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HEME BINDING AND SUBSTRATE-PROTECTED CYSTEINE RESIDUES IN P-450cam

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SUMMARY: Reactions of the Pseudomonas putida cytochrome P-450-substrate complex or enzyme alone with ^{14}C-labeled iodoacetic acid have been investigated at pH 7.0. After subsequent conversion of all of the cysteine residues to S-β-carboxymethylcysteinyl residues, tryptic peptides of the derivative were separated by either high performance liquid chromatography or two dimensional electrophoresis, and their amino acid compositions and partial sequences were determined. All but cysteine residue-134 reacted to some extent. This result implicated residue-134 as the thiol group which is the axial heme ligand since the heme was intact in all of the derivatives made. Reaction of the enzyme-substrate complex with "cold" iodoacetic acid followed by substrate removal and reaction with ^{14}C-labeled iodoacetic acid resulted in radiolabeling of mainly cysteine-240. This suggested cysteine-240 to be an active site cysteine residue.

Recently, the primary structures of several cytochrome P-450 monooxygenases from different sources have been studied by different laboratories (1-5). The Pseudomonas putida cytochrome P-450 (P-450cam)[1] provides biochemists with a model cytochrome P-450 due to its excellent solubility properties. For the elucidation of structure-function relationships, titration with several thiol reagents (6,7) and affinity probes (8,9) have been reported. P-450cam has been reported to contain 6 half cysteine residues (10), and titrations with N-ethylmaleimide (6) and p-chloromercuribenzoate (7) have suggested that they are all in the thiol state. However, the recent sequence study of cytochrome P-450cam indicated the enzyme contained 8 cysteine residues (1). An earlier study suggested the presence of a single highly reactive cysteine residue-355

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[1] The following abbreviations were used: P-450cam for Pseudomonas putida cytochrome P-450; CysCm for S-β-carboxymethylcysteine; HPLC for high pressure liquid chromatography; TLC for thin layer chromatography; TPCK for tosyl phenyl chloromethylketone.

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(11) which is possibly located on the surface of the enzyme and is probably involved in P-450cam dimerization (6). Also Black et al. (5) have suggested from sequence homology that cysteine-134 in P-450cam and cysteine-152 in rat liver microsomal cytochrome P-450b might be the thiols involved in heme binding. However, the subject remains controversial since Fujii-Kuriyama et al. (2) and Heinemann and Ozols (3) have proposed that cysteine-355 in P-450cam and cysteine-436 in rat liver P-450b are the axial ligands to the heme iron. In the present report, chemical data showing which thiol groups in P-450cam are involved in heme-binding and possibly substrate binding are presented.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome P-450cam was isolated from Pseudomonas putida PpG 786 (ATCC 29,607) according to a published procedure (12), and it was crystallized in the orthorhombic I form as previously described (13). Substrate-free enzyme was obtained by gel filtration at 4°C as follows: the P-450cam-camphor solution was chromatographed on a 1 x 20 cm column of Sephadex G-25 equilibrated with 0.1 M sodium phosphate buffer, pH 7.0.

Alkylation of -SH groups. The enzyme solution was diluted with 0.1 M sodium phosphate buffer, pH 7.0, to yield a 2 mg/ml solution. D-camphor (enzyme-camphor ratio of 1:1) was added and incubated at 4°C for one hour. ^{14}C-Iodoacetate dissolved in the above-mentioned buffer was added at an enzyme-reagent molar ratio of 1:60. Reactants were slowly stirred at 4°C for suitable time intervals. The reaction stopped by the addition of excess dithiothreitol (100 nmol). Exhaustive alkylation was carried out as described by Crestfield et al (14) after removal of the heme moiety with 0.3% HCl-acetone.

For the determination of the active site thiol, non-radioactive iodoacetate was used initially for alkylation of reactive -SH groups in the enzyme-camphor complex. After 24 hours of reaction at 4°C, camphor was removed by gel filtration using Sephadex G-25 (1 x 20 cm column) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The camphor-free enzyme was reacted with ^{14}C-iodoacetate (50 μCi/3.6 μmol) New England Nuclear Co. for labeling of the substrate-binding-site-thiol. Finally the sample was exhaustively alkylated with iodoacetate in 6 M guanidine-HCl-0.5 M Tris buffer, pH 8.5.

Trypsin Cleavage. The CysCm-derivatives of P-450cam in 1 ml of 0.2 M NH_4HCO_3 were digested with TPCK-trypsin for 48 hours at 37°C. The enzyme: substrate ratio was 1:50 (w/w). The reaction was stopped by lyophilization of the reaction mixture.

Peptide Separation. Tryptic digests (approximately 10 nmoles) were subjected to an Ultrasphere reverse phase HPLC (4.6 x 250 mm column) (4). Cysteine-containing peptides were detected from radioactivity measurements of 20 μl of aliquots.

Peptide Fingerprinting. Tryptic digests of radio-labeled P-450cam (approximately 10 nmoles) were applied to silica gel TLC plates (20 x 20 cm) and electrophoresis at pH 1.0 (15% acetic acid - 10% formic acid) was performed for 2 hours at 150V. Electrophoresis in the second dimension was performed in pH 3.70 buffer (pyridine:HOAc:water, 1:10:89, v/v), same as the above conditions. Radioactive peptides were detected by autoradiography using Kodak X-ray film. The peptides were cut out and were extracted by overnight shaking with 2 ml of 0.2 N NH_4OH.

Sequencing. One to two nanomoles of peptides were subjected to automated Edman degradation in an updated, modified Beckman 890C Sequencer as described previously (15). Pth-derivatives were identified by reverse phase HPLC on an Ultrasphere (Altex) ODS column on a Waters Associates chromatograph (16).

Amino Acid Analyses. Samples (5-10 μg) were hydrolyzed in 6 N HCl containing 0.02% β-mercaptoethanol for 24 hours at 110°C in sealed evacuated tubes. Amino acid compositions were determined using a one-column Beckman Model 121 MB analyzer.

RESULTS AND DISCUSSION

Identification of Modified Cysteine Residues. The P-450cam-substrate complex (60 nmoles) was alkylated with 3.6 μmoles of ^{14}C-labeled iodoacetic acid at an enzyme:reagent molar ratio of 1:60. After 24 hours of reaction, the P-450cam was treated with 0.3% HCl-acetone. The partially alkylated apocytochrome P-450 was dissolved in 6 M guanidine-HCl - 0.5 M Tris buffer, pH 8.5, and further modified with "cold" iodoacetic acid. After removal of excess reagents by complete dialysis the derivative was digested with TPCK-trypsin. Figure 1 shows the HPLC chromatogram obtained using an Ultrasphere C-8 column (4.6 x 250 nm, 300 Å). Approximately 40 peptides including 7 radiolabeled peptides were separated in a single step. Each radioactive peptide was identified from the NH_2-terminal sequence analyses and from the known sequence of the enzyme (1). Table I shows the list of the reactive cysteine residues in P-450cam. It

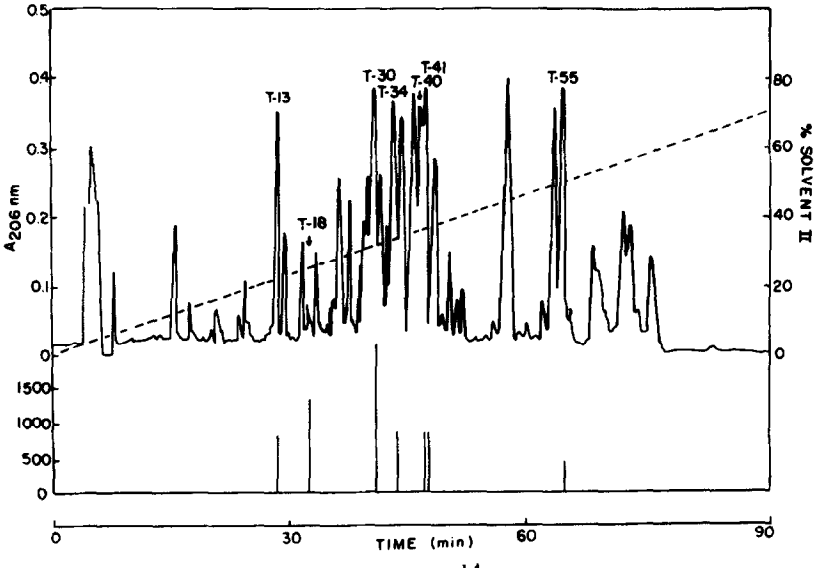

Figure 1. HPLC tryptic peptide map of (^{14}C)-labeled cytochrome P-450cam (10 nmoles). An Ultrasphere (4.6 x 250 nm) column was used. Peptides were eluted with a linear gradient from 0.1% TFA to 70% solvent II (TFA - H_2O - CH_3CN = 0.1:9.9:90, v/v).

Table I. Identification of Radiolabeled Peptides from HPLC and Two Dimensional Electrophoresis

No.	Peptide	NH_2-Terminal Sequence	Cys Residue Number
1	T-13	Cys Asn Gly	Cys 56
2	T-18	Glu Asn Ala	Cys 332
3	T-30	Glu Asn Ala	Cys 332
4	T-34	Val Ser His	Cys 355
5	T-40	Ile Pro Ala	Cys 283
6	T-41	His Phe Ser	Cys 83
7	T-55	Ile Gln Glu	Cys 146
8[a]	T-origin	Met Cys Gly	Cys 240

[a]Detected by TLC peptide mapping.

should be emphasized that the enzyme still contained heme even after alkylation for 24 hours. Reactive cysteine residues identified were cysteine-56, -83, -283, -332 and -355. Cysteine-240 was not identified by HPLC because the peptide containing it was not eluted from the column. Since peptide T-55 contained two cysteine residues it was necessary to show which residue was labeled. For this purpose 2 nmoles of peptide T-55 was further digested with 0.4 µg of thermolysin and the radioactive peptide was isolated by HPLC (Synchropak RPC8 column; 4.1 x 250 mm, 10 µM size) as shown in Figure 2. One radioactive peak, T-55-Th-8 was found which contained cysteine-146 as deduced from its amino acid composition (Table II). The amino acid composition suggested that the peptide was Leu-Arg-Pro-Gln-Gly-Gln-Cys-Asn. Therefore cysteine-146 and not cysteine-134 had react-

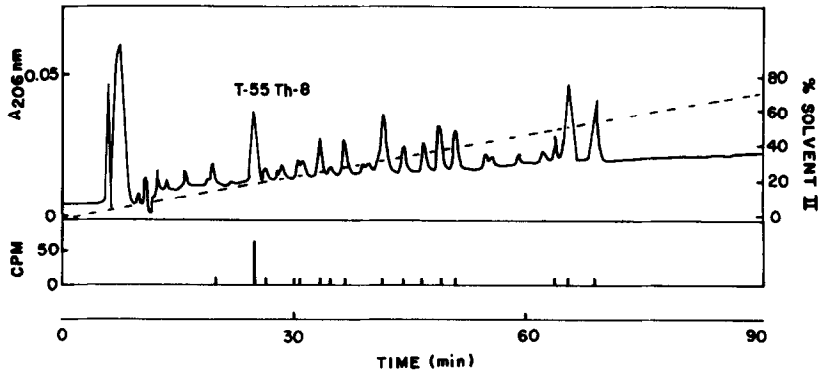

Figure 2. HPLC separation of thermolysin peptides of peptide T-55. Chromatographic conditions were the same as in Figure 1, except that a Synchropak RPC8 column (4.1 x 250 nm) was used.

Table II. Amino Acid Composition of Peptide T-55 Th-8[a]

Amino Acid	Residues/mole of peptide
CysCm	0.78 (1)[a]
Asp	1.09 (1)
Glu	1.92 (2)
Pro	0.82 (1)
Gly	1.15 (1)
Leu	0.87 (1)
Arg	0.83 (1)

[a]See sequence analysis (1) of P-450cam for peptide placement.

ed with iodoacetic acid, as confirmed by sequence analysis of peptide T-55 (Table III). Thus, cysteine residues which reacted with iodoacetic acid under these conditions of the experiment are residues 56, 83, 146, 283, 332 and 355. However, it was not determined whether cysteine-240 had reacted or not because it was not eluted due to its hydrophobic nature and secondly because the peptide containing it was partially precipitated after trypsin digestion.

Kinetics of Labeling of Cysteine Residues Using Fingerprinting. Figure 3 shows the rates of reaction of the various cysteine residues in camphor-bound

Table III. Sequence Analysis of Peptide T-55[a]

Cycle	Amino Acid	Yield (pmol)	Radioactivity (cpm)[b]	
1	Ile	832	23	
2	Gln	645	18	
3	Glu	661	22	
4	Leu	1339	9	
5	Ala	1328	15	
6	Cys	356	56	←---
7	Ser	70	27	
8	Leu	1141	10	
9	Ile	594	15	
10	Glu	354	18	
11	Ser	35	15	
12	Leu	612	20	
13	Arg	+	21	
14	Pro	247	23	
15	Gln	164	15	
16	Gly	239	15	
17	Gln	231	18	
18	Cys	100	340	←
19	Asn	50	170	
20	Phe	155	50	

[a] Approximately 2 nmol of peptide used.

[b] Arrows show the cycles of Edman degradation which released radioactivity.

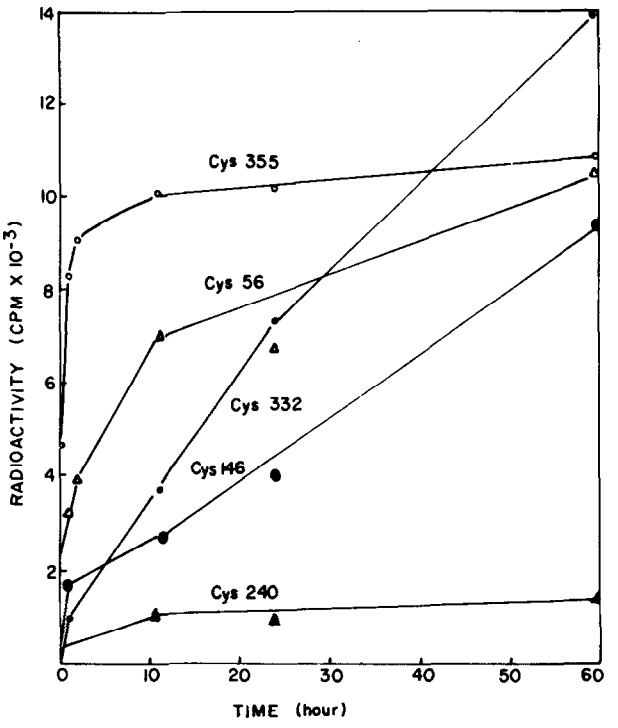

Figure 3. Alkylation of reactive (type I) cysteine residues of P-450cam with 1-(^{14}C)-iodoacetic acid. See experimental section for details.

P-450cam as determined by tne thin layer electrophoretic peptide mapping. At each time interval aliquots were removed and handled as described in the experimental section. Some of the cysteine peptides were not identified by this method because of difficulties with peptide separation. However, it is clear that cysteine-355 was the most reactive thiol. This result was consistent with the data using 2-bromo-acetamido-4-nitrophenol as the thiol reagent (11). It is interesting to note that cysteine-332 reacted slowly initially but after 10 hours of modification the rate of reaction accelerated possibly due to a conformational change. Cysteine-240 reacted only slightly with iodoacetic acid during the same time period. The result suggested that cysteine-240 might be buried in the ES complex or blocked from reacting in the ES complex. The peptide containing cysteine-240 is quite hydrophobic and was only detected by TLC peptide mapping.

Heme Binding Domain. Although two different cysteine residues have been proposed to be heme ligands by different groups (2,3,5), the present chemical

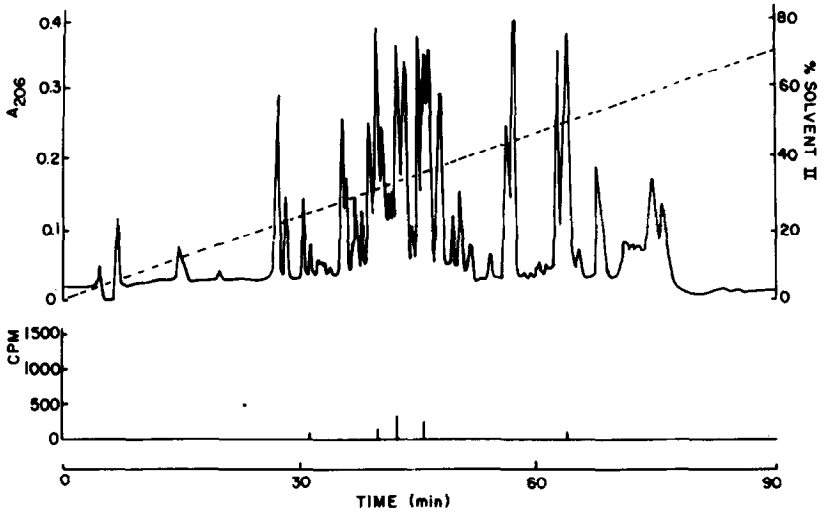

Figure 4. HPLC tryptic peptide map made of P-450cam alkylated with (^{14}C)-iodoacetic acid after removal of camphor. Chromatographic conditions were the same as in Figure 1.

modification experiments implicate cysteine-134. This cysteine residue was unreactive to iodoacetate in both the enzyme-substrate complex and substrate-free enzyme. The heme was still intact under these alkylation conditions. Recent single crystal x-ray diffraction study of P-450cam also has led to the same conclusion[2]. The proposal that cysteine-355 is the potential heme ligand is negated from the observed full reactivity of this cysteine residue with iodoacetate. However, Fujii-Kuriyama et al have observed that one of the isozymes of rat liver cytochrome P-450 is lacking a cysteine residue corresponding to cysteine-152 of cytochrome P-450b[3]. If confirmed, cysteine-152 cannot be a heme ligand in this rat liver cytochrome P-450.

Thiol in the Substrate Binding Site of the Active Center of P-450cam. For the experiment 40 nmol of the P-450cam-substrate complex was first reacted with 4.1 μmol of iodoacetate for 24 hours. The camphor was removed by Sephadex G-25 chromatography and the derivative was labeled with 3.6 μmol of ^{14}C-iodoacetate The tryptic peptide map of the soluble fraction did not show any significantly labeled peptide; three radioactive peptides contained 450∿500 cpm of radio-

[2] Private communication from Dr. T. Poulos.

[3] Private communication from Dr. R. Sato, Protein Research Institute, Osaka, Japan.

Table IV. Amino Acid Composition of Tryptic Core Peptide

Amino Acid	Residues/mole Observed	Theoretical[a]
Asp	1.99	(2)
Thr	1.02	(1)
Ser	1.48	(2)
Glu	1.30	(1)
Gly	3.48	(3)
Ala	1.15	(1)
Val	2.85	(3)
Met	0.89	(2)
Leu	6.16	(6)
Phe	3.12	(3)
Lys	1.02	(1)
Cys	0.69	(1)
Total		26
Radioactivity (^{14}C)	24,770 cpm/nmol of peptide	

[a] Numbers in parentheses are from sequence analysis of P-450cam (1).

activity/nmol of peptide (Figure 4). However, significant radioactivity was observed in the tryptic core as shown in Table IV. Amino acid composition and NH_2-terminal sequence analyses of the radioactive core peptide revealed it contained cysteine-240 as shown in Table IV and V.

This result differs from the report of Dus (17) who reported that cysteine-56 is present in the active center and it possibly forms a thiohemiketal with the carbonyl group of the substrate, camphor. In fact, in our experiment cysteine-56 was significantly labeled in the P-450cam-substrate complex. A

Table V. Sequence Analysis of Tryptic Core Peptide[a]

Cycle	Amino Acid	Yield (pmol)
1	Met	78
2	Cys	44
3	Gly	56
4	Leu	78
5	Leu	79
6	Leu	86
7	Val	88
8	Gly	50
9	Gly	54
10	Leu	56
11	Asp	20
12	Thr	+
13	Val	58
14	Val	61

[a] Approximately 300 pmol of sample were subjected to sequencer analysis.

possible explanation for our result is that cysteine-240 is buried or blocked in the enzyme-substrate complex. However, after removal of camphor, deblocking occurs or a conformational change occurs which makes cysteine-240 accessible to radioactive iodoacetate. Since the peptide containing cysteine-240 is quite hydrophobic, the cyclic hydrocarbon substrate, camphor, can be readily accommodated in such a hydrophobic active site.

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REFERENCES

1. Haniu, M., Armes, L.G., Tanaka, M., Yasunobu, K.T., Shastry, B.S., Wagner, G.C., and Gunsalus, I.C. (1982) Biochem. Biophys. Res. Commun. 105, 889-894.
2. Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., and Muramatsu, M. (1982) Proc. Natl. Acad. Sci. 79, 2793-2797.
3. Heinemann, F.S., and Ozols, J. (1982) J. Biol. Chem. 257, 14988-14999.
4. Yuan, P.-M., Nakajin, S., Haniu, M., Shinoda, M., Hall, P.F., and Shively, J.E. (1983) Biochemistry 22, 143-149.
5. Black, S.D., Tarr, G.E., and Coon, M.J. (1982) J. Biol. Chem. 257, 14616-14619.
6. Lipscomb, J.D., Harrison, J.E., Dus, K.M., and Gunsalus, I.C. (1978) Biochem. Biophys. Res. Commun. 83, 771-778.
7. Yu, C.-A., and Gunsalus, I.C. (1974) J. Biol. Chem. 249, 102-106.
8. Murray, R.I., Gunsalus, I.C., and Dus, K.M. (1982) J. Biol. Chem. 257, 12517-12525.
9. Swanson, R.A., and Dus, K.M. (1979) J. Biol. Chem. 254, 7238-7246.
10. Dus, K.M., Katagiri, M., Yu, C.-A., Erbes, D.L., and Gunsalus, I.C. (1970) Biochem. Biophys. Res. Commun. 40, 1423-1430.
11. Haniu, M., Yasunobu, K.T., and Gunsalus, I.C. (1982) Biochem. Biophys. Res. Commun. 107, 1075-1081.
12. Gunsalus, I.C., and Wagner, G.C. (1978) Methods Enzymol. 52, 166-188.
13. Poulos, T.L., Perez, M., and Wagner, G.C. (1982) J. Biol. Chem. 257, 10427-10429.
14. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) J. Biol. Chem. 238, 622-627.
15. Shively, J.E. (1981) Methods Enzymol. 79, 31-48.
16. Hawke, D., Yuan, P.M., and Shively, J.E. (1982) Anal. Biochem. 120, 302-311.
17. Dus, K.M. (1982) Xenobiotica 12, 745-772.