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Specificity of the cytochrome P-450 interaction with cytochrome b_5

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Abstract

The specificity of the interaction of cytochrome b_5 with different forms of cytochrome P-450 was examined. Immunopurification of cytochromes P-450 1A1, 2B1 and 2E1 from rat liver microsomes resulted in co-purification of cytochrome b_5 with cytochrome P-450 forms 2B1 and 2E1 but not 1A1. This specificity was evaluated in conjunction with multiple sequence alignment of the three cytochrome P-450s and a molecular model of the cytochrome P-450–cytochrome b_5 complex [(1989) Biochemistry 28, 8201–8205]. These analyses suggest two basic residues in the arginine cluster region of P-450, which are present in P-450s 2B1 and 2E1 but are absent in P-450 1A1, as potential binding sites for cytochrome b_5.

Key words: Cytochrome P-450; Cytochrome b_5; Molecular modeling; Protein–protein interaction

1. Introduction

The cytochromes P-450 are a family of enzymes that catalyze the oxidation of a wide array of lipophilic compounds [1–4]. These include xenobiotics such as drugs and carcinogens, as well as the endogenous prostaglandins, fatty acids and steroids. Although the amino acids in the substrate binding site define the substrate specificity of the individual forms of P-450, other undefined regions on the P-450 surface are also functionally active as they interact with NADPH-cytochrome P-450 reductase and cytochrome b_5 [5]. The reductase is an essential component of the mixed function oxygenase system and can transfer two electrons to all forms of microsomal P-450 during the catalytic cycle, In contrast, the role of cytochrome b_5, which may donate only the second of these electrons, is more complex, as its effect on P-450-catalyzed activities exhibits P-450 form-specificity [6–15].

In this study we show that cytochrome b_5 co-purifies to variable extents with different forms of rat liver P-450, thus suggesting differential stabilities of the P-450–cytochrome b_5 complexes. This P-450 form-specificity was used in conjunction with multiple sequence alignment of P-450s and molecular modeling to identify the amino acid residues of mammalian P-450s that interact with cytochrome b_5.

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Abbreviations: P-450, cytochrome P-450; PB, phenobarbital; MC, 3-methylcholanthrene; AC, acetone; MAb, monoclonal antibody.

2. Materials and methods

2.1. Preparation of rat liver microsomes

Male Sprague–Dawley rats (6 weeks old) were injected intraperitoneally daily with 3-methylcholanthrene (MC) (40 mg/kg of body weight for 3 days) to induce P-450 1A1, or with phenobarbital (PB) (80 mg/kg of body weight for 3 days) to induce P-450 2B1. Rats were treated with 25% acetone (AC) in their drinking water for 3 days to induce P-450 2E1. Liver microsomes were prepared by differential centrifugation and stored in 0.25 M sucrose at −80°C. Protein concentrations were determined by the BCA protein assay (Pierce).

2.2. Immunopurification of P-450

Microsomes were solubilized with 0.5% Emulgen 911 (Kao-Atlas Chemical Co.) and P-450s were immunopurified using Sepharose-MAb immunoadsorbents as previously described [16], with the modification that P-450 was eluted from the immunoadsorbent with 40 mM sodium phosphate, pH 3.0. MAb 1-36-1 to P-450 1A1 [17], MAb 2-66-3 to P-450 2B1 [18] and MAb 1-98-1 to P-450 2E1 [19] were used to immunopurify these P-450s from MC-, PB- and AC-microsomes, respectively. These MAbs were obtained from Dr. S.S. Park (National Cancer Institute). Immunopurified P-450s were separated by SDS-PAGE on a 10–20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan), and visualized by Coomassie brilliant blue staining of the gel.

2.3. Immunoblot analysis

Immunopurified P-450s were electroblotted from the gel to polyvinylidene difluoride membranes (Immobilon, Millipore) [20], using a transfer solution of 50 mM Tris-glycine (pH 8.2), 20% methanol. Immunodetection was performed as described [16], using the above anti-P-450 MAbs, or MAb 1-17-1 to cytochrome b_5 [21]. Densitometric analysis of stained bands was performed with a Molecular Dynamics Computing Densitometer.

2.4. Sequence alignment and molecular modeling

Multiple alignment of protein sequences were performed with the MACAW program [22], implemented on an IBM PS/2 Model 80 microcomputer. For visualization of the crystal structures of P-450cam [23] and cytochrome b_5 [24], their coordinates (data sets 2CPP and 3B5C, respectively) were obtained from the Protein Data Bank (Brookhaven National Laboratories, Upton, NY), and displayed on a Silicon Graphics 4D/70G workstation using QUANTA molecular modeling software (Polygen Corp., Waltham, MA).

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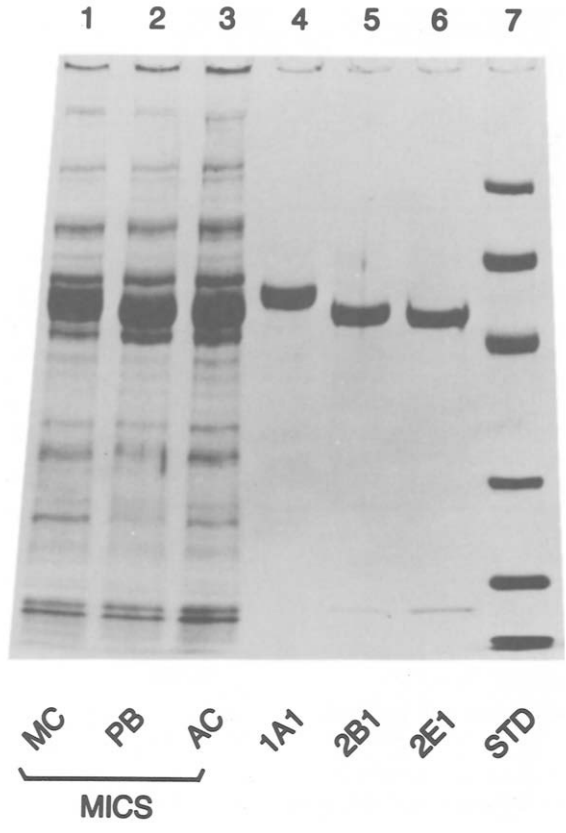

Fig. 1. SDS-PAGE analysis of immunopurified P-450s. Lanes 1–3 contain 20 μg liver microsomes (MICS) from rats treated with MC, PB and AC. Lanes 4–6 contain P-450s 1A1, 2B1 and 2E1 which were immunopurified from these microsomes using MAbs 1-36-1, 2-66-3 and 1-98-1, respectively. Lane 7 contains molecular weight markers. Proteins were stained with Commassie brilliant blue.

3. Results and discussion

P-450s were immunopurified from liver microsomes of rats treated with MC, PB or AC, using MAbs to the major form of P-450 induced by these agents. Fig. 1 shows the total microsomal (lanes 1–3) and immunopurified (lanes 4–6) proteins. The latter yielded single bands in the P-450 region which corresponded to P-450 forms 1A1, 2B1 and 2E1, respectively. However, an additional 17 kDa protein was co-purified with P-450s 2B1 (lane 5) and 2E1 (lane 6) but was undetectable in the lane containing P-450 1A1 (lane 4). Since the size of this protein corresponded to that of cytochrome b_5, immunodetection with MAb 1-17-1 to cytochrome b_5 was employed to verify its identity. The immunoblot (Fig. 2), as expected, revealed cytochrome b_5 in all the microsomal samples (lanes 1–3). The MAb also immunoreacted with the 17 kDa protein that co-purified with 2B1 and 2E1 (lanes 4 and 6, respectively), in accordance with the Commassie brilliant blue-stained gel in Fig. 1. These data show that cytochrome b_5 co-purified with P-450s 2B1 and 2E1 but not 1A1.

The Commassie brilliant blue staining intensities of the immunopurified P-450 and cytochrome b_5 bands were quantitated in order to gauge the relative extent of cytochrome b_5 co-purification with each P-450. The staining intensities of these proteins were first corrected for their different molecular masses, using values of 54.5, 52.2 and 17.0 kDa for P-450s 2B1, 2E1 and cytochrome b_5, respectively. The resultant molecular cytochrome b_5:P-450 ratios were 0.46 ± 0.14 for 2E1 and 0.11 ± 0.04 for 2B1. Based on the sensitivity limit of Commassie brilliant blue staining, we estimate that the cytochrome b_5:P-450 ratio was less that 0.02 for 1A1. The co-purification data thus indicates that, under our immunopurification conditions, the stability of the cytochrome b_5 complex with P-450 varies in the order 2E1 > 2B1 >> 1A1.

Since cytochrome b_5 did not co-purify with P-450 1A1, we sought to determine whether the MAb used for immunopurification of this P-450, 1-36-1, inhibited the P-450–cytochrome b_5 interaction. The immunopurification was thus also carried out using MAb 1-7-1, which binds P-450 1A1 at a different, non-overlapping epitope [25]. The results were the same as with the MAb 1-36-1 immunopurification, as cytochrome b_5 was also unde-

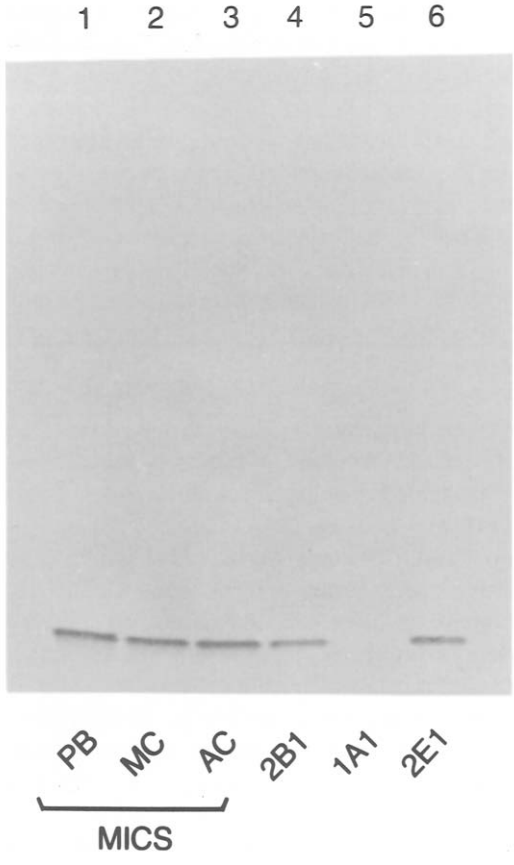

Fig. 2. Immunoblot of cytochrome b_5 in microsomes and immunopurified P-450 samples. Lanes 1–3 contain 20 μg liver microsomes (MICS) from rats treated with PB, MC and AC. Lanes 4–6 contain the P-450s immunopurified from these microsomes using MAbs 2-66-3, 1-36-1 and 1-98-1, respectively. MAb 1-17-1 to cytochrome b_5 was used for immunodetection.

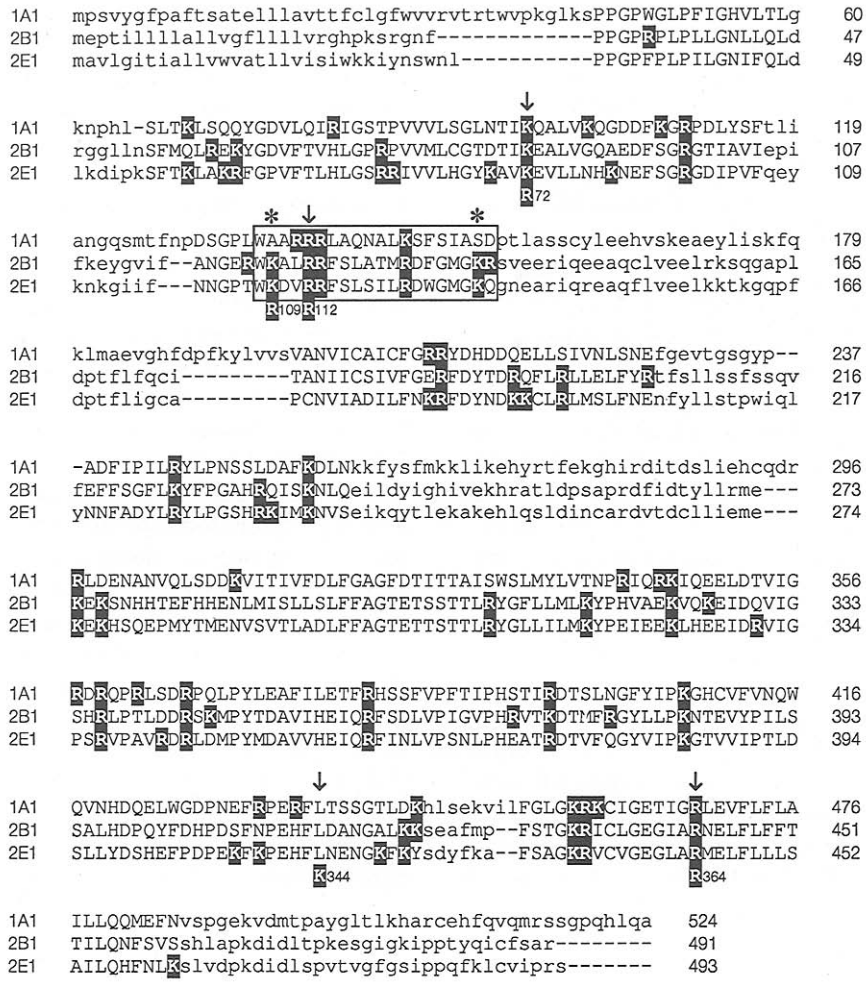

Fig. 3. Multiple sequence alignment of P-450s 1A1, 2B1 and 2E1. The sequences were aligned using the MACAW program. Upper case denotes regions of sequence similarity. Lys and Arg amino acids are shown in reverse type. The aligned residues corresponding to those in P-450cam (R72, R112, K344 and R364) which interact with cytochrome b_5 on the basis of a model of the P-450cam–cytochrome b_5 complex [29] are indicated by arrows. A previously proposed [33] cytochrome b_5 binding region in enclosed by a box. Basic residues in this region which are present in P-450s 2B1 and 2E1 but not 1A1 are denoted by asterisks.

tectable (data not shown). This result indicates that cytochrome b_5 does not co-purity with P-450 1A1 because of low affinity rather than from MAb inhibition of the P-450 1A1–cytochrome b_5 interaction.

We next employed the P-450 specificity of cytochrome b_5 co-purification along with the sequences of these P-450s to identify the P-450 amino acid residues involved in binding to cytochrome b_5. Multiple sequence alignment of the three P-450s was performed and yielded the optimal alignment displayed in Fig. 3. Previous studies have established the importance of electrostatic contacts in the P-450–cytochrome b_5 interaction, involving basic P-450 residues [9,26–28]. The basic residues in homologous regions are thus emphasized in Fig. 3. It is evident that these are conserved at numerous positions which are all candidates for interaction with cytochrome b_5. However, the primary sequence data alone cannot provide further information on which of these residues are involved in binding.

Recent computer modeling studies employed the known crystal structures of the individual proteins to develop a model of the P-450cam–cytochrome b_5 complex [29,30]. In this model, P-450cam basic residues Arg-72, Arg-112, Lys-344 and Arg-364 are suitably positioned for electrostatic contacts with the cytochrome b_5 carboxyls of residues Glu-48, Asp-60, Glu-44 and heme propionate. The equivalent positions of these four P-450cam residues in P-450s 1A1, 2B1 and 2E1 are denoted by the arrows in Fig. 3, and are based on extensive sequence alignments of a large number of P-450s [31]. In all three P-450s, P-450cam Arg-72 is replaced by Lys, P-450cam Arg-112 and Arg-364 are conserved, and P-450cam Lys-344 is replaced by Leu. Acidic–basic residue pairing is thus conserved in three out of the four positions, and the fourth position is also identical in the three P-450s. The Lys-344→Leu substitution may be tolerable and not alter the P-450–cytochrome b_5 interaction since this residue does not have a regular secondary

structure in P-450cam and should thus be more mobile, while the three basic residues are found in helical regions. The three P-450s should thus similarly interact with cytochrome b_5 if these previously identified P-450 residues play a predominant role in binding. Our finding that the binding strength is P-450 form-specific thus indicates a role for an additional P-450 residue(s). Examination of the proposed P-450cam–cytochrome b_5 contact region via molecular modeling revealed that, like Arg-112, Arg-109 is also exposed on the exterior surface and is suitably positioned to interact with Asp-60 of cytochrome b_5. P-450cam Arg-109 is conservatively substituted with Lys in P-450s 2B1 and 2E1, but with hydrophobic Ala in P-450 1A1, as denoted by the asterisk in Fig. 3. Based on our result that cytochrome b_5 binds to P-450s 2B1 and 2E1 but not P-450 1A1, the residue corresponding to P-450cam Arg-109 may also interact with Asp-60 of cytochrome b_5. However, the P-450cam Arg-112 equivalent residue retains a critical role, based on a mutagenesis study which showed that it is essential for binding of mouse P-450 2A5 to cytochrome b_5 [32], and the observation that it is 100% conserved [31].

Although the electrostatic interaction model [29] thus suggests P-450cam Arg-109 as a potential interaction site, our data may also be interpreted in terms of a comparative sequence analysis which has identified the region corresponding to P-450cam residues 108–124 as a putative cytochrome b_5 binding site [33]. Examination of the basic residues in this region, which is indicated by the box in Fig. 3, reveals an additional position (asterisk) with Lys in P-450s 2B1 and 2E1 and Ser in P-450 1A1. These analysis thus yield two sites in P-450s 2B1 and 2E1 that potentially interact with cytochrome b_5. Although these were derived on the basis of charge-pairing, additional residues probably also stabilize the P-450–cytochrome b_5 complex by hydrophobic and hydrogen bonding interactions.

A basic question in P-450 structure–function concerns the binding of various functional modulators to P-450. Lipids and NADPH-cytochrome P-450 reductase are essential cofactors for all P-450s, and their interactions with P-450s have been extensively studied. While it is non-essential, cytochrome b_5 regulates P-450 activity in a more complex manner since it may both transfer the second electron required for P-450-mediated substrate oxidations and change P-450 conformation [9,27,34]. The complexity of its action is illustrated by the differential effect of cytochrome b_5 on the activity of a single P-450 towards different substrates [14,35–37], and that it can enhance or inhibit P-450 activities in artificially reconstituted systems [7,8,12,13,38–41]. Thus one cannot simply infer the relative affinities of cytochrome b_5 for different forms of P-450 from its effect on P-450 catalyzed activities.

The interactions of P-450s with cytochrome b_5 have accordingly been examined from various perspectives. Previous work established that cytochrome b_5 (i) changes the spin state of rat P-450s 2C11 [9] and 2B1 [34] and rabbit P-450 2B4 [9,27]; (ii) changes the magnetic circular dichroism of rabbit 2B4 [43]; (iii) complexes with rabbit P-450 2B4 via chemical crosslinking [26]; (iv) binds rat P-450 2C7 [43] and mouse 2A5 [32,41] by affinity chromatography; (v) inhibits phosphorylation of rabbit P-450 2B4 by cAMP-dependent protein kinase [44,45]; and (vi) inhibits phosphorylation and stabilizes P-450 2B1 from denaturation [45]. Since various laboratories have employed a variety of criteria and different P-450s to evaluate the P-450–cytochrome b_5 interaction it is difficult to assess the relative strengths of this interaction for different P-450s. However, our conclusions concerning P-450 form-specificity are based on the application of a single criterion to three P-450s. We point out, however, that although cytochrome b_5 did not co-purify with P-450 1A1 during our immunopurification procedure, cytochrome b_5 has been found to stimulate P-450 1A1-catalyzed oxidation of diethylstilbestrol under the different experimental conditions found in a reconstituted system [46]. However, since only P-450 1A1 was evaluated for activation by cytochrome b_5 one cannot make conclusions regarding P-450 specificity from this study.

The P-450 specificity of cytochrome b_5 action may derive from variations in (i) affinity of P-450 for cytochrome b_5, (ii) cytochrome b_5-induced changes in P-450 conformation, which alters binding to substrate or interaction with NADPH-cytochrome P-450 reductase, or (iii) the rate of electron transfer from cytochrome b_5 to P-450. In this study we examined the first of these factors and found that, under the conditions of co-purification, a P-450 in the 1A subfamily interacts more weakly than P-450s in the 2B and 2E subfamilies. Although sequence alignment of these P-450s suggested several amino acids which potentially interact with cytochrome b_5, further localization of the interacting residues requires additional studies which employ a single criterion to measure cytochrome b_5 binding to a variety of P-450s from different families.

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