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RECONSTITUTION OF CYTOCHROME P4502B4 (LM2) ACTIVITY WITH CAMPHOR AND LINALOOL MONOOXYGENASE ELECTRON DONORS

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SUMMARY: The P4502B4 (LM2) monooxygenase was shown to bind the P450cam and P450lin redoxins with respective dissociation constants of ca. 500 and 100 μM. When the redoxin reductases are added, the heterogeneous complexes demethylate benzphetamine with HCHO yields and Vmax similar to results with the homologous microsomal reductase. Implications of evolutionary conserved P450 redox electron donor binding site residues are discussed.

The cytochrome P450 superfamily, now exceeding 180 individual proteins (20), was recognized with the first cell extracts, to comprise two classes of redox protein energy couples from reduced pyridine nucleotides. The 'microsomal reductase', was identified as a flavoprotein with one FAD as NADPH-dehydrogenase, coupled to FMN as reductant to the P450-hemethiolate catalytic center in two sequential single electron events. A second 'mitochondrial-microbial' P450 class was found to function with two redox proteins, a FAD-NADH, or NADPH, reductase, about half the size of the microsomal reductase, and smaller acidic protein, soon identified as an iron sulfur redoxin of the $Fe_2S_2Cys_4$ type. The latter retain system specificity and differ in redox potential from the plant and clostridial ferredoxins.

The first tests of cross reactions of the microsomal P450LM2, now 2B4, with P450cam redox proteins revealed a lack of activity at the homologous protein levels (Coon and Gunsalus, unpublished data). Similarly as reviewed (18, 6) the adrenal- and CAM-redoxins lacked reciprocal activity. Thus grew the concept of selectivity for redox couples along with strict microbial-mitochondrial P450 substrate stereo- and regio- choice, and a

ABBREVIATIONS: P450: 2B4 (LM2), from rabbit liver microsomes; Scc = adrenal cortex side-chain cleavage; Cam = P450101; Lin = P450111.
Redoxins: Pd = putida; Ld = linalool. Reductases: PdR = putida; LdR: lin;
FITC = fluorescein isothiocyanate.

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contrasting microsomal P450 common FAD-FMN-NADPH reductase and promiscuous substrate selectivity (25).

Two observations on the P450cam microbial system tended to alter the perceived level of strict specificity: the ferrous substrate state of P450cam, m^{rs}, prepared by photochemical reduction, yielded a half mole of product in a single turnover with an abundant variety of iron-sulfur ferredoxins, rubredoxins, and cytochrome b5 (17). Second, on isolation of two additional microbial P450 proteins, P450lin and P450cym, and the linredoxin and linredoxin-reductase, heterologous partial activities were noted between redox proteins and the hemethiolate catalytic centers --- they in fact aided the isolation of P450lin and -cym components (12). Stringency is considered less between flavo- and iron-sulfide proteins (12). More recent studies with Streptomyces systems extended these findings to the plant ferredoxins (see for example reference 26, 27). Thus the sharp selectivities deduced from the first examples yields to the reality that nature seldom partitions rigidly along taxonomic lines.

This paper considers redox component selectivity of the P450 monoxygenases with dioxygen as co-substrate. The shortened peroxidase cycle, by-passing redox steps with organo- or hydrogen-peroxide, and single oxygen donors, such as iodoso-benzene, may gain less from these considerations.

MATERIALS AND METHODS: Optical difference and fluorescence spectroscopy were used to measure binding constants of Pd and Ld by the microsomal P4502B4, (LM2), it's FITC-modified form, and the procaryote P450's, P450lin, and P450cam. The difference spectra induced upon Pd- and Ld-P450 binding were recorded in tandem cuvettes with a Varian Cary 219 split beam UV-visible spectrophotometer. The P450 concentrations, in 50 mM K^+PO_4 buffer, pH 7.0, were 14 μM, and the redoxins were varied over a range from 2 to 50 μM. The fluorescence measurements used a 480 nm excitation, and emission recorded with a 490-620 nm bypass filter. The P4502B4 concentrations, and those of the FITC derivative were 1.4 μM, (FITC ratios were 0.6 to 1.4 mol/P4502B4). The data were corrected for inner filter effect of the redoxins, with the free redoxin concentrations taken into account for K_D calculations, using direct parabolic data fits. The more optimum buffer 0.1 M K^+PO_4, pH 7.4, with 10 % glycerin was used (7, 15). The rates of NADH oxidation, and HCHO formation (19) were measured at 0.6 μM P4502B4 with the respective redoxin reductases at 0.6 μM (cam) or 0.7 μM (lin), and redoxins Pd and Ld varied from 40-100 and 7-35 μM. The rate of NADH-oxidation was monitored for 3 minutes, benzphetamine added at 1 mM, and the substrate dependent rate recorded. The rates with FITC modified P4502B4 were as indicated previously (3, 4).

RESULTS

With P4502B4 (LM2), the binding of Pd, Ld, by the microsomal reductase, induces a high spin shift in the heme iron, whereas for P450101 (P450cam) redoxin binding lowers the spin state; with P450lin, Ld induces only an atypical decrease in the absorption around 400 nm without a predominant maximum, a behavior not yet understood.

TABLE 1. Optical Measurement of P450 Redox Affinities

P450 used	Electron Donor		
	microsomal	Pd	Ld
	K_D*, μM		
LM2	0.115**	~500	100
CAM	--	17	--
CAM-FITC (1:1.44)	--	70	125
LIN	--	--	10
LIN-FITC (1:0.70)	--	76	40

50 mM K phosphate buffer, pH 7, 25° C;
P450 = 14 μM for optical; 1.4 μM fluorescence.
Redoxin; Pd 5-55 μM; Ld 2-26 μM.

* From direct parabolic plots; ** Dilauroyl phosphatidylcholine added, data from (7).

Table 1 indicates the respective P4502B4 dissociation constants for Ld as 100 μM and Pd 500 μM; 10 and 30 fold lower affinities than for their native procaryote P450's, Lin and Cam. The data with FITC-P4502B4, (0.6 mol/mol), are similar with slight decrease in binding, (data not shown). With the FITC-P450cam and -P450lin heme proteins, as Table 1 indicates, the affinity is decreased about 4 fold with the homologous redoxins and with the heterologous ones by another 2 fold.

The microsomal P4502B4:procaryote redoxin complexes, indicated by optical spectra were monitored for catalytic effectiveness in the rates of NADH oxidation, product yield and time course, vs those with the native reductase, using NADPH. For the microsomal heme protein the conditions were optimized by including glycerin and dilauroyl-phosphatidyl-choline in the buffer (7, 15). The time course of HCHO formation from benzphetamine is shown in Figure 1, and the product yield vs pyridine nucleotide oxidation in Table 2.

The HCHO formation is linear over the first 4-5 minutes, and decreases thereafter. The efficiency of product yield, that is whether NADH oxidation is partially abortive, leading to hydrogen peroxide or water formation as with the native microsomal reductase, was examined. Table 2 indicates a slight disfavor in product formation with the procaryote replacements; to about one third mole per NADH, compared with about one half per mole with native reductase. The product forming efficiency can be compared with varying levels of the electron donor and extrapolating to Vmax. Although the redoxins and reductases were available currently in sufficient quantity, their optical

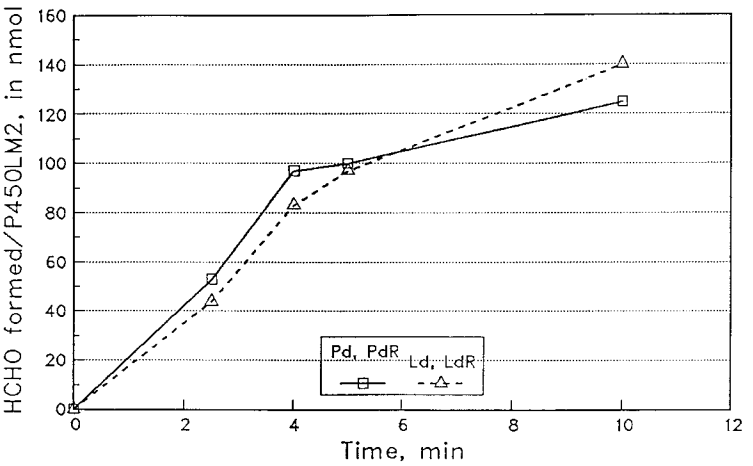

Figure 1, Time-dependent formation of HCHO with P4502B4:redoxin:reductase. Conditions as in Table 2. With P4502B4 0.60μM; Pd/PdR =105, 0.6μM; Ld/LdR = 24, 0.7μM.

density precluded measurements to saturation; thus the Vmax values, approximated from measurements below the Km require caution. Conversely, the complexes of microsomal reductase with the procaryote P450 heme proteins were not found effective in camphor or linalool hydroxylation, (data not shown).

Figure 2 contains data on the effect on catalytic activity of increasing substitution of P4502B4 with FITC. They indicate, as with the native reductase (3, 4), a disturbance of redox function on substitution of residues in, or near, the binding sites. At 1.3:1, FITC:P4502B4, activity was decreased by half, above which increased scattering precluded measurements.

TABLE 2: P4502B4 Benzphetamine Demethylase Activity

	Redox Donor μM	Vmax min^{-1}	Product Yield HCHO/NAD(P)H
microsomal		41*	0.48
PdR: Pd	0.6: 42-105	42	0.37
LdR, Ld	0.7: 7-34	52	0.30

100mM K phosphate buffer, 10% glycerol, pH 7, 25°C; 1mM benzphetamine added to initiate reaction.

* With dilauroyl phosphatidylcholine, data from (15).

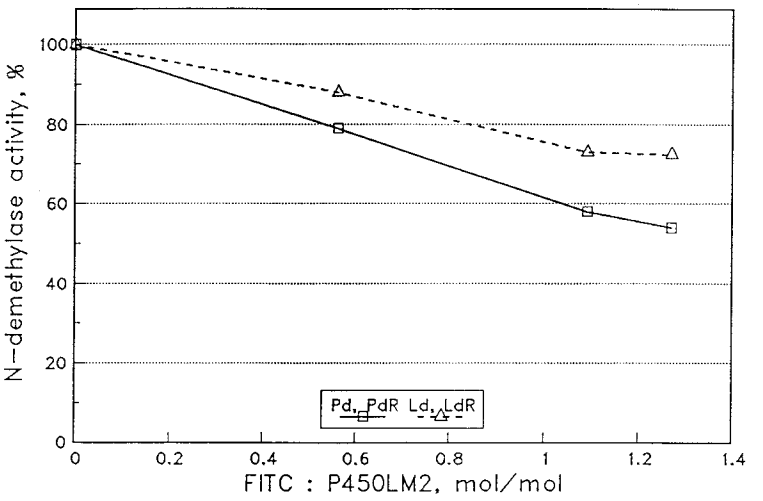

Figure 2. FITC-P4502B4-activity vs native = 100, Conditions as Figure 1.

DISCUSSION

The first experiments with the P450 mooxygenases of microsomal and microbial origin, made clear a lack of equimolar activity in replacements, and differences in the redoxin:reductase couples from the mitochondrial and microbial sources (6, 18). However, as primary structures emerged homologies began to appear, first for the small iron sulfide proteins, adrenodoxin and putidaredoxin (32), and soon among P450's, as first made evident in publications of Fujii-Kuriyama (8, 10). The domains in common lead to recognition of retained residues surrounding the heme and the later identified axial thiol ligand. Particularly as the tertiary structure of P450cam was added to the primary structures, computer simulations, aided by site specific modifications have gained, though slowly, predictive value of more general similarities and their catalytic consequences (11, 22).

Thus Bernhardt and colleagues (1-4), examined the structure-activity influences of chemical modification of the microsomal P450 LM2, 2B4. We have applied these techniques to the procaryote P450cam and P450lin of Pseudomonas origin, and made comparison to the microsomal results (2). These data need to be viewed also against the backdrop of product formation via modulation of the second electron reduction step by a variety of proteins from photochemically prepared ferrous P450cam-substrate complex as cited earlier. Additional evidence of modified and heterologous complex activities are the Pd-desTrpGln, 50 fold lowered affinity for P450cam but recovery of full Vmax on saturation (28), and the preliminary data, now in extension of marked, though limited, native substrate activities of P450lin and P450cym with the P450cam redoxin-reductase (12).

From the current experiments several conclusions can be drawn: Mammalian P4502B4 activity can be reconstituted *in vitro* with the bacterial electron donors (Pd/PdR, or Ld/LdR, and NADH) replacing microsomal NADPH-dependent reductase. The efficiency of the prokaryotic/eukaryotic complexes are in the order of magnitude of the native microsomal system (Table 2). In this bypass of the perceived barriers to activity between prokaryotic-eukaryotic (2 vs 3 protein) systems, and the soluble vs membrane bound hangup, the abundant Pseudomonad and Streptomyces proteins thus afford additional potent tools to solution of the selectivity and redox mechanism puzzles. So far unexplained are the limited heterologous redoxin hemethiolate couples of the procaryote systems, and moreover, the more nearly quantitative product yields from NADH oxidation (12).

The heterologous reconstitution of P4502B4 activity might be due either to a conserved electron donor binding region on evolutionary distinct P450s, or to unspecified of P450/electron donor interactions.

The first explanation is supported by conservation of interacting residues and regions (12), as shown by computer modeling (22, 30, 31), the chemical modification of amino acid groups (1-4), sequential alignment in different P450's (5, 8-10, 14, 21-24), sutcide substrate studies (16) and heterologous binding of various electron donors to different P450's (6, 12, 29).

In summary, data in this paper implicate an evolutionarily conserved P450 binding site for different electron donors. Electron donor binding , however, does not necessarily lead to product formation thus each heterologous P450 system requires individual analysis. Further physical-chemical and protein engineering analyses are necessary for insight to constraints of heterologous electron transfer and product formation.

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