al.*, 1990). No binding of the anti-peptide antibodies directed against cytochrome P450IA1 was observed in a hepatic microsomal preparation obtained from untreated rats (Fig. 4), and this is in accord with the report that cytochrome P450IA1 is undetectable in such animals (Sesardic *et al.*, 1990b). These observations were confirmed by e.l.i.s.a. using both of the antibodies against cytochrome P450IA1 (Fig. 5). In addition, neither of the antibodies bound to any other rat cytochrome P450, as determined by both immunoblotting and e.l.i.s.a. using hepatic microsomal fractions prepared from rats following treatment with sodium

Table 2. Immunoinhibition of cytochrome *P450*-catalysed reactions by anti-peptide antibodies directed against cytochrome *P450IA1* and *P450IA2*

The amino acid sequences of rat cytochrome *P450IA1*, residues 174–182, is identical to that of rat cytochrome *P450IA2*, residues 171–179, and the region at rat cytochrome *P450IA1*, residues 356–363, is identical to rat cytochrome *P450IA2*, residues 350–357. The effects of the addition of various antisera are compared with addition of pre-immune serum. The mean values and s.d.s obtained from three experiments are shown. The results were analysed using Student's *t* test: **P* < 0.01, ***P* < 0.001 versus control. n.d., not determined.

Peptide to which antiserum was raised	Cytochrome <i>P450</i> activity inhibition by antibody (%)		
	AHH	POD	2-AAF
Pre-immune serum	0 ± 4	0 ± 4	0 ± 6
Rat <i>P450IA1</i> -(294–301)			
<i>N</i> -Terminal coupled	65 ± 5**	0 ± 2	50 ± 10*
<i>C</i> -Terminal coupled	65 ± 4**	1 ± 4	52 ± 9*
Rat <i>P450IA2</i> -(290–296)	0 ± 5	65 ± 5**	72 ± 13*
Rat <i>P450IA1</i> -(174–182)	–2 ± 3	–3 ± 9	n.d.
Rat <i>P450IA1</i> -(356–363)	2 ± 3	3 ± 3	n.d.

phenobarbitone, pregnenolone 16 α -carbonitrile, acetone or clofibrate (results not shown).

The two anti-peptide antibody preparations were tested for binding to hydrocarbon-inducible cytochromes *P450* in species other than the rat. Immunoblots of hepatic microsomal fractions prepared from MC-treated animals developed with the antiserum produced against the *N*-terminal-coupled peptide showed binding to rabbit and mouse cytochrome *P450IA1* (Fig. 4). The antibody also bound to a protein of *M_r* 50 000 in hepatic microsomal fraction prepared from guinea pig (Fig. 4), but it is not clear if this protein is a cytochrome *P450*. There was no binding of the antibody to hepatic microsomal protein prepared from either the hamster or man (Fig. 4). With hepatic microsomal protein prepared from untreated animals, the antibody bound only to the rabbit and guinea pig samples (results not shown). In the rabbit, the antibody bound to cytochrome *P450IA1*, albeit with a decreased intensity compared with that in the MC-treated animal. This is in accord with reports on the levels of cytochrome *P450IA1* in untreated and hydrocarbon-treated rabbits (Schwab & Johnson, 1989). In the untreated guinea pig, the antibody bound to a protein with the same electrophoretic mobility and intensity as that seen in the MC-treated animals. The lack of induction of cytochrome *P450* in the guinea pig following treatment with MC has been reported previously (Thorgeirsson *et al.*, 1979). There was no binding to microsomal proteins from the untreated mouse or hamster (results not shown).

In e.l.i.s.a., the antibody was shown to bind more strongly to hepatic microsomal protein prepared from MC-treated rats than to that from the other MC-treated animals examined (Fig. 5). Binding to microsomes from the rabbit, guinea pig, and mouse was observed, but there was no detectable binding to the hamster or human samples. In untreated animals, binding was seen only to the guinea pig and rabbit preparations (Fig. 5). In contrast, anti-peptide antibody raised against the *C*-terminal-coupled peptide was species-specific, binding only to rat cytochrome *P450IA1* and to no other cytochromes *P450* or other hepatic microsomal protein in immunoblotting (Fig. 4) or e.l.i.s.a. (Fig. 5).

Both of the anti-peptide antibodies directed against cytochrome *P450IA1* inhibited AHH activity in hepatic microsomal fractions obtained from rats treated with MC (Table 2). However, neither

of the two antisera inhibited high-affinity POD activity (Table 2). Both antibodies were also able to inhibit the mutagenic activation of 2-AAF (Table 2). This is in contrast to the results with the anti-peptide antibody directed to the equivalent region of cytochrome *P450IA2*, which inhibited 65% of POD activity, but not AHH activity (Table 2). However, this antibody did inhibit the mutagenic activation of 2-AAF. Neither AHH nor POD was inhibited by anti-peptide antibodies which were directed to two other regions common to both cytochromes *P450IA1* and *P450IA2* (Table 2).

DISCUSSION

A model for the three-dimensional structure of eukaryotic cytochromes *P450* is currently being developed (Edwards *et al.*, 1989a, 1991). This model is based on the structure of cytochrome *P450CI*, which is the only form of cytochrome *P450* to have had its structure elucidated (Poulos *et al.*, 1987). The α -helices of cytochrome *P450CI* comprise about half of the secondary structure, and regions of the eukaryotic isoenzymes equivalent to these α -helices have been identified (Edwards *et al.*, 1989a). Thus there is a high degree of structural similarity between the eukaryotic isoenzymes and cytochrome *P450CI*. A number of anti-peptide antibodies targeted towards cytochrome *P450* have been produced (Frey *et al.*, 1985; Edwards *et al.*, 1988, 1990, 1991) and their binding sites have been mapped on to this model (Edwards *et al.*, 1991). All of the anti-peptide antibody binding sites were located on the surface of the model, and their distribution over the surface of the protein suggests that the globular domain of eukaryotic cytochromes *P450* is largely exposed to the cytosol and in an orientation such that the plane of the haem of the enzyme is more perpendicular than horizontal to the plane of the membrane (Edwards *et al.*, 1991). This model of the structure of cytochrome *P450* is based on the hypothesis that all cytochromes *P450* have a similar tertiary structure, despite considerable variability in their primary structures. The conservation of the structure of functionally related proteins at the expense of amino acid composition has been observed in other protein families, e.g. the serine proteases, aspartic proteinases (Blundell *et al.*, 1988) and the GTPase superfamily (Bourne *et al.*, 1990).

When bound to cytochrome *P450*, some of the anti-peptide antibodies have been shown to inhibit enzyme activity. If the structure of cytochrome *P450* is conserved in all isoenzymes, then it would be expected that the identification of a target site for inhibitory antibodies on one cytochrome *P450* should enable the identification of the corresponding sites on other isoenzymes, even when there is considerable variability of the primary structure of such sites between different isoenzymes. This theory was tested using cytochromes *P450IA1* and *P450IA2*, which have 68% sequence similarity (Black & Coon, 1986). We have previously identified an inhibitory site on rat cytochrome *P450IA2* (Edwards *et al.*, 1990). The equivalent region of cytochrome *P450IA1* was identified by sequence alignment (Table 1). Antibodies were raised against this peptide and were shown to bind specifically to cytochrome *P450IA1*. There is little similarity between the amino acid structure of this peptide and that occurring in cytochrome *P450IA2*. Nevertheless, the antibodies inhibited cytochrome *P450IA1* activity, demonstrating that this surface site plays a similar role in the two isoenzymes.

Three different immunogens were used to raise anti-peptide antibodies against the target region of cytochrome *P450IA1*. Anti-peptide antibodies were raised successfully when the synthetic peptide was chemically coupled to KLH through a cysteine residue introduced at the *N*- or *C*-terminus of the peptide. However, no anti-peptide antibodies were detected after immuniz-

ation with the MAP. The lack of antibodies against the MAP could not be attributed to a poor immunological response of the rabbits to the peptide sequence, as subsequent immunization of one of these rabbits with the peptide conjugated to KLH successfully elicited anti-peptide antibodies. The MAP was also shown to possess antigenic peptide that was accessible to antibodies, as anti-peptide antibodies produced from rabbits immunized with peptide coupled to KLH in either orientation bound to the MAP in the e.l.i.s.a. (Fig. 1).

Antibodies have been raised successfully against other MAPs which consisted of different peptide sequences of between 9 and 21 residues in length (Tam, 1989; Posnett & Tam, 1989). The reason for the poor immunogenicity of the MAP used here is unknown, but it is possible that an eight-residue peptide may result in a MAP which is too small to be strongly immunogenic. Interestingly, the antiserum obtained from the rabbit which was immunized first with the MAP and subsequently with peptide coupled to KLH bound more strongly to the MAP in e.l.i.s.a. than did the antiserum obtained from rabbits which had been immunized with the peptide-protein conjugate alone (Fig. 1). It is possible, therefore, that the MAP did produce some immune response, although it was unable to induce anti-peptide IgG antibody production *per se*.

The anti-peptide antibodies failed to bind to microtitre plates coated with the peptide in an e.l.i.s.a. This has been observed previously for some other peptides (Edwards *et al.*, 1989b), although binding to small peptides used to coat e.l.i.s.a. plates has also been demonstrated (Edwards *et al.*, 1988). However, when the MAP was used to coat the microtitre plates, the antibodies did bind successfully. Tam & Zavala (1989) have previously shown that MAPs are better antigens in e.l.i.s.a. than their corresponding peptides. Presumably, when some peptides bind to the microtitre plate their amino acid side chains are unavailable for interaction with antibody, but in the case of the MAP the repeated antigenic structure allows binding to both the microtitre plate and antibody.

Both of the anti-peptide antibodies raised against the peptide-KLH conjugates bound specifically to cytochrome P450IA1 in the rat, as determined by e.l.i.s.a. and immunoblotting. There was no binding to cytochrome P450IA2, the most closely related cytochrome P450 isoenzyme. Both anti-peptide antibodies also inhibited enzyme activities associated with cytochrome P450IA1. With both antibodies the AHH activity of hepatic microsomes prepared from MC-treated rats was decreased by 65%; this reaction is catalysed principally (approx. 80%) by cytochrome P450IA1 (Thomas *et al.*, 1984). The activation of 2-AAF was inhibited by approx. 50%. In previous studies it has been shown that both purified reconstituted cytochromes P450IA1 and P450IA2 can catalyse this reaction (Åström & DePierre, 1985). The results of the present study demonstrate that, unlike in MC-treated rabbit (Johnson *et al.*, 1980), the two isoenzymes contribute almost equally to the activation of this aromatic amide in the hepatic microsomal fraction from MC-treated rats. High-affinity POD activity, which is catalysed specifically by cytochrome P450IA2 in the rat (Sesardic *et al.*, 1990c), was not affected by either of the antibodies. Conversely, an antibody raised against the corresponding region of cytochrome P450IA2 (residues 290–296) did not affect AHH activity, but inhibited both POD activity and 2-AAF activation (Table 2). Thus the inhibitory effects of the antibodies are entirely consistent with their specificity of binding to cytochromes P450. The inhibitory effect of the antibodies was due to the specific location of the binding site, as two other anti-peptide antibodies, directed to different regions of cytochromes P450IA1 and P450IA2, did not affect either AHH or POD activities (Table 2).

The two anti-peptide antibodies against cytochrome P450IA1 differed in their relative binding to the MAP in e.l.i.s.a., the yield of antibody following affinity purification, the binding of the purified preparations to *N*-terminal- and *C*-terminal-coupled peptide conjugates in e.l.i.s.a., and their binding to cytochromes P450IA1 from different species. These observations can all be explained by the orientation of the coupled peptide used for immunization. The importance of orientation in the coupling of peptides to protein carriers has been recognized previously. Dyrberg & Oldstone (1986) raised antibodies against an octa-peptide by coupling through either its *N*- or *C*-terminus to a carrier protein. Both of the antisera bound to unconjugated peptide, but they recognized the peptide-protein conjugate only when the peptide was coupled in the same orientation as that used for immunization. Further, only the antiserum raised against the peptide coupled through its *C*-terminus bound to a larger peptide fragment which represented a region of the human acetylcholine receptor, or to a purified preparation of the acetylcholine receptor from *Torpedo californica*. Lipkin *et al.* (1988) found that an antiserum raised against a 12-residue peptide, which was coupled through its *N*-terminus to carrier protein, bound to somatostatin in mouse brain slices. However, when the peptide was coupled through its *C*-terminus, the anti-peptide antibodies raised did not bind to somatostatin. By comparing the binding of anti-peptide antibodies to a series of structurally related peptides, Schaaper *et al.* (1989) have shown that, in general, the antibodies were directed towards the uncoupled ends of these peptides.

Our results are consistent with these observations. The pattern of binding of the antibodies raised against the conjugated peptide was such that the antibodies always bound more strongly to the peptide which was in the same orientation as that used for immunization. Hence the antibody raised against the peptide coupled through its *C*-terminus bound more strongly to the MAP, in which the peptide was also coupled through its *C*-terminus, than that raised against the peptide coupled through its *N*-terminus. Conversely, the antibody raised against the peptide coupled through its *N*-terminus bound better to peptide immobilized by coupling through its *N*-terminus to CNBr-activated Sepharose, and consequently yielded a greater quantity of purified antibody than the antiserum raised against the *C*-terminal-coupled peptide. As expected, the purified antibody raised against the peptide coupled through its *N*-terminus bound better to peptide which was coupled to KLH through its *N*-terminus than peptide coupled through its *C*-terminus. However, after purification, the antibody raised against the peptide coupled through its *C*-terminus bound equally to the peptide coupled in either orientation. This was because the affinity purification of the antibody preparation was achieved using peptide coupled through its *N*-terminus to CNBr-activated Sepharose, and hence would select for those immunoglobulin species that best recognize the peptide in this orientation.

The antibody raised against the peptide coupled through its *C*-terminus bound only to rat cytochrome P450IA1, and this can be explained by the variability in the known sequences of cytochromes P450IA1 at the region equivalent to the *N*-terminus of the peptide (Table 1). In mouse cytochrome P450IA1 there was just one amino acid residue different (Lys-294) from the peptide sequence, and in the rabbit there were three differences, at residues 294–296. The human cytochrome P450IA1 sequence also varied from the peptide sequence at the *N*-terminal region (residues 291–293), and the trout sequence contained two differences at residues 294 and 297. In contrast, the antibody raised against the peptide coupled through its *N*-terminus bound to rat, mouse and rabbit cytochrome P450IA1, and this can be explained by the occurrence of the sequence Leu-Asp-Glu-Asn in

all of these cytochromes P450IA1 in the region equivalent to the C-terminus of the peptide (Table 1). Human cytochrome P450IA1 also contains this sequence, but as cytochrome P450IA1 is undetectable in human liver (McManus *et al.*, 1990) the lack of binding of the anti-peptide antibody to human hepatic microsomes in immunoblotting and e.l.i.s.a. is most likely a consequence of the low levels or absence of this protein. From the sequence similarities it is predicted that this antibody should also bind to trout cytochrome P450IA1, but this has yet to be tested. Binding to a hepatic microsomal protein, most likely a cytochrome P450 isoenzyme, in the guinea pig was also observed, but the amino acid sequences of guinea pig cytochromes P450 are at present unknown.

Despite all of the above differences, both of the antibody preparations bound equally well to the target cytochrome P450. This indicates that in the native protein the whole of the peptide region is accessible for binding by the antibodies. Further, Parkinson *et al.* (1986a) have shown that when the cysteine residue (Cys-293, Table 1) in cytochrome P450IA1 adjacent to this region is chemically modified by a number of alkylating reagents, there is inactivation of the isoenzyme. Modification by 4,4'-dithiopyridine was shown to be reversible upon the addition of functional cytochrome P450 reductase (Parkinson *et al.*, 1986b). Therefore the extended region comprising residues 293–301 is likely to be exposed on the surface of the protein, and possibly in a loop conformation. The function of this region may involve the interaction of cytochrome P450 with cytochrome P450 reductase or in the transfer of electrons from the reductase to the haem of cytochrome P450.

In conclusion, anti-peptide antibodies directed towards rat cytochrome P450IA1 have been produced. These antibodies bound specifically to this isoenzyme in the rat and inhibited enzyme activities associated with the isoenzyme. This site of inhibition is equivalent in position to an inhibitory site identified on rat cytochrome P450IA2 (Edwards *et al.*, 1990), and although the amino acid sequences of the two regions are quite different, they appear to have a common function. The exact nature of this site has yet to be determined, although a role in the transfer of electrons from cytochrome P450 reductase is indicated. It should be possible to expand the use of anti-peptide antibodies to investigate the specificity of hydrocarbon-inducible isoenzymes in other species, particularly the human, where the primary structure of cytochrome P450 has been determined. Also, the investigation of the corresponding site in other families of cytochrome P450 will establish if the functional role of this region is conserved. This approach allows the prospect that specific inhibitory antibodies may be produced against any cytochrome P450 isoenzyme of known primary structure.

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