# P-450 HEMEPROTEINS OF RHIZOBIUM JAPONICUM
## Purification by Affinity Chromatography and Relationship to P-450$_{CAM}$ and P-450$_{LM-2}$*

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Summary: Rhizobium hemeproteins P-450a, b, and c cross react with antibodies to P-450$_{CAM}$ and P-450$_{LM-2}$. Anti P-450$_{CAM}$ IgG and phenobarbital, each bound to Sepharose 4B, were effective in purification of Rhizobium P-450c; the latter was more convenient. The amino acid composition of highly purified Rhizobium P-450c resembles the compositions of P-450$_{CAM}$ and P-450$_{LM-2}$. These results suggest that P-450 heme proteins of unrelated substrate specificities may nevertheless contain similar structural features.

P-450 hemeproteins are usually not found in $N_2$-fixing organisms. The unexpected appearance of a family of soluble P-450's in both symbiotic Rhizobia (1,2) and cells grown anaerobically in a nitrate-organic carbon medium (3) therefore requires an explanation. Involvement of these cytochromes in an oxidative phosphorylation pathway which supports $N_2$-fixation in R. japonicum has recently been suggested (4) but their exact function remains unclear.

The wide distribution of P-450 hemeproteins in nature and their participation in seemingly unrelated metabolic processes has prompted us to raise the question of structural similarity between these b-type cytochromes. It is already known that P-450$_{CAM}$ of the 5-exo hydroxylase of D (+) camphor of P. putida and P-450$_{LM-2}$ of the phenobarbital-induced drug hydroxylase of rabbit liver microsomes resemble each other as judged from immunochemical and compositional data (5). The present communication extends this comparison of related P-450 hemeproteins to Rhizobial proteins. A first account of this work has been reported recently (6).

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## Materials and Methods

Hemeproteins: Symbiotic $N_2$-fixing cells of Rhizobium japonicum, strain CC705 (syn. Wisconsin 505), were isolated from "Lincoln" strain soybean root nodules (1). Partially purified Rhizobium hemeproteins P-450a, b, and c were obtained from cell extracts by ion exchange chromatography on DE-52 cellulose (2). Partial purification of P-450$b_1$ which is found in non-symbiotic cells of Rhizobium was carried out using extracts of cells grown anaerobically in pure culture in the presence of nitrate (2,3). Crystalline P-$450_{CAM}$ of P. putida (7) was generously provided by Dr. I. C. Gunsalus, and phenobarbital-induced liver microsomal P-$450_{LM-2}$ containing 15 nmoles P-450 per mg protein (8) was kindly donated by Dr. M. J. Coon.

Antibodies and Radioimmunoassays: Rabbit antibodies to P-$450_{CAM}$ and P-$450_{LM-2}$ were obtained and purified as described previously (5). For competitive binding assays P-$450_{CAM}$ and P-$450_{LM-2}$ were labeled with $^{125}I$ exculsively at tyrosines (9) and without apparent loss of catalytic activity (10).

Affinity Chromatography: ω-Aminohexyl Sepharose 4B and ω-carboxypentyl Sepharose 4B were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. m-Aminophenobarbital was synthesized (11) and bound either to Sepharose 4B via BrCN coupling or to ω-carboxypentyl Sepharose 4B by carbodiimide dehydration (12). The capacity of these Sepharose derivatives was estimated to be 15-18 μmoles/ml for cytochrome P-$450_{CAM}$. Routinely, volumes of 0.25-0.35 ml of swollen resin were transferred into Pasteur pipettes, equilibrated with 0.05 M phosphate, pH=7.0, and immediately used for affinity chromatography of 3-5 mg of P-450 hemeprotein.

Isoelectric Focusing in Acrylamide Gel: The apparatus, constructed from Lucite to accommodate a gel bed of 25x15x0.5 cm, was cooled at 4° C. Filter paper strips (Whatman No. 3, 3x9 mm) soaked with sample solution to contain ~ 5 μg protein were placed on the gel ~ 1 cm from the cathode. Electrofocusing was carried out in acrylamide gel (7% cross linkage, 2% ampholytes pH 3-10) for 2 h maintaining a constant current of < 35 mA. After the run the protein was fixed in 7% TCA.

## Results and Discussion

Immunochemical Experiments: As seen in Fig. 1, the family of symbiotic Rhizobium P-450 hemeproteins showed substantial cross reactivity with anti P-$450_{CAM}$ antibodies, namely, 54% for P-450c, 50% for P-450b, and 45% for P-450a. This compares to 60% cross reactivity of P-$450_{LM-2}$ with anti P-$450_{CAM}$ antibodies reported earlier (5). It is deemed significant that the extent of displacement of Rhizobium hemeproteins by P-$450_{CAM}$ is not the same for all members and that P-450c shows the closest relationship to P-$450_{CAM}$ in this assay. These immunochemical data are in agreement with a closer correlation of P-450c and P-$450_{CAM}$ with regard to absorption maxima (2), good solubility in aqueous buffers, and fairly acidic isoelectric points, at pH=4.9 and 4.5 for P-450c and P-$450_{CAM}$, respectively (13). P-450c also happens to be the most abundant

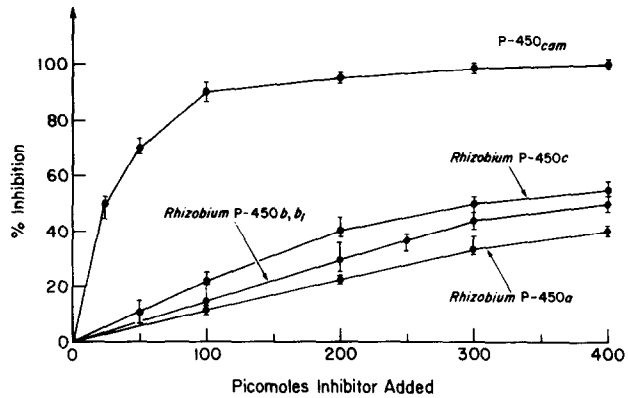



Fig. 1. Cross Reactivity of Rhizobium P-450 Hemeproteins with Antibodies to P-450$_{CAM}$.

Radioimmunoassays of competition of unlabeled Rhizobium P-450 hemeproteins with $^{125}$I-labeled P-450$_{CAM}$ for binding to anti P-450$_{CAM}$ antibodies were performed in triplicate using 0.15 M NaCl-0.05 M sodium phosphate buffer, pH=7.0, as described previously (3). Measurements of radioactivity in the resulting precipitates carried out either with the $^{14}$C window of a Beckman Model LS-30 Liquid Scintillation Counter or with a Packard Tricarb Liquid Scintillation Spectrometer 3375, with $\beta$-$\gamma$-control, yielded identical sets of curves. The inhibition of binding by Rhizobium P-450 hemeproteins is based on maximal interaction of unlabeled P-450$_{CAM}$ to anti P-450$_{CAM}$ antibodies; these values were normalized to 100%.

Rhizobium P-450 component and the one most readily purifiable by ion exchange chromatography (2). P-450b$_1$ of anaerobic-nitrate grown cells, showing elution patterns in DE-52 ion exchange chromatography identical to that of symbiotic P-450b (2), could not be distinguished from P-450b by cross reactivity with anti P-450$_{CAM}$ antibodies. Preliminary data not included here also point to strong cross reaction between Rhizobium P-450 hemeproteins a, b, and c and anti P-450$_{LM-2}$ antibodies. In this case P-450a seems to be the component most closely related to P-450$_{LM-2}$.

As outlined in Table I, the similarity in antigenic site structures was exploited for further purification of P-450c via specific complexing with anti P-450$_{CAM}$ IgG antibodies which had been immobilized on Sepharose 4B. In this case the batch procedure was preferred over column chromatography because of the need to incubate the P-450c with the immobilized antibodies for several hours to guarantee complete interaction. Although this immunochemi-

Table I
Purification of Rhizobium P-450c Using
Immobilized Anti-P-450$_{CAM}$ Antibodies

A. Preparation of Specific Antibodies:
   1.) binding of P-450$_{CAM}$ to Sepharose 4B via BrCN coupling
   2.) application of IgG fraction
   3.) removal of non-specific antibodies by washing with 2 volumes of 50 mM $PO_4^=$ at pH 7.0
   4.) elution of 2 specific antibody fractions
      a) 1 M NaCl, 50 mM $PO_4^=$, pH 7.0
      b) 1 M NaCl, 10% dioxane, 50 mM $PO_4^=$, pH 7.0
   5.) dialysis of combined fractions; 80% recovery of anti-P-450$_{CAM}$ antibodies.

B. Batch Purification of P-450c:
   1.) binding of anti-P-450$_{CAM}$ IgG to Sepharose 4B via BrCN coupling
   2.) incubation with Rhizobium P-450c ($A_{280}/A_{417}$ = 1.02): 18 hrs. at 4° C
   3.) removal of unbound protein contaminants by washing with 50 mM $PO_4^=$, pH 7.0
   4.) elution of P-450c with 1 M NaCl; 60% recovery of P-450c ($A_{280}/A_{417}$ = 0.86)

cal affinity procedure yielded P-450c preparations of significantly increased purity as evidenced by the improvement of the spectral ratio, $A_{280}/A_{417}$, normally used as purity index, from 1.02 to 0.86 (Table I), several difficulties were encountered with this procedure. First, large amounts of pure P-450$_{CAM}$ bound to Sepharose 4B via BrCN coupling were required for the preparation of specific antibodies, and second, only P-450$_{CAM}$ antibodies of medium affinity toward P-450c could be used effectively in the subsequent purification step. This selection was necessary to avoid losses due to loose binding of P-450c or formation of complexes too tight to dissociate at moderately high salt concentrations.

Affinity Chromatography on Sepharose Derivatives: A more convenient and less costly purification procedure is summarized in Fig. 2. P-450c bound only moderately strongly to ω-aminohexyl Sepharose 4B and was recovered in similar yields and even higher purity, $A_{280}/A_{417}$ = 0.74 - 0.78, than from the immobilized antibody resin. m-Aminophenobarbital bound directly to Sepharose 4B via BrCN coupling was apparently not sufficiently exposed to permit good binding of P-450 hemeproteins but if it was attached to an extended alkyl arm

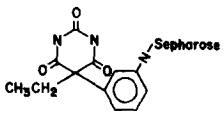
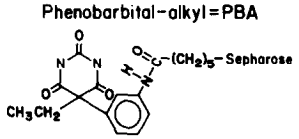
| Column | P-450*a* | P-450*b* | P-450*c* |
|---|---|---|---|
| ω-aminohexyl — $H_2N-(CH_2)_6$-Sepharose | + | + + $A_{414}^{280}$ 3.1→2.6 73% | + + $A_{418}^{280}$ 1.02→0.78 54% |
| Phenobarbital —  | + | + | + |
| Phenobarbital-alkyl=PBA  | + + + $A_{416}^{280}$ 3.3→1.44 64% | + + + $A_{414}^{280}$ 3.1→2.05 80% | + + + $A_{418}^{280}$ 2.05→0.95 65% |

Fig. 2. Affinity Chromatography of Symbiotic Rhizobium P-450.

Pasteur pipettes containing 0.25-0.35 ml of swollen Sepharose 4B derivatives were employed as miniature affinity columns. The resin was equilibrated with 0.05 M sodium phosphate, pH=7.0, and charged with 3-5 mg of P-450 dissolved in the same buffer. The columns were washed with 20 void volumes of buffer to remove contaminating proteins and the affinity-bound hemeproteins were then eluted with buffers of higher ionic strength: Weakly bound P-450s (+) eluted with 0.5 M phosphate, moderately strongly bound P-450s (++) eluted with 1.0 M phosphate-2.0 M NaCl, and very strongly bound P-450s (+++) eluted with 2.0 M urea, all at pH=7.0. All eluates were immediately dialyzed against 0.05 M phosphate.

(12) strong binding was observed for all Rhizobium P-450s as well as for P-$450_{CAM}$ and P-$450_{LM-2}$. As shown by the improved spectral ratios, P-450s a, b, and c were substantially purified (Figs. 2+3). Better resolution of components was achieved by isoelectric focusing in polyacrylamide gel shown in Fig. 4. Each of the DE-52 fractions (2) was resolved into many bands including several hemeprotein bands with different isoelectric points. Thus we conclude that symbiotic Rhizobia contain at least 6 distinct P-450 hemeproteins. Affinity chromatography removed most of the non-heme contaminants (Fig. 4). Yet this preparation of P-450c still contained two hemeproteins. Its spectral ratio, $A_{280}/A_{417}$ was 0.72-0.75. Since P-450c contains fewer tyrosines than P-$450_{CAM}$ or P-$450_{LM-2}$ (see Table II), we estimate a hemeprotein content of about 70% for the purified preparation and assume that apo P-450c contributes considerably to the remaining 30%.

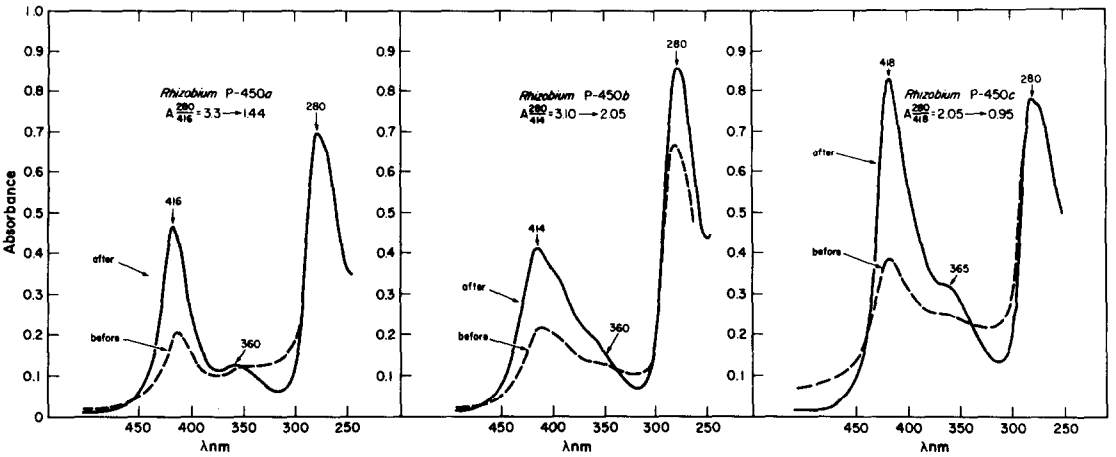



Fig. 3. Spectral Properties of Rhizobium P-450 Hemeproteins.

All absorption spectra were recorded with a Cary spectrophotometer, model 14, in 0.05 M phosphate, pH=7.0. The dashed and solid traces refer to the same protein preparations before and after affinity chromatography on PBA-Sepharose 4B respectively. The UV traces of P-450a and c before and after purification were matched but those of P-450b differed.

As expected, $P\text{-}450_{LM\text{-}2}$ preparations (8) bound to $\omega$-phenobarbital alkyl (PBA) Sepharose very tightly and, after extensive washing with .5M NaCl in 50 mM phosphate pH 7.0, the hemeprotein eluted with 2 M NaCl in 50 mM phosphate pH 7.0 showed an improved spectral ratio, $A_{280}/A_{417}$ of about 0.72 (about 95% pure).* It was surprising, however, to find that all Rhizobium P-450's bound very tightly to PBA-Sepharose as well and had to be eluted with 1 M phosphate - 2 M NaCl. Even $P\text{-}450_{CAM}$ bound quite tightly to this resin. It should be noted that none of these hemeproteins was retarded on Sepharose 4B or its - carboxypentyl or $\omega$-aminohexyl-p-aminobenzamidyl derivatives, and neither cytochrome c nor bovine serum albumin bound to PBA-Sepharose. Also, the P-450s did not bind at high salt concentrations as is preferred for sample

*R. Goewert and K. Dus, unpublished data

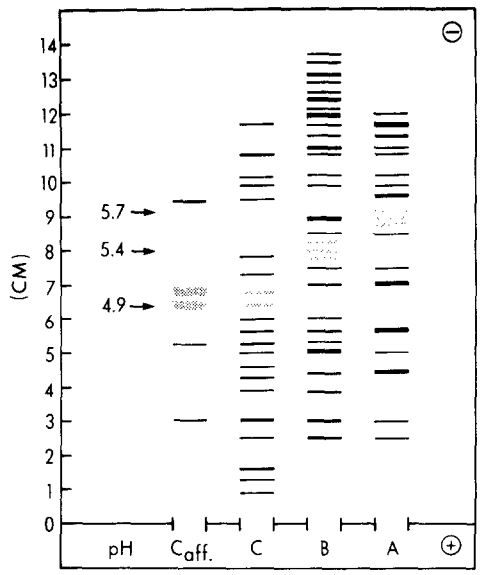



Fig. 4. Isoelectric Focusing of Rhizobium P-450 Hemeproteins in Polyacrylamide Gel.

Electrofocusing was carried out in gels of 7% cross linkage using 2% ampholyte solutions, pH 3-10, for 2h at 4° C maintaining a constant current below 35 mA. Rhizobium P-450 hemeproteins a, b, and c refer to fractions obtained from chromatography on DE-52 cellulose (2) while P-450 $c_{aff}$ refers to P-450c purified by affinity chromatography on PBA-Sepharose.

application in hydrophobic chromatography (14). On the other hand, P-450s bound tightly to PBA-Sepharose exhibited the Soret maxima of the substrate-free forms rather than those of the substrate complexes.

The amino acid composition of the purified P-450c preparation (Table II) is based on a molecular weight of 46,000 daltons as estimated by gel filtration on Sephadex G-75 (2). This composition shows close correlation to the compositions of $P\text{-}450_{CAM}$ (13) and $P\text{-}450_{LM\text{-}2}$ (5). All three P-450 hemeproteins are acidic and show an unusually high content of hydrophobic residues. The only significant deviations are the occurrence of only 2 cysteines and the much larger content of alanine in Rhizobium P-450c. This protein seems to be more closely related to $P\text{-}450_{CAM}$ than to $P\text{-}450_{LM\text{-}2}$, but whenever it deviates from $P\text{-}450_{CAM}$, as in the contents of Gly, Ile, Leu, and Arg, it tends to approach

TABLE II AMINO ACID COMPOSITION OF CYTOCHROMES

| Amino Acids | P-450$_{cam}$ Pseudomonas putida | P-450$_{LM-2}$ phenobarbital-induced rabbit liver microsomes | P-450$_{c}$ Rhizobium japonicum |
|---|---|---|---|
| Asx | 36 | 35 | 33 |
| Thr | 19 | 23 | 14 |
| Ser | 21 | 26 | 20 |
| Glx | 55 | 43 | 54 |
| Pro | 27 | 24 | 25 |
| CySH | 6 | 6 | 2 |
| Gly | 26 | 30 | 33 |
| Ala | 34 | 23 | 54 |
| Val | 24 | 27 | 25 |
| Met | 9 | 8 | 8 |
| Ile | 24 | 19 | 14 |
| Leu | 40 | 46 | 46 |
| Tyr | 9 | 11 | 6 |
| Phe | 17 | 28 | 17 |
| His | 12 | 11 | 12 |
| Lys | 13 | 19 | 14 |
| Trp | 1 | 1 | 1 |
| Arg | 24 | 29 | 29 |
| Total | 397 | 409 | 407 |
| Heme | 1 | 1 | 1 |
| PI | 4.5 | 4.2 | 4.9 |

the values found for P-450$_{LM-2}$. Based on the resemblances among Rhizobium P-450$_{c}$, P-450$_{CAM}$ and P-450$_{LM-2}$ in immunochemical reactivities and amino acid compositions we suggest that these are structure-related P-450 hemeproteins.

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