Probing the Putative Cytochrome P450- and Cytochrome *c*-Binding Sites on NADPH–Cytochrome P450 Reductase by Anti-Peptide Antibodies†

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ABSTRACT: Two regions (amino acid residues 110–130 and 204–218) of NADPH–cytochrome P450 reductase (reductase) have been shown to be the putative binding sites for the interaction with cytochrome P450 or cytochrome *c*. To obtain further insight into the molecular mechanism of protein–protein interaction between these proteins, three anti-peptide antibodies (1A, 2A, and 3A) were generated against the peptides corresponding to these two regions on rat reductase to study the interaction between the reductase and cytochrome P450 or cytochrome *c*. All three anti-peptide antibodies have high affinity for their peptide antigens on ELISA (titre $> 1 \times 10^{-6}$ g/L), and they also bind to rat reductase on ELISA under both denatured and native conditions, suggesting that these regions are on the surface of the protein. 1A and 3A also bind to rabbit and human reductase, though 1A binds to human reductase with lower affinity. Antibody 2A does not bind to rabbit or human reductase. Western blot analysis using these anti-peptide antibodies showed similar results. Antibodies 1A and 3A inhibit both cytochrome P4501A1-dependent ethoxycoumarin hydroxylation activity and P4502B1-dependent pentoxyresorufin dealkylation activity, but the inhibition by 1A and 3A was not additive. Antibodies 1A and 3A also have inhibitory effects on the activity of P4501A1-dependent ethoxycoumarin hydroxylation reconstituted with reductase from rabbit and human. However, none of the three anti-peptide antibodies inhibits cytochrome *c* reduction by rat reductase. These data suggest that reductases from rat, rabbit, and human share similar structure in at least two regions which appear to be on the surface of the protein. These two regions (110–119 and 204–218) of rat reductase both seem to be involved in the interaction with cytochrome P450, while the association of reductase with cytochrome *c* may depend on different mechanisms.

NADPH–cytochrome P450 reductase (reductase) is an FAD- and FMN-containing flavoprotein, which provides reducing equivalents from NADPH to cytochrome P450, the terminal oxidase of the microsomal mixed-function oxidase system. Reductase has been purified to homogeneity from various sources, and its mechanism of electron transfer has been elucidated (Strobel & Dignam, 1978; Vermilion & Coon, 1978; Yasukochi & Masters, 1976). Rat microsomal reductase has been cloned, and subsequent sequence analysis has enabled the prediction of sites for NADPH binding and possible FAD and FMN binding (Porter & Kasper, 1985, 1986). More complete knowledge of the structure of reductase awaits solution of its crystal structure. It is believed that the cytochrome P450:reductase ratio is approximately 20:1 *in vivo*. However, studies have shown that an equimolar complex of P450 and reductase has optimal functional activity in a reconstituted microsomal system *in vitro* (Estabrook et al., 1976). Both stable complex formation and transient complex formation between cytochrome P450 and reductase have been suggested (French et al., 1980; Nisimoto & Otsuka, 1988; Gut et al., 1983; Wagner et al., 1984). As for many other electron transfer protein complexes, it is proposed that the electrostatic charge pairing interaction is one of the major forces for the association between the two enzymes (Bösterling & Trudell, 1982; Bernhardt et al., 1988; Nadler & Strobel, 1988; Strobel et al., 1989; Stayton & Sligar, 1990; Shimizu et al., 1991; Shen & Strobel, 1992), though other forces of interaction are also proposed (Voznesensky & Schenkman, 1992a,b). Using chemical modification approaches, Nadler and Strobel (1991) identified a region including amino acids 110–130 of rat reductase as participating in interaction with cytochrome P450. Another region including amino acids 204–218 was suggested by Nisimoto (1986) to be the site for cytochrome *c* association. The data from a kinetics study have suggested that cytochrome P450 and cytochrome *c* may share the same binding site on reductase (Davydov, 1992). It was known not only that the reductase can reduce cytochrome P450, but also that it can reduce other heme proteins such as cytochrome *c* (Phillips & Langdon, 1962), cytochrome b_5 (Enoch & Strittmatter, 1979), and ferric heme oxygenase (Yashinaga et al., 1982). The question still remains whether reductase associates with P450 and other exogenous heme proteins by the same mechanisms. We have therefore prepared three specific antibodies against peptides which correspond to the two regions of rat reductase described above. The results from experiments using these antibodies in the *in vitro* reconstituted system support the important roles of amino acids 110–119 and 204–118 of rat reductase in the association with cytochrome P450.

MATERIALS AND METHODS

Materials. Keyhole limpet hemocyanin (KLH), maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), and Freund's adjuvants for immunization of rabbits were purchased from Sigma. DEAE Affi-Gel Blue, peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase (HRP) color development reagent were obtained from Bio-Rad. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was

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bought from Boehringer Mannheim GmbH. CH-activated Sepharose 4B was obtained from Pharmacia. All other Chemicals were purchased from Sigma and were of analytical grade or better.

Synthesis of Peptides. Peptides for this study were synthesized by Dr. Robert Moore at the Analytical Chemistry Center of The University of Texas Houston Health Science Center. The synthesis utilized *t*-Boc/benzyl solid-phase methods in an Applied Biosystems Model 430A automatic peptide synthesizer. The peptides were cleaved and deblocked with HF and purified by reverse-phase HPLC on a 1 × 10 cm C-8 ABI/Brownlee column using a gradient of 0 – 100% of 70% acetonitrile in 0.1% trifluoroacetic acid. Amino acid analysis of the synthesized peptide was accomplished by fast atom bombardment mass spectrometry (Moore & Caprioli, 1991). The purity was determined to be >90%.

An additional cysteine (for the purpose of coupling the peptide to KLH) was added to the N-terminus of the sequences.

Coupling of Synthetic Peptides to Carrier Protein. Peptides were coupled to keyhole limpet hemocyanin (KLH) using maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) through the cysteine of the peptides as described by Green et al. (1982) and Lin and Guidottiet (1989); 8 mg of KLH in 0.25 mL of 10 mM sodium phosphate buffer (pH 7.2) was reacted with 1.4 mg of MBS which was dissolved in dimethylformamide and stirred for 30 min at room temperature. This reaction mixture was then fractionated using a 20-mL G-25 Sephadex column equilibrated with 50 mM sodium phosphate (pH 6.0) to remove the unreacted MBS. The fractions containing KLH were combined (4 mL) and mixed directly with 10 mg of peptide dissolved in 100 μL of dimethyl sulfoxide. The reaction was continued overnight with stirring at room temperature. The reaction mixture was used immediately to immunize the rabbit or stored at –70 °C.

Preparation of Anti-Peptide Serum. Rabbits were immunized according to the following schedule: first, 1 mg of peptide-coupled KLH in complete Freund's adjuvant (1:1) was injected subcutaneously on day 0; preimmune serum was obtained before the first inoculation. Second, 1 mg of peptide-coupled KLH in incomplete Freund's adjuvant (1:1) was injected intramuscularly on day 14. Animals were boosted again on day 28 with a mixture of KLH–peptide and incomplete adjuvant intramuscularly and bled from the ear artery 1 week after the injection. The serum was checked for antibody titer by ELISA assay against the peptide used as antigen. Intramuscular injection of peptide conjugate with incomplete adjuvant (1:1) was continued every other week until a good antibody titer was detected. Immediately after bleeding, the blood was allowed to stand at room temperature for 1 h, and then refrigerated at 4 °C overnight. After centrifugation at 2000*g* for 10 min, the supernatant layer was poured through four layers of cheesecloth into clean tubes and then heated at 56 °C for 20 min to inactivate the complement. The serum was centrifuged for 10 min at 10000g at 4 °C to remove residual red blood cells. The final antiserum preparation was stored at –20 °C.

Purification of IgG by DEAE Affi-Gel Blue. Total serum IgG was purified using a protocol provided by Bio-Rad. The serum sample was first dialyzed overnight against 0.02 M Tris-HCl buffer (pH 8.0) containing 0.028 M NaCl and 0.02% NaN_3 before being loaded onto a DEAE Affi-Gel Blue column. IgG was washed through the column, and a total volume equivalent to 8 times the volume of the initial serum sample was collected and concentrated with an Amicon concentration apparatus. The IgG preparation was subsequently dialyzed against phosphate-buffered saline (PBS) buffer (pH 7.4) and stored at –70 °C.

Purification of IgG by Using a Peptide-Coupled Affinity Column. CH-activated Sepharose 4B was used as the affinity matrix for the anti-peptide antibody purification. The peptide was coupled to the resin according to the procedure provided by Pharmacia. Ten milligrams of peptide dissolved in 0.1 M $NaHCO_3$ buffer (pH 8.0) containing 0.5 M NaCl was mixed with pretreated CH-activated Sepharose 4B for 2 h at room temperature in an end-over-end mixer. After the resin was washed with the coupling buffer, the remaining active groups were blocked using 0.1 M Tris-HCl buffer (pH 8.0) for 1 h. The peptide–Sepharose was then washed with 3 cycles of 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and finally with PBS buffer (pH 7.4).

For affinity purification, serum dialyzed overnight against PBS buffer (pH 7.4) was mixed end-over-end with affinity gel at 4 °C overnight. Following the incubation, the mixture was poured into a column and washed with PBS buffer until no further protein could be detected in the eluate at A_{280nm}. Nonspecific or weakly bound protein was eluted with 10 mM potassium phosphate buffer (ph 7.4) containing 1.0 M NaCl. The absorbed antibodies were then eluted by washing the column with 200 mM glycine hydrochloride buffer (pH 2.5) and collected into 1 M Tris-HCl (pH 8.45). The antibody preparation was concentrated with an Amicon apparatus, dialyzed twice against 100 volumes of PBS, and stored at –70 °C.

Purification of Reductase and Cytochrome P450. Reductases from rat, rabbit, and human were purified from the liver microsomes using a modified procedure of Dignam and Strobel (1977). Cytochromes P4501A1 and P4502B1 were purified from liver microsomes of rats treated with β-naphthoflavone and phenobarbital, respectively (Saito & Strobel, 1981).

Enzyme-Linked Immunosorbent Analysis (ELISA) for Anti-Peptide Antibodies. ELISA assays were conducted as follows: peptides or purified reductases used as antigens were diluted with 50 mM sodium carbonate buffer (pH 9.6) to a final concentration of 1 μg/mL and incubated in ELISA microplate wells in a volume of 0.1 mL at 4 °C overnight. Plates were washed 5 times with PBS plus 0.5% Tween 20 (washing buffer), blocked with 0.2 mL of 2% BSA for 1 h at room temperature, washed 5 times with washing buffer, and incubated with 0.1 mL of rabbit serum or purified IgG diluted to different fold dilutions in PBS containing 2% BSA at 37 °C for 2 h. The plates were washed 5 times with washing buffer, and then 0.1 mL of affinity-purified peroxidase conjugated goat anti-rabbit IgG diluted 1:1000 in 2% BSA in PBS was added and incubated at 37 °C for 1 h. The plates were washed 5 times with washing buffer and 1 time with PBS buffer and developed for 30 min at room temperature with 0.1 mL of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in 0.1 M sodium citrate buffer (pH 4.2) containing 2 mM H_2O_2. The reaction was stopped by the addition of 0.1 mL of 10% SDS. The absorbance at 405 nm was read in a Multiskan Plus Version plate reader.

Western Blot Analysis. Purified NADPH–cytochrome P450 reductase and hepatic microsomes from phenobarbital- or β-naphthoflavone-treated rats were electrophoresed in a 9% polyacrylamide gel using a Bio-Rad minigel system. The protein samples were boiled in 5% SDS and reduced with DTT before being loaded onto the gel. Proteins were transferred to nitrocellulose filter paper by electroblotting at

Table 1: Putative Protein-Binding Sites on Reductase

peptide	species	sequence
1	rat (110–119)[a]	SADPEEYDLA
	rabbit	AADPEEYDLA
	human	SADPEEYDLA
2	rat (118–130)	LADLSSLPEIDKS
	rabbit	LADLSSLPEINNA
	human	LADLSSLPEIDNA
3	rat (204–218)	GLGDDDGNLEEDFIT
	rabbit[b]	GMGDDDANLEEDFIT
	human	GLGDDDGNLEEDFIT

[a] Nadler and Strobel (1991). [b] Nisimoto (1986).

100 V and 200 mA for 1 h, and blocked with 1% milk in Tris-buffered saline (TBS) (pH 7.5) solution for 45 min. The filter was washed with TBS containing 0.5% Tween 20 (washing buffer) and incubated with anti-peptide antibodies for 2 h at 37 °C. The paper was washed 5 times with the washing buffer and incubated with 1:1000 peroxidase-conjugated goat anti-rabbit antibody for 1 h at 37 °C. After being washed 5 times with washing buffer and 2 times with TBS buffer, the filter was developed with mixture of 12 mg of HRP color development reagent (from Bio-Rad) in 4 mL of cold methanol and 12 μL of cold 30% H_2O_2 in 20 mL of TBS. The filter was rinsed well with water and dried between paper towels.

Inhibition of P450 Substrate Hydroxylation by Anti-Peptide Antibodies. Cytochrome P4501A1 ethoxycoumarin hydroxylation and P4502B1 pentoxyresorufin dealkylation assays were run according to the procedure as described previously (Ryan et al., 1979; Shen & Strobel, 1992) with fluorescence measurements obtained with a Perkin-Elmer LS-5 spectrofluorometer. In assays which were performed in the presence of anti-peptide antibodies, the antibodies to reductase were added to the enzyme before reconstitution with cytochrome P450 and dilauroylphosphatidylcholine and mixed for 10 min. Other components were added and the constituents then allowed to reconstitute.

Inhibition of Cytochrome c Reduction by Anti-Peptide Antibodies against Reductase. The cytochrome *c* reduction assay by reductase was run in 300 mM potassium phosphate buffer (pH 7.5). For assays which were performed in the presence of anti-peptide antibodies, the reductase and antibodies were incubated at 22 °C for 10 min, and then cytochrome *c* was added. The reaction was initiated with 0.1 mM NADPH, and the rate of cytochrome *c* reduction was measured at 550 nm. The extinction coefficient for cytochrome *c* used in the calculation of the activity was 21 mM^{-1} cm^{-1}.

RESULTS

Two regions on rat reductase have been implicated in the interaction with cytochrome P450 or cytochrome *c*. The amino acid sequence of the peptides derived from rat reductase and the homologous sequences of reductase from rabbit and human are shown in Table 1. Peptides 1 and 2 are regions which were identified as participating in the association with cytochrome P450 by Nadler and Strobel (1991). Peptide 3 is believed to be the site of cytochrome *c* association based on cross-linking studies (Nisimoto, 1986). Note that there is high degree of homology between the amino acid sequence of rat reductase and reductase from rabbit and human. There is a common feature among these three peptide sequences: they contain clustered carboxyl groups and are highly hydrophilic. Three peptides were synthesized and cross-linked to keyhole limpet hemocyanin and used as primary antigens

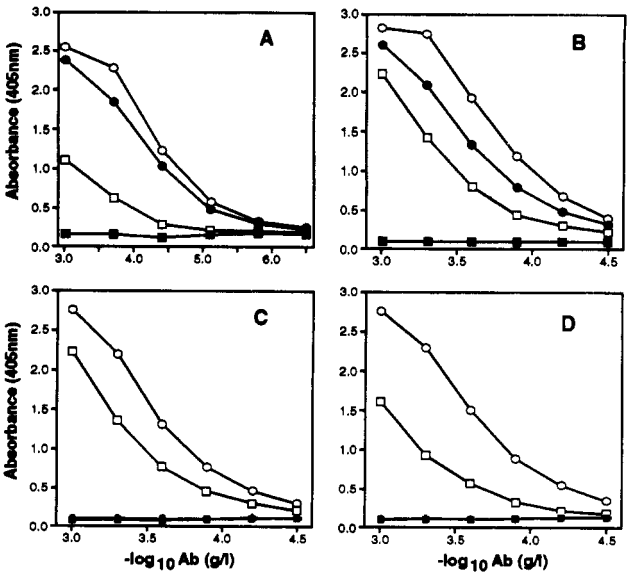

FIGURE 1: Relative binding of anti-peptide (reductase) antibody to various antigens in ELISA. Mirotiter plates were coated with 100 ng of antigen in sodium carbonate buffer (pH 9.6). (A) Corresponding peptides; (B) purified rat P450 reductase; (C) purified rabbit P450 reductase; and (D) purified human P450 reductase. (■) Represents preimmune IgG, (□) 1A, (●) 2A, and (○) 3A. To each group of wells was added a series of dilutions of affinity-purified anti-peptide antibodies, and its binding was determined as described in the text.

in rabbits. The antisera titers were monitored by ELISA assay using either synthetic peptides or purified reductase as antigens. Three anti-peptide antibodies were generated corresponding to the sequences of peptides 1, 2, and 3, which are designated as 1A, 2A, and 3A. The antibodies for all the studies were purified using a peptide–Sepharose 4B affinity column. The preimmune IgG was purified with a DEAE Affi-Gel Blue column.

Figure 1 shows the binding of anti-peptide antibodies to their respective synthetic peptides and purified reductases which were adsorbed directly to the ELISA plates. Antibodies 2A and 3A have a high reactivity with their respective peptide antigens, while 1A has lower reactivity with its peptide (Figure 1A). The binding of each of the antibodies to its peptide is specific; there is no detectable cross-reactivity with unrelated peptides (data not shown). There is no binding of any peptide to the preimmune IgG.

All three anti-peptide antibodies also have high affinity to pure rat reductase with detectable binding at an antibody concentration of 3.12×10^{-5} g/mL. 1A binds to rat reductase with a lower affinity than 2A and 3A. These results are illustrated in Figure 1B. Antibodies 1A and 3A bind equally as well to rabbit and human NADPH–cytochrome P450 reductase as to rat reductase; however, no binding was detected between antibody 2A and reductase from rabbit or human (Figure 1C,D). Similar results were obtained when phosphate-buffered saline (pH 7.4) was used as the coating buffer for antigens to reduce denaturation of the antigen (Figure 2).

Western blot analysis was also performed to determine the reactivity of three anti-peptide antibodies with NADPH–cytochrome P450 reductase from rat, rabbit, and human. As shown in Figure 3, all three antibodies bind with pure rat reductase and to reductase protein present in phenobarbital-treated rat liver microsomes. Antibodies 1A and 3A recognize the reductase from rabbit and human, but no binding was detected between antibody 2A and reductase from rabbit or human, consistent with the results of ELISA assays. Antibody raised against the rat reductase has higher cross-reactivity

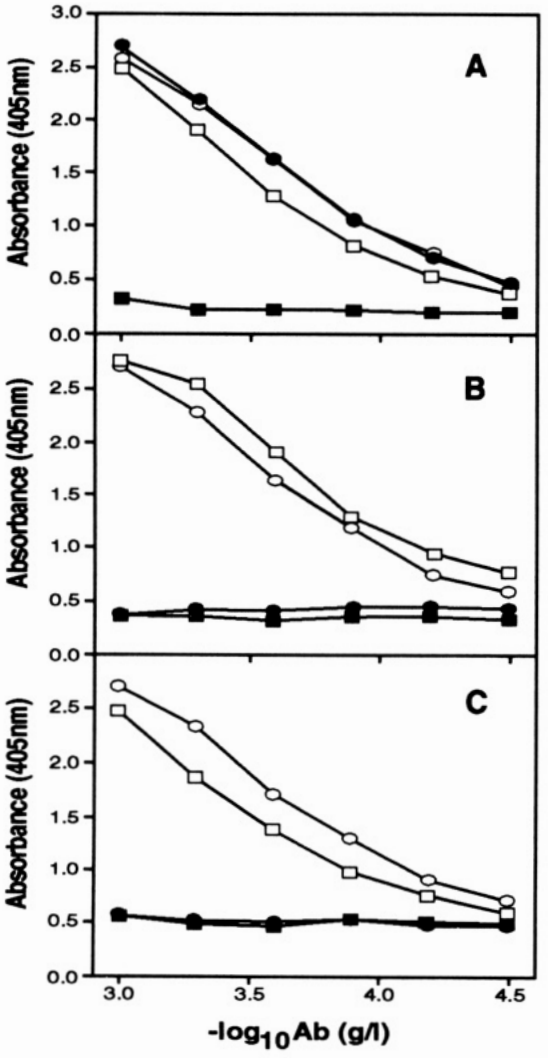

FIGURE 2: Relative binding of anti-peptide (reductase) antibody to various antigens in ELISA. Mirotiter plates were coated with 100 ng of antigen in phosphate-buffered saline (pH 7.4). (A) Purified rat P450 reductase; (B) purified rabbit P450 reductase; and (C) purified human P450 reductase. (■) Represents preimmune IgG, (□) 1A, (●) 2A, and (○) 3A. To each group of wells was added a series of dilutions of affinity-purified anti-peptide antibodies, and its binding was determined as described in the text.

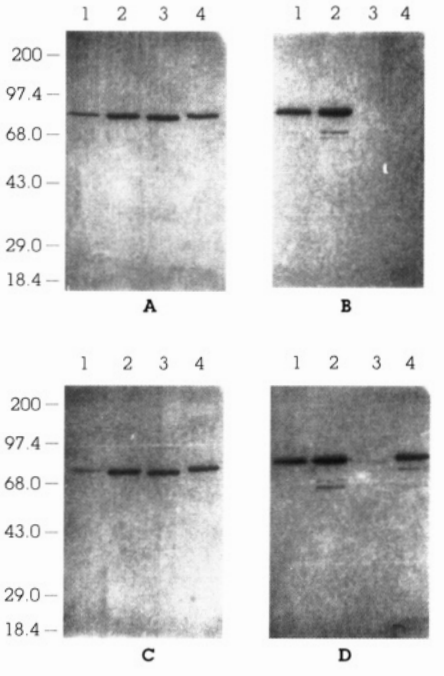

FIGURE 3: Western blot showing the binding of anti-peptide (reductase) antibodies to purified reductase from various species and microsomal preparations. (A) Blotting with 1A; (B) blotting with 2A; (C) blotting with 3A; (D) blotting with anti-rat reductase polyclonal antibody. Lane 1 was loaded with 5 μg of phenobarbital-treated rat liver microsomal protein; lanes 2 through 4 were loaded with 0.5 μg of purified reductase from rat, rabbit, and human liver microsomes, respectively.

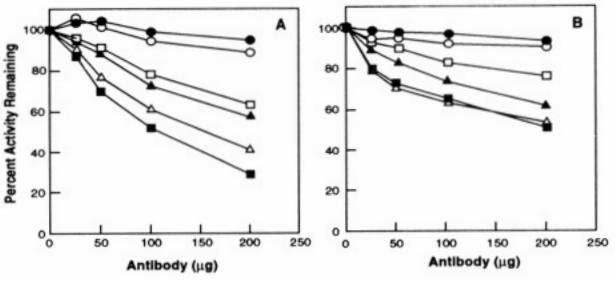

FIGURE 4: Effect of anti-peptide antibodies on the activity of cytochrome P4501A1 7-ethoxycoumarin hydroxylation (A) and P4502B1 pentoxyresorufin dealkylation (B) in a reconstituted system. Prior to their addition of other assay mixtures prepared as described in the text, reductase was allowed to incubate with varying amounts of antibodies for 10 min at 37 °C. (●) Preimmune IgG, (□) 1A, (○) 2A, (△) 3A, (▲) 1A+3A, and (■) antibody to rat detergent-solubilized reductase.

with human reductase than with rabbit reductase (Figure 3D). This result suggests that the rat enzyme is more closely related to the human enzyme than to the rabbit enzyme in terms of antigenic determinants. These data are consistent with the conclusion of a close relationship between rat and human reductase reported by Guengerich et al. (1981) and by McManus et al. (1989).

The data of Figure 4A show the effect on cytochrome P4501A1-dependent ethoxycoumarin hydroxylation by the three anti-peptide antibodies against rat NADPH–cytochrome P450 reductase. Increasing amounts of the anti-peptide antibodies were incubated with rat reductase for 10 min before the reductase was reconstituted with cytochrome P4501A1 and lipid. The P4501A1 7-ethoxycoumarin hydroxylation activity was determined and plotted as the percentage of activity in the absence of antibody (Figure 4A). At 200 μg antibody concentration, 3A and 1A have 60% and 38% inhibition of P4501A1-dependent ethoxycoumarin hydroxylation activity. Antibody 2A does not have any significant effect on the P450 activity. When 1A and 3A were added together, they had no additive effect. Anti-peptide antibodies 3A and 1A also inhibit cytochrome P4502B1 pentoxyresorufin dealkylation activity (Figure 4B). At 200 μg antibody concentration, 3A and 1A inhibit P4502B1 activity by 57.6 and 26.4%, respectively. An inhibition of 40% was detected if 100 μg each of 1A and 3A were added together. However, no effect on P4502B1 activity was observed for antibody 2A. The inhibition by antibodies 3A and 1A was also observed in a hepatic microsomal preparation when assayed by ethoxycoumarin hydroxylation, suggesting the effect of the antibodies is on the P450/reductase interaction during catalysis. At 200 μg antibody concentration, 3A and 1A inhibit microsomal ethoxycoumarin hydroxylation activity by 25.4 and 12.3%, while polyclonal antibody against rat reductase inhibits the activity by 30%.

In the rat cytochrome P4501A1 reconstituted system with reductase from rabbit or human, ethoxycoumarin hydroxylation activity was also inhibited by anti-peptide antibodies 1A and 3A, but not by 2A (Figure 5). At 200 μg antibody concentration, 3A and 1A inhibit P4501A1 7-ethoxycoumarin

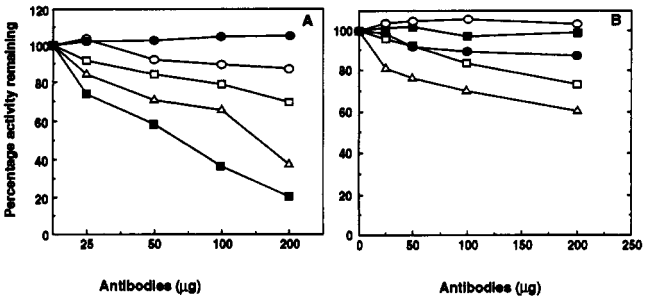

FIGURE 5: Effect of anti-peptide antibodies on rat cytochrome P4501A1 7-ethoxycoumarin hydroxylation reconstituted with reductase from rabbit (A) and human (B). Prior to their addition of other assay mixtures prepared as described in the text, reductase was allowed to incubate with varying amounts of antibodies for 10 min at 37 °C. (●) Preimmune IgG, (□) 1A, (○) 2A, (△) 3A, and (■) antibody to rat detergent-solubilized reductase.

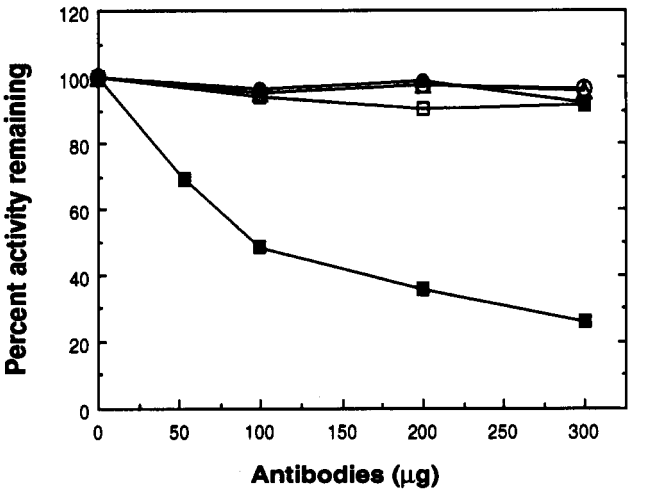

FIGURE 6: Effect of anti-peptide antibodies on the cytochrome *c* reduction assay by NADPH–cytochrome P450 reductase. Prior to their addition of other assay components prepared as described in the text, reductase was allow to incubate with antibodies at 22 °C for 10 min. (●) Preimmune IgG, (□) 1A, (○) 2A, (△) 3A, and (■) antibody to rat detergent-solubilized reductase.

hydroxylation activity reconstituted with rabbit reductase by 62.7% and 30.3%, respectively. However, they exhibit less inhibition of P4501A1 activity reconstituted with human reductase, only 39.5% and 26.7%, respectively. We observed that the catalytic efficiency of the rat P4501A1 system reconstituted with human reductase is much lower that that of the P450 system reconstituted with reductase from rat and rabbit. This experiment indicates that a very similar cytochrome P450 binding site seems to exist on reductases from rat, rabbit, and human.

In the presence of up to 300 μg of anti-peptide antibodies, cytochrome *c* reduction by NADPH–cytochrome P450 reductase was not affected under the assay conditions used, but 100 μg of anti-reductase polyclonal antibody inhibited the cytochrome *c* reduction by 50% (Figure 6). The results indicate that cytochrome *c* may associate with reductase by different mechanisms, or at another critical site.

DISCUSSION

The development of specific immunological probes allowed us to study the protein–protein interaction between reductase and cytochrome P450 or cytochrome *c*. The high hydrophilicity of the peptide antigens suggests that they are probably exposed on the surface of the protein. The three anti-peptide antibodies we prepared have high affinities for purified reductase protein under either denatured or native conditions. The ELISA binding assay shows that antibody 2A against peptide from 118–130 can only bind with rat reductase, but not to reductase from rabbit and human. The sequence of peptide 2 among reductases from rat, rabbit, and human shows a high degree of homology, with the only difference being a three amino acid stretch at the N-terminus of the peptide. These results suggest that the anti-peptide antibodies have a high degree of specificity.

The ELISA and Western analysis indicated that anti-peptide antibodies against rat reductase sequences 1 and 3 cross-react with the reductases from rabbit and human. This suggests that the reductases from these three species share similar structural features at two regions which are both likely to be on the surface of the protein since they constitute epitopes. Both regions (110–119 and 204–218) of rat reductase seem to be involved in the interaction with cytochrome P450 as judged by the specific anti-peptide antibody inhibition studies. While antibody against detergent-solubilized rat reductase efficiently inhibits the cytochrome *c* reduction by reductase, none of the three anti-peptide antibodies have any effect on the cytochrome *c* reduction by reductase. Perhaps the association of rat reductase with cytochrome P450 or cytochrome *c* relies on different mechanisms. Even though Nisimoto (1986) has successfully cross-linked cytochrome *c* and reductase and further identified the EDC cross-linked peptide (amino acids 204–218), he also observed that the loss in P450LM2 reductase activity is more remarkable than that in cytochrome *c* reductase activity after EDC modification. His results suggested that the inhibitory effects are greater in the interactions between the P450LM2 and carboxyl-modified reductase than in the interactions between cytochrome *c* and modified flavoprotein. Nadler and Strobel (1988) have demonstrated that modification of NADPH–cytochrome P450 reductase by EDC increased the apparent K_m by 1.3- or 5.2-fold in reconstituted P4501A1 or P4502B1 substrate hydroxylation systems, respectively, but modification of carboxyl amino acid residues of reductase decreased its K_m by $^1/_2$ to $^2/_3$ of the control value in the reduction of cytochrome *c*. Thus, the same modification of the reductase making it less negatively charged made the reductase less able to interact with cytochrome P450 (increased K_m), but more able to interact with cytochrome *c* (decreased K_m). A resonable interpretation of these results would suggest at least a different degree of charge–charge pairing and perhaps even different amino acid residue sites are involved. These observations may provide a basis to explain the difference between the flavoprotein reductase with association of P450 or cytochrome *c*. While regions 110–119 and 204–218 on reductase are important for the electrostatic interaction with P450, they may not be the major binding sites for cytochrome *c*.

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REFERENCES

Bernhardt, R., Kraft, R., Otto, A., & Ruckpaul, K. (1988) *Biomed. Biochim. Acta 47*, 581–592.

Bösterling, B., & Trudell, J. R. (1982) *J. Biol. Chem. 257*, 4783–4787.

Davydov, D. R. (1992) *Arch. Biochem. Biophys. 297*, 304–314.

Dignam, J. D., & Strobel, H. W. (1977) *Biochemistry 16*, 1116–1123.

Estabrook, R. W., Werringloer, J., Masters, B. S. S., Jonen, H., Matsubara, T., Ebel, R., O'Keefe, D., & Peterson, J. A. (1976) in *The Structure Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 429–445, Academic Press, New York.

French, J. S., Güengerich, F. P., & Coon, M. J. (1980) *J. Biol. Chem. 255*, 4112–4119.

Green, G., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Sutcliffe, J. G., & Lerner, R. A. (1982) *Cell 28*, 477–487.

Guengerich, F. P., Wang, P., & Mason, P. S. (1981) *Biochemistry 20*, 2379–2385.

Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1983) *J. Biol. Chem. 258*, 8588–8594.

Lin, S. H., & Guidotti, G. (1989) *J. Biol. Chem. 264*, 14408–14414.

McManus, M. E., Huggett, A., Burgess, W., Robson, R., & Birkett, D. J. (1989) *Clin. Exp. Pharmacol. Physiol. 16*, 121–134.

Moore, W. T., & Caprioli, R. M. (1991) *Techniques in Protein Chemistry II* (Villafranca, J., Ed.) pp 511–528, Academic Press, Inc., San Diego, CA.

Nadler, S. G., & Strobel, H. W. (1988) *Arch. Biochem. Biophys. 261*, 418–429.

Nadler, S. G., & Strobel, H. W. (1991) *Arch. Biochem. Biophys. 290*, 277–284.

Nisimoto, Y. (1986) *J. Biol. Chem. 261*, 14232–14239.

Nisimoto, Y., & Otsuka, M. H. (1988) *Biochemistry 27*, 5869–5876.

Porter, T. D., & Kasper, C. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 973–977.

Porter, T. D., & Kasper, C. B. (1986) *Biochemistry 25*, 1682–1687.

Ryan, D. E., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1374.

Saito, T., & Strobel, H. W. (1981) *J. Biol. Chem. 256*, 984–988.

Shen, S., & Strobel, H. W. (1992) *Arch. Biochem. Biophys. 294*, 83–90.

Shimizu, T., Tateishi, T., Hatano, M., & Fujii-Kuriyama, Y. (1991) *J. Biol. Chem. 266*, 3372–3375.

Stayton, P. S., & Sligar, S. G. (1990) *Biochemistry 29*, 7381–7386.

Strobel, H. W., & Dignam, J. D. (1978) *Methods Enzymol. 52*, 89–96.

Strobel, H. W., Nadler, S. G., & Nelson, D. R. (1989) *Drug Metab. Rev. 20*, 519–533.

Vermilion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 2694–2704.

Voznesensky, A. I., & Schenkman, J. B. (1992a) *J. Biol. Chem. 267*, 14669–14676.

Voznesensky, A. I., & Schenkman, J. B. (1992b) *Eur. J. Biochem. 210*, 741–746.

Wagner, S. L., Dean, W. L., & Gray, R. D. (1984) *J. Biol. Chem. 259*, 2390–2395.

Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.