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Steroid 21-Hydroxylase (Cytochrome P-450) from Porcine Adrenocortical Microsomes: Microsequence Analysis of Cysteine-Containing Peptides†

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ABSTRACT: The steroid 21-hydroxylase cytochrome P-450 from porcine adrenocortical microsomes was purified to homogeneity. The protein exhibited two NH_2-terminal sequences, one of which was identical with the first but lacking the NH_2-terminal methionine. The sequence was extremely hydrophobic but had little homology to the 17α-hydroxylase/$C_{17,20}$-lyase isolated from neonatal porcine testes or to rat or rabbit liver microsomal cytochromes P-450. The cysteine-containing fragments of the S-carboxymethylated protein were purified by high-performance liquid chromatography and sequenced. Three of the cysteine-containing peptides exhibited significant sequence homology with peptides from the major phenobarbital-induced rat liver cytochrome P-450 (P-450b) and two with peptides from cytochrome $P\text{-}450_{cam}$ (camphor methylene hydroxylase from *Pseudomonas putida*). The presence of conserved regions in the primary sequences of these proteins appears likely to provide clues to the nature of their heme-binding domains.

The conversion of cholesterol to active steroid hormones requires a number of enzymatic reactions of which several are catalyzed by cytochromes P-450 (Ryan & Engle, 1957; Estabrook et al., 1963). The steroidogenic cytochromes P-450 are characterized by well-defined substrate specificities and absolute specificity for the site on the substrate at which catalysis occurs (Nakajin & Hall, 1981). Because of the interest in enzymology of P-450 and the difficulty of working with drug metabolizing cytochromes P-450 in which substrate and position specificity are ill-defined, we have purified a number of the steroidogenic cytochromes P-450 in preparation for determination of their primary structures. To facilitate comparison between different steroidogenic cytochromes P-450, we have confined our studies to a single species, namely, the pig. Microsomal 21-hydroxylase was previously purified from beef adrenal (Kominami et al., 1980). We now report initial structural studies of cytochrome P-450, 21-hydroxylase from microsomes of pig adrenal glands.

Experimental Procedures

Materials. Acetonitrile (HPLC grade) was obtained from J. T. Baker Chemical Co. Trifluoroacetic acid was distilled first over chromium trioxide and then over alumina. Iodoacetic acid (Sigma) was recrystallized from petroleum ether prior to use, and iodo[1-^{14}C]acetic acid (50 μCi/0.7 mg) was obtained from New England Nuclear. 3-Sulfophenyl isothiocyanate (sodium salt) and *o*-phthaldialdehyde were from Pierce Chemical Co. Trypsin (TPCK treated; 232 units/mg) was obtained from Worthington Biochemical Co. *Staphylococcus aureus* protease (strain V8, specific activity 500–700 units/mg) was from Pierce Chemical Co. The sources of various chemicals for enzyme purification have been given elsewhere (Nakajin & Hall, 1981) except for the following: [4-^{14}C]-progesterone (lot no. 965-209; 55.7 mCi/mmol) and 17α-hydroxy[4-^{14}C]progesterone (lot no. 467-128; 50 mCi/mmol) were obtained from New England Nuclear Co. and hydroxylapatite was from Bio-Rad (Bio-Gel HTP).

Purification of 21-Hydroxylase. Pig adrenal glands were homogenized in 5 volumes of sucrose (0.25 M) containing 0.1 mM EDTA[1] and centrifuged at 9000*g* for 30 min. The su-

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pernatant was centrifuged at 105000*g* for 60 min to give a microsomal pellet which was resuspended in sucrose–EDTA and resedimented at 9000*g* for 30 min; the supernatant was centrifuged at 105000*g* for 60 min to give the microsomal pellet which was washed in potassium phosphate buffer (100 mM; pH 7.2) containing EDTA (0.1 mM). The final microsomal pellet was suspended at a concentration of 12–14 mg of protein/mL in the same buffer containing 20% (w/v) glycerol. Sodium cholate and dithiothreitol were added to the microsomal suspension at final concentrations of 2.5 mg of cholate/mg of protein and 0.1 mM, respectively. The turbid mixture was stirred at 4 °C for 60 min. The mixture was then centrifuged at 105000*g* for 60 min, and the supernatant was dialyzed against potassium phosphate (50 mM; pH 7.2) with sodium cholate (0.4% w/v), glycerol (20% w/v), EDTA (0.1 mM), and dithiothreitol (0.1 mM) (buffer A). The dialyzed preparation was applied to a column (21 × 2.0 cm) of ω-amino-*n*-octyl-Sepharose 4B prepared as described by Kominami et al. (1980). Cytochrome P-450 was eluted by means of a linear gradient of Emulgen 913 (0–0.18% w/v) in buffer A (total volume 500 mL). The gradient was followed by 800 mL of Emulgen 913 (0.18% w/v in buffer A). CO-difference spectroscopy revealed two peaks containing cytochrome P-450 of which the second peak contained the 21-hydroxylase. This fraction was dialyzed against buffer A and reapplied to a column of ω-amino-*n*-octyl-Sepharose 4B (10 × 2.0 cm). Elution was performed with a linear gradient of Emulgen 913 in buffer A (0–0.22% w/v) with a total volume of 500 mL. Cytochrome P-450 was eluted in a single peak. Fractions containing P-450 were pooled and dialyzed against potassium phosphate (10 mM; pH 7.2) containing glycerol (20% w/v), Emulgen 913 (0.2%), EDTA (0.1 mM), and dithiothreitol (0.1 mM) (buffer B). The dialyzed solution was applied to a column of hydroxylapatite (10 × 2.0 cm) equilibrated with buffer B. Elution was performed with a linear gradient of potassium phosphate 10–250 mM in buffer B by using a total volume of 400 mL. Cytochrome P-450 was eluted as a single peak, and the appropriate fractions were pooled and stored at −20 °C. When the P-450 was used in the experiments to be reported, Emulgen was removed by means of a column of hydroxylapatite (Nakajin & Hall, 1981).

Analytical Methods. Steroid C21-hydroxylation was measured by incubating the steroid substrate ([4-^{14}C]progesterone or 17α-hydroxy[4-^{14}C]progesterone: 10 nmol, 25000 cpm) in ethanol (20 μL) with cytochrome P-450 (20 pmol), P-450 reductase from adrenal microsomes (200 pmol), and potassium phosphate (50 mM; pH 7.2) in a final volume of 1.0 mL for 10 min at 37 °C. Following incubation the following carrier steroids (50 μg each) were added: progesterone and 11-deoxycorticosterone or the two corresponding 17α-hydroxy steroids. The incubation medium was extracted 3 times with methylene chloride. Extracts were taken to dryness and applied to thin-layer chromatograms developed in the system acetone–benzene (1:7 v/v). The product of the reaction was localized by means of a UV light, and ^{14}C was measured by liquid scintillation spectrometry. Electrophoresis in polyacrylamide gels was performed according to the method of Laemmli (1970). Protein was determined by the method of Lowry or by a suitable modification of this method when detergent was present (Dulley & Grieve, 1975). Methods for measuring heme and P-450 by CO difference followed standard procedures (Nakajin & Hall, 1981). ω-Amino-*n*-octyl-Sepharose 4B was prepared as described by Cuatrecasas (1970). P-450 reductase was purified from both pig liver and adrenal according to Yasukochi et al. (1979). Molecular weight determinations by gel filtration were performed in sodium phosphate buffer (0.1 M; pH 7.2) containing sodium dodecyl sulfate (2% w/v) and 2-mercaptoethanol (5% w/v). Samples were heated in boiling water for 2 min and then dialyzed overnight against the above buffer. Samples were applied and eluted on a column of Sephadex G-150 (1.6 × 60 cm) in the above buffer.

Amino Acid Analysis. Samples (5–10 μg) were reduced, ^{14}C-labeled S-carboxymethylated, and hydrolyzed in 6 N HCl containing 0.02% β-mercaptoethanol for 24, 48, and 72 h in sealed, evacuated tubes at 110 °C. Amino acid compositions were determined on a two-column Beckman 121MB analyzer according to Del Valle & Shively (1979). Peptides were hydrolyzed for 24 h.

Isolation of Cysteine-Containing Tryptic Peptides. One milligram of ^{14}C-labeled S-carboxymethylated protein was treated with TPCK-treated trypsin (1 mg/mL, 0.2 M ammonium bicarbonate, 24 h at 37 °C, enzyme to protein ratio 1/50 w/w). Soluble tryptic peptides were separated by HPLC on an Ultrasphere RP-8 column as described by Yuan et al. (1982a) by using a linear gradient from 100% solvent I (0.1% TFA) to 70% solvent II (0.1:9.9:90 = TFA–H_2O–CH_3CN). Insoluble peptides were further digested with *Staphylococcus aureus* V8 protease (1 mL of 0.2 M ammonium bicarbonate, 24 h at 37 °C, enzyme 5 μg). The final digest was completely soluble, and the resulting peptides were separated by HPLC on a SynChropak RP-P C-8 column (250 × 4.6 mm, 300 Å pore size) as described above. Peptides were detected by absorbance at 206 nm and manually collected, and 20 μL from each peak fraction was counted for radioactivity. Carboxymethylcysteine content in each radioactive peak fraction was quantitated by amino acid analysis.

NH_2-Terminal Sequence. Two to four nanomoles of protein or peptide were subjected to automated Edman degradations on an updated modified Beckman 890C sequencer as previously described by Shively (1981). S-PITC modification of protein was performed in the spinning cup according to Nakajin et al. (1981). PTH derivatives were identified by high-pressure liquid chromatography on an Ultrasphere (Altex) ODS column by using a Waters Associates chromatograph (Hawke et al., 1982).

COOH-Terminal Sequence. COOH-Terminal sequences of peptides were determined by time course analysis of amino acids released by carboxypeptidase Y. The amino acids were monitored as their *o*-phthaldialdehyde derivatives by HPLC according to the method of Jones et al. (1981).

Results

Cytochrome P-450 C21-Hydroxylase. The enzyme purified from pig adrenal microsomes by the method described here shows a single band on electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (Figure 1A). Good agreement is seen in the determination of molecular weight by electrophoresis and by chromatography on Sephadex with sodium dodecyl sulfate (Figure 1B); both methods gave a value of 54000. The immunochemistry of the 21-hydroxylase will be published in detail elsewhere when it will be shown that the enzyme gives a single band on double diffusion against immune IgG prepared from rabbit antiserum. The purified P-450 contains 0.8 mol of heme/mol of peptide. The spectral properties of the enzyme show a typical low-spin P-450, and the reduced P-450–CO complex shows an absorbance maximum at 450 nm. Other spectral properties of the enzyme are

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; S-PITC, 3-sulfophenyl isothiocyanate; IgG, immunoglobulin G.

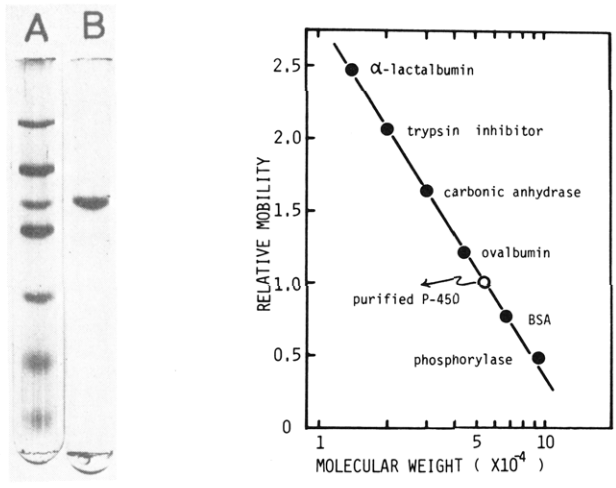


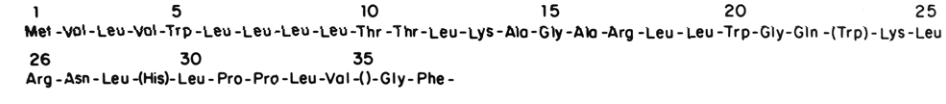
FIGURE 1: Gel electrophoresis (left) and column chromatography of adrenocortical microsomal steroid 21-hydroxylase (right). Electrophoresis on $NaDodSO_4$–polyacrylamide gels. Gel A shows the enzyme with six standard proteins (see below). Gel B shows 21-hydroxylase (3.1 μg). The gels were stained with Coomassie brilliant blue. Chromatography (right) of 21-hydroxylase (purified P-450) with six standard proteins on Sephadex G-150. The column was packed and eluted with a buffer containing 2% sodium dodecyl sulfate (w/v) (see Experimental Procedures).

not remarkable (not shown). This P-450 catalyzes C21 hydroxylation of progesterone and 17α-hydroxyprogesterone (K_m = 5.5 μM for both substrates). The disappearance of substrate in both cases was quantitatively accounted for by appearance of the corresponding 21-hydroxy steroid so that under the conditions used, no other reactions were catalyzed by this enzyme.

Amino Acid Analysis and NH_2-Terminal Sequence. The amino acid composition of porcine 21-hydroxylase is contrasted, in Table I, with the amino acid composition of the corresponding bovine enzyme and with that of the C21 side-chain cleavage enzyme (17α-hydroxylase/$C_{17,20}$-lyase) from porcine testicular microsomes. It is interesting to note that there is no obvious similarity in the compositions of the three proteins. Apparently there are significant interspecies differences in the 21-hydroxylase system. Moreover, the porcine 21-hydroxylase shows little similarity to the testicular C21 side-chain cleavage enzyme of the same species.

The NH_2-terminal sequence analysis for the 21-hydroxylase is shown in Figure 2. The sample (4 nmol) was treated with a 50-fold molar excess of S-PITC to retain the protein in the spinning cup. The derivative showed two sequences that were identical except that the minor sequence lacks the NH_2-terminal methionine. The two sequences showed yields of 1.3 and 0.5 nmol (total 45%). Apart from residue 35 (unidentified) and residue 23 (tentatively identified as tryptophan), 37 residues have been identified. Starting from residue 26 it was difficult to independently identify the two sequences, especially in view of the large number of leucine residues encountered. Thus, in this analysis residues 1–25 only could be identified with high confidence. Residues 26–37 were later confirmed by sequence analysis of a tryptic fragment (see later discussion) and are shown here for purpose of comparison. A second sequence determination was performed on 2 nmol of protein and gave a total yield of 48% with an identical sequence.

Cysteine-Containing Peptides. The HPLC tryptic map of the S-carboxymethylated protein is shown in Figure 3. Approximately 30 peptides were resolved by means of a linear program between 0.1% TFA and a mixture of TFA and CH_3CN. Three peptides containing cysteine were identified; the amino acid compositions and NH_2-terminal sequences of these peptides are shown in Tables II and III, respectively. The peptide map from *S. aureus* protease digest of tryptic core material is shown in Figure 4. Five additional cysteine-



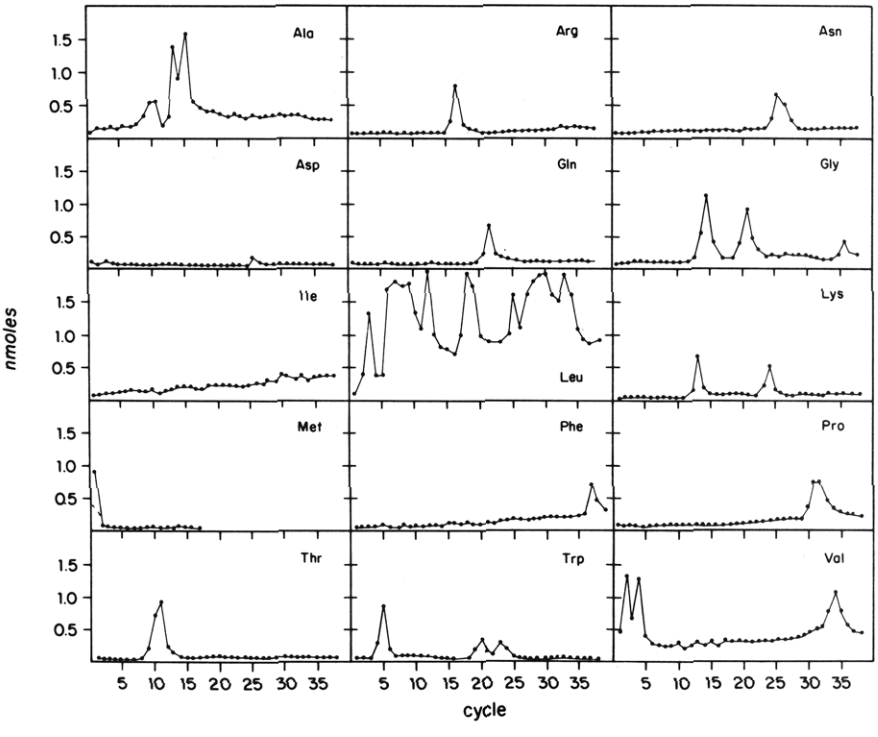


FIGURE 2: NH_2-Terminal amino acid sequence of steroid 21-hydroxylase (see Experimental Procedures for details).

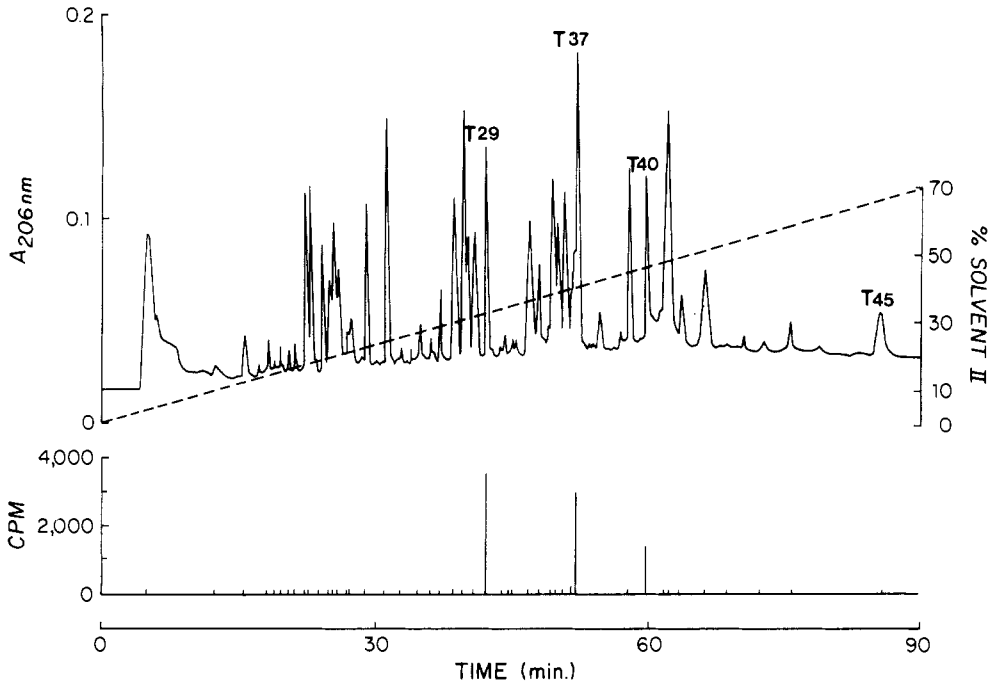


FIGURE 3: HPLC mapping of soluble tryptic peptides. Approximately 7 nmol of tryptic digest was spun down to remove the insoluble core material and loaded onto an Ultrasphere RP-8 column (250 × 4.6 mm, 5 μm particle size). A 90-min linear gradient program from 100% solvent I (0.1% TFA) to 70% solvent II (TFA–H_2O–CH_3CN = 0.1:9.9:90 v/v/v) at a flow rate of 0.9 mL/min. Fractions were manually collected, and 20 μL of aliquot from each peak fraction was counted for ^{14}C radioactivity as shown in the bottom panel.

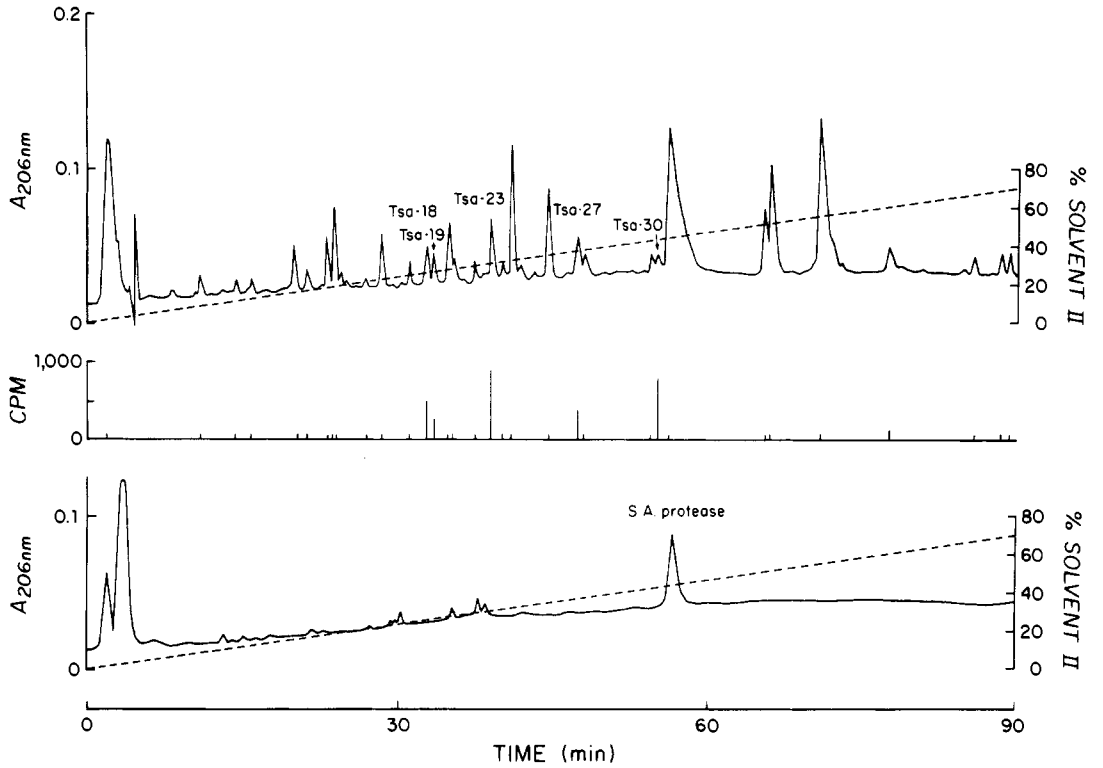


FIGURE 4: Peptide mapping of *S. aureus* protease digest of tryptic core material. Tryptic core material was digested with *S. aureus* protease and applied to SynChropak RP-P C-8 column (250 × 4.1 mm, 10 μm particle size). Chromatographic conditions were same as in Figure 3. Enzyme was also chromatographed under the same HPLC conditions as the control.

containing peptides (Tsa-18, Tsa-19, Tsa-23, Tsa-27, and Tsa-30) were obtained. The amino acid compositions and NH_2-terminal sequences of these peptides (Tables II and III) show that the Tsa-18 has the same sequence as Tsa-19 with the exception of the COOH terminus which has glutamic acid instead of glutamine. The amino acid sequence of these two peptides also overlapped with Tsa-27. COOH-terminal sequence analysis was performed enzymatically to complete the sequence of some peptides as shown in Table IV. These results account for nine cysteine residues, roughly twice that predicted by amino acid analysis. Evidently, quantitative S-carboxymethylation was not obtained, a commonly encountered problem with integral membrane proteins.

Comparisons with Other Cytochromes P-450. Figures 5 and 6 show homologies between the enzyme that forms the subject of this paper and other cytochromes P-450 from rat

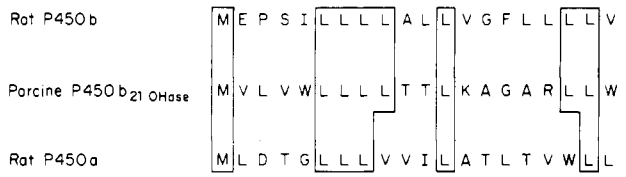


FIGURE 5: Comparison of NH_2-terminal sequences of porcine 21-hydroxylase and two rat liver cytochromes P-450. Sequences of P-450b and P-450a were taken from Botelho et al. (1979). Note that the variable presence of NH_2-terminal methionine for P-450b was described by Yuan et al. (1982b).

Table I: Comparative Amino Acid Compositions of Porcine and Bovine Adrenal Microsomal 21-Hydroxylase and Pig Testes Microsomal 17α-Hydroxylase/$C_{17,20}$-Lyase

	no. of residues/molecule		
amino acid	porcine 21-hydroxylase	porcine C21 side-chain cleavage P-450[a]	bovine 21-hydroxylase[b]
Asx	24	47	36
Thr	19	26	19
Ser	25	30	23
Glx	55	53	45
Pro	37	43	23
Gly	38	38	25
Ala	29	38	28
Val	21	33	21
Met	8	13	9
Ile	16	25	19
Leu	77	62	50
Tyr	8	14	11
Phe	16	29	25
Lys	16	32	24
His	17	12	11
Arg	32	30	25
Trp	1[c] (7)[e]	4	2
Cys	4[d] (9)[e]	6	4
total	454	535	400

[a] Data reported by Nakajin et al. (1981). [b] Data reported by Hiwatashi & Ichikawa (1981). [c] Low yield due to destruction of Trp on HCl hydrolysis. [d] Determined as *S*-carboxymethyl derivative. [e] Determined from preliminary sequence data.

liver and that from *Pseudomonas putida* ($P\text{-}450_{cam}$) with respect to NH_2-terminal sequences and cysteine-containing peptides, respectively. It is clear that Tsa-23 and Tsa-30 show

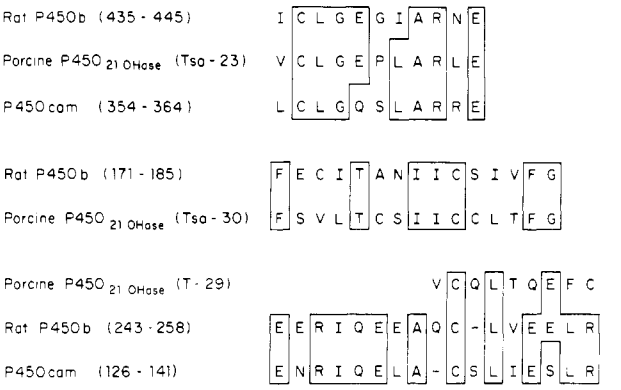


FIGURE 6: Comparison of cysteine-containing peptides from the 21-hydroxylase of porcine adrenal cortex microsomes, rat liver P-450b (Fujii-Kuriyama et al., 1982; Yuan et al., 1982b), and $P\text{-}450_{cam}$ from *Pseudomonas putida* (Haniu et al., 1982).

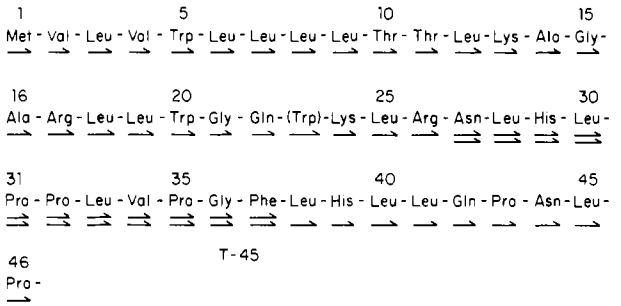


FIGURE 7: Extended NH_2-terminal amino acid sequence of steroid 21-hydroxylase by overlapping with one tryptic fragment sequence T-45.

significant homologies to the cysteine-containing peptides from P-450b and $P\text{-}450_{cam}$.

Extended NH_2-Terminal Sequence of 21-Hydroxylase. Table V shows the partial sequence of a hydrophobic tryptic peptide T-45 which overlaps with the NH_2-terminal sequence of protein. When the sequence result shown in Figure 2 is overlapped, an extended NH_2-terminal sequence of 21-hydroxylase was obtained through residues 46 (Figure 7) which contributes approximately 10% of the polypeptide chain. Only six basic amino acid residues appeared in this region; the rest of them were either hydrophobic or neutral residues. Sixteen leucines were found which contributed more than 30% of the amino acid residues in the amino-terminal region.

Table II: Amino Acid Compositions of Cysteine-Containing Peptides

amino acid	T-29	T-37	T-40	Tsa-18	Tsa-19	Tsa-23	Tsa-27	Tsa-30
Asx		1.01 (1)	2.22 (2)	2.06 (2)	2.11 (2)		2.38 (2)	
Thr	0.83 (1)[b]			0.50 (1)	0.66 (1)		1.08 (1)	1.34 (2)
Ser		1.07 (1)	2.43 (3)				0.91 (1)	1.62 (2)
Glx	3.75 (4)		1.31 (1)	1.09 (1)	1.15 (1)	2.13 (2)	1.36 (1)	1.31 (1)
Pro		1.88 (2)	0.88 (1)			1.11 (1)		
Gly		2.70 (3)	1.36 (1)			1.03 (1)	1.35 (1)	1.36 (1)
Ala		3.70 (5)	1.13 (1)	0.63 (1)	0.96 (1)	1.00 (1)	1.14 (1)	
Val	0.51 (1)			1.13 (2)	0.94 (2)	0.73 (1)	2.00 (2)	0.97 (1)
Met							0.23 (1)	
Ile			0.73 (1)				0.63 (1)	0.90 (2)
Leu	1.15 (1)	1.55 (2)	2.46 (3)	1.38 (2)	1.97 (2)	2.66 (3)	2.59 (3)	1.92 (2)
Tyr			0.83 (1)					
Phe	0.74 (1)	1.31 (2)	0.89 (1)					1.38 (2)
Lys			0.85 (1)				1.29 (1)	1.36 (1)
His			0.88 (1)	1.21 (2)	1.22 (1)		2.00 (2)	
Arg	1.14 (1)	1.21 (1)				0.78 (1)		
Trp			0.48 (1)					trace (1)
Cys[a]	0.88 (2)	0.50 (1)	0.88 (1)	0.30 (1)	0.40 (1)	0.33 (1)	0.48 (1)	1.18 (3)
total	11	18	19	12	12	11	18	18

[a] Carboxymethylcysteine derivative. [b] Values in parentheses were obtained by sequence analyses.

Table III: Amino Acid PTH Derivatives

cycle	T-29 (3.5 μg)	T-37 (5.1 μg)	T-40 (3.1 μg)	Tsa-18 (1.0 μg)	Tsa-23 (1.4 μg)	Tsa-30 (1.0 μg)
1	Val (865)[a]	Phe (1526)	Leu (279)	Asp (76)	Val (573)	Phe (220)
2	CmCys (928)	Leu (1240)	Ala (271)	Thr (48)	CmCys (274)	Ser (45)
3	Gln (1070)	Ala (1339)	Ser (17)	Leu (214)	Leu (638)	Val (119)
4	Leu (753)	Pro (752)	Gln (119)	Val (237)	Gly (335)	Leu (179)
5	Thr (646)	Gly (867)	His (15)	His (69)	Glu (328)	Thr (30)
6	Gln (489)	Ala (1186)	CmCys (16)	Ala (151)	Pro (144)	CmCys (48)
7	Glu (399)	Asn (1064)	Pro (94)	Leu (108)	Leu (340)	Ser (29)
8	Phe (338)	Pro (422)	Asp (29)	His (22)	Ala (278)	Ile (83)
9	CmCys (338)	Ser (277)	Ile (201)	Asp (30)	Arg (58)	Ile (147)
10	Glu (392)	Ala (879)	Ser (10)	CmCys (40)	Leu (187)	CmCys (32)
11	Arg (102)	Leu (997)	Leu (105)	Val (74)	Glu (62)	CmCys (65)
12		Ala (970)	Gly (34)	Gln (24)		Leu (103)
13		Phe (982)	Asp (10)			Thr (17)
14		Gly (645)	Tyr (84)			Phe (132)
15		CmCys (571)	Ser (5)			Gly (36)
16		Gly (447)	Leu (64)			ND[b]
17		Ala (653)	Phe (27)			Lys (31)
18		Arg (366)				Glu (31)

[a] Values in parentheses are in picomoles. [b] ND, not determined.

Table IV: COOH-Terminal Sequence Analysis of Cysteine-Containing Peptides[a]

peptide	sequence
T-29	Glu-Arg
T-37	Ala-Arg
T-40	Leu-Phe-Trp-Lys
Tsa-18	Val-Gln
Tsa-23	Ala-Arg-Leu-Glu
Tsa-27	(Lys, Gly)-Leu-Met

[a] Approximately 1 nmol of each peptide was treated with carboxypeptidase Y (1/100 enzyme to substrate molar ratio and 5% of sample aliquot was analyzed at $T = 0$ min, $T = 10$ min, $T = 20$ min, $T = 60$ min, and $T = 24$ h by the precolumn Fluoropa derivatization method with HPLC (Jones et al., 1981).

Table V: Amino-Terminal Sequence Analysis of T-45

	T-45 (6.4 μg)	
	amino acid PTH derivative	recovery (pmol)
1	Asn	223
2	Leu	385
3	His	75
4	Leu	225
5	Pro	195
6	Pro	199
7	Leu	373
8	Val	268
9	Pro	127
10	Gly	122
11	Phe	150
12	Leu	151
13	His	58
14	Leu	251
15	Leu	175
16	Gln	95
17	Pro	60
18	Asn	56
19	Leu	97
20	Pro	23

Discussion

The NH_2-terminal sequence data are consistent with the results of electrophoresis in polyacrylamide gels and Ouchterlony double diffusion in demonstrating homogeneity of the porcine 21-hydroxylase. Since the two sequences (with and without NH_2-terminal methionine) are identical except for the methionine and since all 34 of the identified residues were analyzed as single amino acids, it is extremely likely that the enzyme has been prepared in a homogeneous form.

The presence of two NH_2-terminal sequences identical except that one lacks an NH_2-terminal methionine has been reported for one P-450 from rat liver (P-450b) (Yuan et al., 1982b) and one P-450 from rabbit liver (LM_{3b}) (Ozols et al., 1981). In addition, the C21 side-chain cleavage P-450 and three cytochromes P-450 from rat liver (a, b, and e) have NH_2-terminal methionine. In the case of P-450b, cell-free translation studies yield a protein with NH_2-terminal methionine (Bar-Nun et al., 1980). It would appear that microsomal cytochromes P-450 commonly possess an NH_2-terminal methionine. Presumably the NH_2-formyl group is removed enzymatically which permits analysis of the unblocked proteins. In some cases proteolytic cleavage of the enzyme partly or completely removes the NH_2-terminal amino acid (methionine). The findings of Bar-Nun et al. (1980) make it likely that the true NH_2-terminal amino acid in vivo is methionine so that, at least for P-450b, proteolysis is confined to the NH_2-terminal methionine; there is therefore no reason to believe that additional residues are also missing from the purified protein. In addition, the extemely hydrophobic sequences at the NH_2 terminus of the cytochromes P-450 so far studied suggest that these enzymes begin with a signal peptide for insertion into the microsomal membranes (Blobel & Dobberstein, 1975).

Comparison between the porcine testicular microsomal C21 side-chain cleavage P-450 and the present enzyme (21-hydroxylase from porcine adrenal microsomes) is instructive. The enzymes have been purified from one species (pig) and from the same organelle (microsomes). Moreover, both enzymes use progesterone as substrate. In spite of this there is little homology between the proteins in NH_2-terminal amino acid sequence [see Results and Nakajin et al. (1981)].

Interest in the peptides that contain cysteine arises from evidence that the heme iron of cytochromes P-450 is thiolated presumably through a cysteine [for review of the evidence, see White & Coon (1980)]. Since the fundamental spectral properties common to all cytochromes P-450 are largely attributed to this attachment, it is presumed to be present in all of these enzymes. Moreover, this cysteine attached to the heme must form part of the active sites of these enzymes. In addition, as the basis for comparisons between different cytochromes P-450, regions involving cysteine disulfide bonds are frequently important for tertiary structure and for this reason are likely to be conserved during evolution. It will be of interest to determine whether any of the peptides shown in Figure 6

show homology with similar peptides from other steroidogenic cytochromes P-450.

Homologies between the 21-hydroxylase and nonsteroidogenic cytochromes P-450 are also of interest. Figure 5 shows some homology between the present enzyme and two cytochromes P-450 from rat liver. Homology with P-450b is significant in view of the observation by Hiwatashi & Ichikawa (1981) that immunological cross-reactivity was seen between bovine adrenal 21-hydroxylase and liver P-450_{BPA}.

It would be premature to attempt any general interpretation of these various similarities and differences. It is, however, clear that sequence information is likely to offer a major contribution to resolving the structure–function relations seen in these interesting enzymes.

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Registry No. Steroid 21-hydroxylase, 9029-68-9; cytochrome P-450, 9035-51-2.

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