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Purification and Characterization of Two Unique Forms of Cytochrome P-450 from Rabbit Nasal Microsomes†

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ABSTRACT: Two forms of cytochrome P-450, designated P-450NMa and P-450NMb, were purified to electrophoretic homogeneity from rabbit nasal microsomes. The purified cytochromes, which contained 14–16 nmol of P-450/mg of protein, exhibited apparent monomeric molecular weights of 49 500 and 51 000, respectively. As indicated by several criteria, including the amino acid composition, absorption spectra, and peptide maps, the two nasal forms of P-450 are distinct from each other. Furthermore, as judged by the NH_2-terminal amino acid sequences, they are distinct from all other P-450 cytochromes described to date. In the ferric form, P-450NMa is in the low-spin state, whereas P-450NMb is predominantly in the high-spin state. When reconstituted with NADPH–cytochrome P-450 reductase and phospholipid, P-450NMa is very active in the oxidation of ethanol as well as several nasal procarcinogens, including the N-deethylation of *N*-nitrosodiethylamine, the O-deethylation of phenacetin, and the N-demethylation of hexamethylphosphoramide. P-450NMb also metabolizes these substrates, but at lower rates. Both nasal forms are also active with testosterone, with P-450NMa oxidizing the substrate in the 17-position to give androstenedione and P-450NMb catalyzing hydroxylation in the 15α-, 16α-, and 19-positions. The two cytochromes represent the major portion of the total P-450 in nasal microsomes, but the corresponding forms could not be detected in hepatic microsomes.

The involvement of cytochrome P-450 in the oxidative metabolism of numerous xenobiotics as well as endogenous substances is well recognized. As reviewed recently (Black & Coon, 1986, 1987), more than 60 P-450s have been purified to electrophoretic homogeneity from various species, mostly from liver microsomes. The tissue-specific distribution of some of these isozymes has been established. For example, P-450 isozymes that are immunochemically indistinguishable from form 3a, the alcohol-inducible rabbit liver microsomal cytochrome P-450, have been identified in microsomal preparations of rabbit kidney and nasal mucosa, but not in other tissues, including adrenal, brain, heart, intestine, lung, ovary, spleen, testis, and uterus (Ding et al., 1986). Most P-450s that oxygenate xenobiotics have some distinguishing and some overlapping substrate specificities, and the genetically determined level of each of the expressed forms is at least partly responsible for the tissue-specific toxicity of many foreign compounds.

The mammalian olfactory tissue functions to detect odorants as well as pheromones (Gower et al., 1981). To fulfill these functions, it is constantly exposed to the external environment and is one of the major targets for the toxicity of many airborne xenobiotics. The specific content of P-450 in nasal microsomes is relatively high, being second only to that of hepatic microsomes among all rabbit tissues examined (Ding et al., 1986), and is higher in the olfactory mucosa than in the respiratory mucosa (Dahl et al., 1982). The P-450-dependent metabolism of a variety of substances in nasal mucosa (Ding et al., 1986; Brittebo, 1982, 1987; Hadley & Dahl, 1983; Dahl & Hadley, 1983; Brittebo et al., 1983; Löfberg & Tjälve, 1984; Brittebo & Rafter, 1984; Dahl & Brezinski, 1985; Reed et al., 1986; Longo et al., 1986) may be related to the ability of the organism to maintain olfactory sensitivity (Dahl et al., 1982) as well as to the susceptibility of this particular tissue to the toxic effects of many foreign compounds (Dahl & Hadley, 1983). A striking feature of the monooxygenase system of the nasal mucosa is that its catalytic activity, expressed as the turnover number for total P-450, is much higher than that of hepatic microsomes with many substrates, such as butanol (Ding et al., 1986), hexamethylphosphoramide (HMPA)[1] (Dahl & Brezinski, 1985), or ethoxycoumarin (Reed et al., 1986). The higher concentration of NADPH–cytochrome P-450 reductase in rabbit and rat nasal microsomes as compared to liver microsomes (Ding et al., 1986;

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[1] Abbreviations: HMPA, hexamethylphosphoramide; NDEA, *N*-nitrosodiethylamine; PA, phenacetin; PEG, poly(ethylene glycol); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P-450NM and -LM, nasal microsomal and liver microsomal cytochrome P-450, respectively; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IgG, immunoglobulin G.

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Reed et al., 1986) may contribute to the magnitude of these activities, as may the presence of tissue-specific forms of P-450.

In the present paper, evidence is presented for the occurrence of two unique forms of rabbit nasal microsomal cytochrome P-450, designated NMa and NMb, and procedures for the purification of these constitutive isozymes are described. A variety of techniques, including amino acid analysis, NH_2-terminal sequence determination, spectral analysis, and peptide mapping, have been employed to identify these cytochromes as distinct forms. A comparison of the catalytic properties of the two purified nasal forms with those of previously purified P-450s from liver microsomes indicates that P-450NMa is the most active toward two compounds previously shown to be nasal procarcinogens, NDEA (Lijinsky, 1984) and PA (Isaka et al., 1979), and that both nasal forms are highly active toward a third nasal procarcinogen, HMPA (Lee et al., 1981), as well as toward ethanol, butanol, and testosterone. In addition, evidence is presented for the partial purification and identification of a nasal homologue of P-450 form 3c.

Materials and Methods

Spectral Analyses. The concentration of cytochrome P-450 in microsomal suspensions and in crude fractions was determined from the CO difference spectrum of the reduced protein with use of an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference between the maximum absorbance in the 450-nm region and at 490 nm (Omura & Sato, 1964). Heme was determined as the reduced pyridine hemochrome (Antonini & Brunori, 1971), and the concentration of the purified hepatic isozymes was determined from the absolute spectra of the ferrous carbonyl complexes as previously described (Koop et al., 1982). All spectra were recorded with a Cary 219 spectrophotometer.

Amino Acid Analysis and Sequence Determinations. Samples of enzyme were precipitated in 1.5–mL microtubes by the addition of cold acetone to a final concentration of 70% (v/v). The precipitates were washed with 50% acetone, dried in vacuo, dissolved in 88% formic acid, and then subjected to manual Edman degradation (Tarr, 1982) or to analysis with use of an Applied Biosystems Model 470A gas-phase protein sequencer (Hewick et al., 1970). Cysteine residues were determined after reduction with dithiothreitol and alkylation by 4-vinylpyridine (Friedman et al., 1970; Tarr et al., 1983). The amino acid composition of purified NMa and NMb was determined according to the procedures described by Tarr (1986). The amino acid and sequence analyses were performed in the University of Michigan Protein Sequencing Facility.

Peptide Maps. Following proteolysis, peptide maps were prepared according to the procedure of Cleveland et al. (1977) as follows. Purified hepatic and nasal cytochromes were dialyzed against 10 mM Tris-HCl buffer, pH 6.8, containing 0.2% SDS, and boiled for 5 min. Aliquots of 50 μL each (containing approximately 2 μg of protein) were added to incubation mixtures that contained 0.75 μg of *Staphylococcus aureus* V_8 protease and 0.1% SDS in a final volume of 100 μL. The mixtures were incubated at 37 °C for 10 h, boiled for 3 min to stop the reaction, lyophilized, resuspended in 40 μL of SDS–PAGE sample buffer (Laemmli, 1970), and submitted to electrophoresis on a 12.5% acrylamide gel as described below. Control experiments were run in which the protease or the P-450 was omitted from the mixture.

Gel Electrophoresis. Polyacrylamide slab gel electrophoresis was carried out with use of the discontinuous buffer system of Laemmli (1970). The slab separating gel (9.0 × 17.5 × 0.075 cm) contained 7.5% or 12.5% acrylamide with 0.20% or 0.34% bis(acrylamide) cross-linking, respectively. After electrophoresis, the gels were fixed in 50% (v/v) methanol and stained with silver according to Wray et al. (1981).

Catalytic Activities. Enzyme assays were carried out for lengths of time that represented the initial linear rate of product formation. The formaldehyde formed from HMPA N-demethylation was measured by a fluorometric modification (Belman, 1963) of the method of Nash (1953), and the formation of acetaldehyde from O-deethylation of PA or N-deethylation of NDEA was determined by gas chromatography of the headspace gas of the reaction mixture as described previously for alcohol oxidation assays (Koop et al., 1984). The oxidation of ethanol and butanol was also assayed as described earlier by this laboratory.

For the oxidation of testosterone, the assay was carried out essentially as described by Wood et al. (1983). Testosterone metabolites were analyzed by high-pressure liquid chromatography by a modification of the procedure described by van der Hoeven (1981), using a 5-μm octadecylsilane reverse-phase column obtained from Supelco (150 × 4.6 mm i.d.) and preceded by a guard column containing octadecylsilane (40-μ particles). The metabolites were eluted with a methanol/water/acetonitrile mobile phase as described. A second system was used to confirm the identification of the reaction products, with a Waters 5-μm octadecylsilane reverse-phase column (150 × 4.6 mm i.d.) and elution by a tetrahydrofuran/water mobile phase (Wood et al., 1983). All analyses were performed at room temperature with a Waters Model 600 multisolvent delivery system, and column effluents were monitored at 254 nm with a Waters Model 490 multiwavelength detector and a FLO-ONE/Beta Model CT radioactivity flow detector (Radiomatic Instruments). The metabolites were quantitated by determination of the radioactivity of the individual peaks.

Other Methods. Hepatic P-450 cytochromes were purified to electrophoretic homogeneity according to published procedures (Coon et al., 1978; Koop et al., 1981, 1982; Koop & Coon, 1984), from untreated rabbits (for the isolation of LM_{3b}) or from rabbits treated with phenobarbital (for LM_2), ethanol (for LM_{3a}), isosafrole (for LM_4), or imidazole (for LM_{3c} and LM_6). The specific contents of the preparations ranged from 16 to 20 nmol of P-450/mg of protein. The NADPH–cytochrome P-450 reductase purified from microsomes obtained from phenobarbital-treated rabbits (French & Coon, 1979) had a specific activity of 45–60 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$.

Materials. NADPH, S-Sepharose, PEG 8000, NDEA, HMPA, and PA were obtained from Sigma. Dilauroylglyceryl-3-phosphocholine was from Calbiochem, Bio-Gel HTP was from Bio-Rad, CHAPS was from Pierce, and Polybuffer 74, Polybuffer 96, and Polybuffer Exchanger 94 were from Pharmacia. Testosterone and [4-^{14}C]testosterone were obtained from Aldrich and New England Nuclear, respectively. 15β-Hydroxytestosterone was a gift from Dr. T. A. van der Hoeven (Albany Medical College), and 15α-hydroxytestosterone was a gift from The Upjohn Co. 7α-, 16β-, and 19-hydroxytestosterone and 16-ketotestosterone were obtained from Steraloids, Inc. (Wilton, NH), and 6β-, 11β-, and 16α-hydroxytestosterones and androstenedione were from Sigma.

Purification of Cytochromes P-450NMa and -NMb. Frozen tissues containing the nasal area from adult New Zealand White male rabbits were obtained from Pel-Freez Biologicals (Rogers, AR). The tissues were allowed to thaw at 4 °C for about 2 h in 0.1 M Tris–acetate buffer, pH 7.4, containing 0.15 M KCl and 1 mM EDTA. The nasal ethmoturbinoids and septum were then removed by dissection, pooled, and homogenized with a Brinkman Polytron Model PT 10/35

homogenizer, and pyrophosphate-washed microsomes were prepared as previously described (Coon et al., 1978).

The pooled nasal microsomes from 180 animals were suspended in 0.1 M Tris–acetate buffer, pH 7.4, containing 0.1 M KCl, 1 mM EDTA, and 20% glycerol at a protein concentration of 3.5 mg/mL. Tergitol NP-10 and CHAPS were then added as 10% (w/v) solutions to final concentrations of 0.3% and 0.5%, respectively. The mixture was stirred for 1 h at 4 °C and fractionated with PEG 8000 as described earlier (Coon et al., 1978). The fraction precipitating from 7% to 14% PEG was suspended in 25 mM Tris–acetate buffer, pH 8.3, containing 0.1 mM EDTA and 20% glycerol (buffer A) and dialyzed overnight against 200 volumes of the same buffer. Unless otherwise indicated, the following fractionation procedures and pH measurements were conducted at 4 °C, all buffers contained 20% glycerol, and phosphate buffers were prepared as the potassium salts.

The 7–14% PEG fraction (containing 188 nmol of P-450 and having a protein concentration of 4.0 mg/mL) with Tergitol and CHAPS added to final concentrations of 0.6% and 1.0%, respectively, was applied to a Polybuffer Exchanger 94 column (1.5 × 48 cm) previously equilibrated with buffer A containing 0.6% Tergitol NP-10. Elution was carried out at a flow rate of 25 mL/h with a mixture of Polybuffer 96 and Polybuffer 74 (final concentrations 3% and 7%, respectively, v/v) adjusted to pH 6.0 with acetic acid and containing 0.6% Tergitol and 1.0% CHAPS, and 4.3-mL fractions were collected. The concentration of heme proteins was estimated by the 417-nm absorbance, and the samples were pooled into two fractions as shown in Figure 1a. Fraction I, which represented about 30% of the P-450 applied to the Exchanger column, was enriched in P-450NMa, and fraction II, which represented about 25% of the P-450, was enriched in P-450NMb as determined by SDS–PAGE. The two small peaks between fractions I and II were contaminated with hemoglobin as determined spectrally, and the shoulder in the elution profile after fraction II represented about 7% of the applied P-450. The chromotofocusing procedure used is a modification of the system described by Marriage and Harvey (1986) for the resolution of mouse hepatic P-450 isozymes.

P-450NMa was isolated as follows. Fraction I was concentrated to 10 mL by ultrafiltration with an Amicon PM-30 membrane, dialyzed overnight against 10 mM phosphate buffer, pH 7.4, containing 0.1% NP-10 and 0.01 mM EDTA (buffer B), and applied to a Bio-Gel HTP column (0.7 × 7.6 cm) previously equilibrated with buffer B. The column was washed at a rate of 20 mL/h with 6 mL of buffer B and then with 30 mL of buffer B with the phosphate buffer increased to 30 mM to remove Polybuffer, and protein was eluted with 200 mM phosphate buffer, pH 7.4, containing 0.2% Tergitol NP-10 and 0.01 mM EDTA. The eluate was dialyzed against 20 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA (buffer C), and Tergitol NP-10 was added as a 10% (v/v) solution to a level of 1 μmol of the detergent/nmol of P-450. The solution (approximately 42 nmol of P-450 in 14 mL) was then applied to a CM-Sepharose column (0.7 × 15 cm) previously equilibrated with 20 mM phosphate buffer, pH 7.4, containing 0.2% Tergitol NP-10 and 1.0 mM EDTA (buffer D), and the column was washed with 25 mL of buffer D, followed by elution of the protein by a 110-mL linear gradient from 20 to 120 mM phosphate in buffer D at a flow rate of about 8 mL/h. P-450 form 3c (NM3c) was eluted at approximately 35 mM phosphate to give fraction A (see Figure 1b), which is consistent with the results reported previously for the purification of P-4503c from liver microsomes (Koop

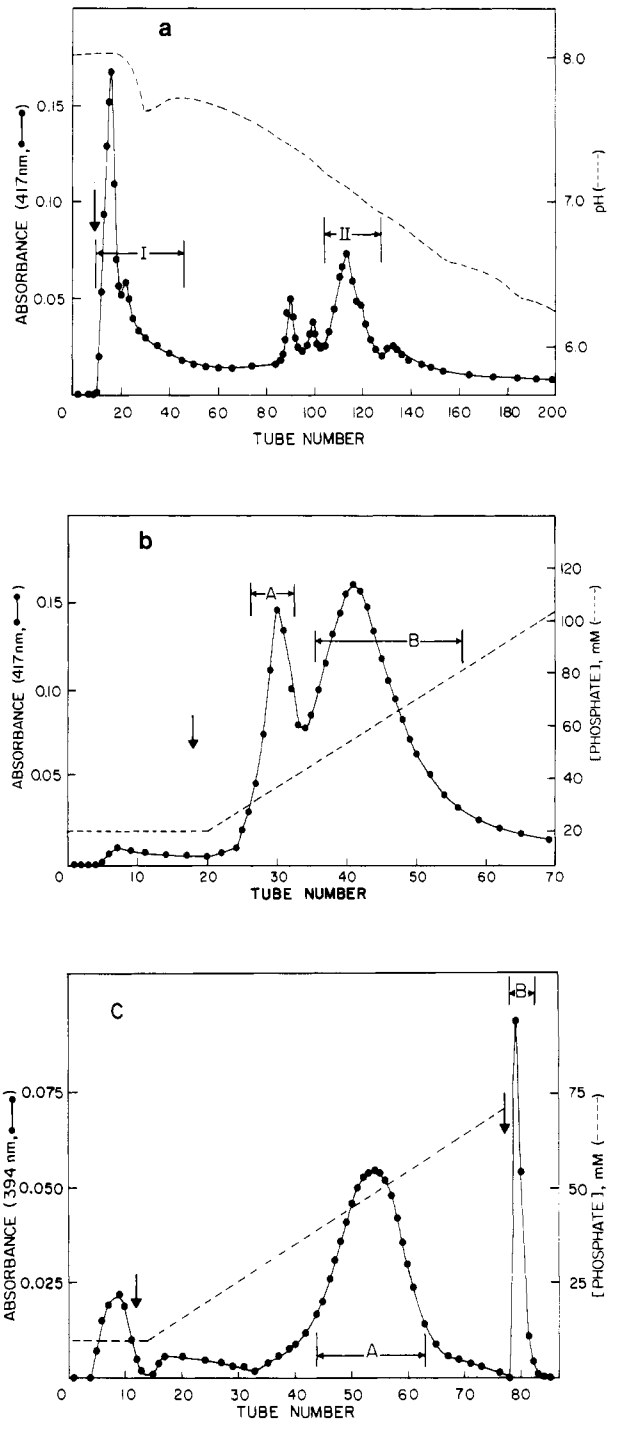

FIGURE 1: Column chromatography of hemoproteins in the purification of P-450NMa and P-450NMb. (a) Profile of cytochromes eluted from the Polybuffer Exchanger column during chromatofocusing at 4 °C, with the pH gradient determined subsequently at 22 °C. Elution with Polybuffer began as indicated by the *vertical arrow*. Fractions I and II, obtained by pooling samples as indicated by the *vertical lines*, were used for subsequent purification of P-450NMa and -NMb, respectively. (b) Profile of cytochromes eluted from the CM-Sepharose column. The gradient elution began as indicated by the *vertical arrow*. Fractions were collected (2 mL/tube) and analyzed for absorbance at 417 nm. Aliquots of the various fractions were analyzed by SDS–PAGE and pooled into fractions A (P-450NM3c-rich) and B (P-450NMa-rich) as indicated by the *vertical lines*. (c) Profile of cytochromes eluted from the HTP column. Phosphate gradient elution began at the first *vertical arrow*, and elution with 500 mM phosphate began at the second *vertical arrow*. The elution profile was monitored by the absorbance at 394 nm rather than 417 nm, because P-450NMb is a high-spin form.

Table I: Purification of P-450NMa and P-450NMb from Nasal Microsomes of Untreated Rabbits

preparation	protein (mg)	cytochrome P-450 content (nmol/mg of protein)			yield (%)
		P-450NM	P-450NMa	P-450NMb	
pyrophosphate-treated microsomes	857	0.6			100
PEG precipitate (7–14%)	145	1.3			37
Polybuffer Exchanger column eluate, fraction I	9.9	5.6			10.8
CM-Sepharose column eluate					
fraction A[a]	0.7	7.3			1.0
fraction B	2.3	8.7			3.9
S-Sepharose column eluate	1.8	9.7			3.4
first hydroxylapatite–cellulose column eluate	1.3	10.7			2.7
second hydroxylapatite–cellulose column eluate	0.87		14.1[b] (NMa)		2.4
fraction II	7.4	6.3			9.1
HTP column eluate, fraction A	2.6	12.3			6.2
CM-Sepharose column eluate	1.7	13.4			4.4
hydroxylapatite–cellulose column eluate	1.3			16.4[b] (NMb)	4.1

[a] Fraction A, which was enriched in P-450 form 3c, was not used for further purification of P-450NMa. [b] The values for the purified proteins are based on the heme content. All other values are based on the determination of P-450 as the CO complex.

et al., 1981), whereas P-450NMa was eluted at about 55 mM phosphate to give fraction B. The latter fraction, which represented 48% of the P-450 applied to the CM-Sepharose column, was dialyzed and concentrated in a Micro-ProDiCon apparatus containing buffer C, resolubilized by the addition of 10% Tergitol to a level of 1 μmol/nmol of P-450, and applied to an S-Sepharose column (0.7 × 14 cm) previously equilibrated with buffer D in which the Tergitol NP-10 had been increased to 0.3% (buffer E). The S-Sepharose column was washed with 12 mL of buffer E to remove residual P-450 forms 2 and 3c, and additional protein was eluted with a 110-mL linear gradient from 0 to 200 mM KCl in buffer E at a rate of 10 mL/h. The eluate obtained at about 65 mM KCl exhibited, upon SDS–PAGE analysis, P-450NMa as the predominant band, with only a few other bands in the higher molecular weight region.

The P-450NMa-rich fractions were dialyzed against buffer B and applied to a hydoxylapatite–cellulose (1:1, w/w) column (0.7 × 1.7 cm) previously equilibrated with buffer B containing 0.2% Tergitol NP-10 (buffer F). The column was washed with 10 mL of buffer F containing 30 mM phosphate and then eluted with a 110-mL linear gradient from 45 to 200 mM phosphate in buffer F at a flow rate of 10 mL/h. P-450NMa, which was eluted at a phosphate concentration of approximately 80 mM, was homogeneous as judged by SDS–PAGE. The eluate was dialyzed against buffer F and applied to a second hydroxylapatite–cellulose column (0.7 × 1.6 cm) to remove the detergent. The column was washed with 5 mL of buffer F containing 30 mM phosphate, followed by a sufficient amount of the same buffer without detergent so that the absorbance of the eluate at 276 nm was less than 0.01. The detergent-free enzyme was then eluted with 250 mM phosphate buffer, pH 7.4, containing 0.1% sodium cholate and 0.01 mM EDTA, and the preparation was dialyzed against 100 mM phosphate buffer, pH 7.4, containing 0.05 mM EDTA, to remove the cholate. The final preparation had a specific content of 14.1 nmol of P-450/mg of protein, indicating some loss of heme during the purification process.

For the purification of P-450NMb, fraction II eluted from the chromatofocusing column was concentrated and dialyzed as described above for fraction I (see Figure 1a), and an aliquot of 7.5 mL (approximately 24 nmol of P-450) was applied to a Bio-Gel HTP column (0.7 × 13 cm) previously equilibrated with buffer F. The column was washed with 10 mL of buffer F, followed by a 110-mL linear gradient from 10 to 70 mM phosphate in buffer F at 5 mL/h. P-450NMb was eluted at a phosphate concentration of approximately 50 mM (fraction A, Figure 1c). Further treatment of the column with 500 mM phosphate buffer, pH 7.4, containing 0.01 mM EDTA, gave fraction B that was enriched in P-450 form 3a as determined by SDS–PAGE and immunoblot analysis with a monoclonal anti-P-450 3a antibody (Ding et al., 1986) (data not shown). The P-450NMb-rich fraction, which represented about 70% of the P-450 applied to the Bio-Gel HTP column, was dialyzed against 10 mM phosphate buffer, pH 6.0, containing 0.1% Tergitol NP-10 and 0.1 mM EDTA, and Tergitol was than added to the preparation to a final concentration of 0.5%. The sample was then applied to a CM-Sepharose column (0.7 × 15 cm) previously equilibrated with 10 mM phosphate buffer, pH 6.0, containing 0.5% Tergitol NP-10 and 1.0 mM EDTA (buffer G), and the column was washed with 30 mL of buffer G containing 50 mM phosphate, followed by elution with a 110-mL linear gradient from 50 to 200 mM phosphate in buffer G at a flow rate of about 10 mL/h. P-450NMb, which was eluted at a phosphate concentration of approximately 100 mM, was homogeneous as determined by electrophoretic analysis. The enzyme preparation was then dialyzed against buffer F and applied to a hydroxylapatite–cellulose column to remove Tergitol NP-10 as described above for P-450NMa, except that the column was washed with 10 mM instead of 30 mM phosphate buffer. The final preparation had a specific content of 16.4 nmol of P-450/mg of protein.

Results

Purification of P-450NMa and P-450NMb. A summary of the purification of cytochromes P-450NMa and P-450NMb from nasal microsomes of untreated rabbits is shown in Table I. In the typical experiment shown, the overall yield based on starting total P-450 was 2.4% for NMa and 4.1% for NMb. In view of the generally low yields obtained in the purification of such membrane proteins and the occurrence of only trace amounts of known P-450s in this tissue, it appears that these two isozymes are the major forms of P-450 in nasal microsomes of untreated rabbits. The SDS–PAGE profile of various P-450 preparations obtained during the purification is shown in Figure 2, experiment 1. Three major bands appear in the P-450 region of nasal microsomes (lane e). The two lower bands, which correspond to NMa and NMb, were completely separated by chromatofocusing as shown in Figure 1a, resulting in fractions I (lane f) and II (lane d). Further purification of fraction I on a CM-Sepharose column resolved forms NMa (lane g) and a form tentatively called NM3c (lane h) because of the similar electrophoretic mobility of the latter to that of hepatic isozyme 3c (lane i). Subsequent NH_2-terminal se-

Table II: Amino Acid Analysis of Purified Cytochromes

amino acid	no. of residues/molecule of P-450		amino acid	no. of residues/molecule of P-450	
	P-450NMa	P-450NMb		P-450NMa	P-450NMb
Asx	24	29	Leu	45	48
Thr	32	28	Tyr	11	13
Ser	31	28	Phe	41	32
Glx	38	37	His	11	21
Pro	27	31	Lys	30	28
Gly	34	41	Arg	38	38
Ala	26	22	$Cys(O_3H)$	5	6
Val	25	29	Trp	3	3
Met	17	11	total	458	471
Ile	20	26			

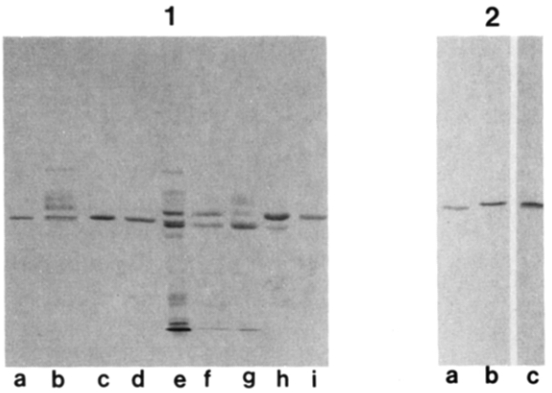

FIGURE 2: SDS–PAGE of nasal microsomes and various P-450 preparations. Migration was from *top* to *bottom*. In *experiment 1*, the samples and amounts of protein were as follows: (a) purified LM3a, 0.075 μg, as a standard; (b) fraction B from Figure 1c, 0.2 μg; (c) fraction A from Figure 1c, 0.19 μg; (d) fraction II from Figure 1a, 0.34 μg; (e) nasal microsomes, 10 μg; (f) fraction I from Figure 1a, 0.36 μg; (g) fraction B from Figure 1b, 0.37 μg; (h) fraction A from Figure 1b, 0.21 μg; and (i) purified LM3c, 0.10 μg, as standard. In *experiment 2*, the amounts of purified nasal P-450 protein were as follows: (a) NMa, 0.11 μg; (b) NMb, 0.11 μg; and (c) NMa and MNb, 0.07 μg of each.

quence determination of NM3c showed that the first seven residues are identical with those in hepatic form 3c. Thus, the top band is probably NM3c. Chromatography of fraction II on an HTP column (shown in Figure 1c) resulted in the partial purification of nasal isozyme 3a (fraction B in Figure 1c) (see Figure 2, experiment 1, lane b), as determined by immunoblot analysis with monoclonal anti-3a antibodies (data not shown), and extensive purification of NMb (fraction A in Figure 1c) (see Figure 2, experiment 1, lane c). Purified LM3a is shown as a standard (lane a). The amount of nasal isozyme 3a present was not sufficient to allow further purification.

As shown in Figure 2, experiment 2, the final preparations of NMa (lane a) and of NMb (lane b) each appear as a single band on SDS–PAGE, and the two proteins are resolved into a dark upper band a light lower band when mixed (lane c). A plot of the relative mobility versus the log of the molecular weight of protein standards (data not shown) indicated an apparent molecular weight of 49 500 for NMa and 51 000 for NMb, but it should be noted that the values for membrane proteins determined in this manner tend to underestimate the actual molecular weights (Black & Coon, 1986). The specific contents of various preparations ranged from 12.6 to 14.1 for NMa and from 14.8 to 16.4 for NMb without correction for the amino acid composition of the proteins.

In experiments not presented, hepatic microsomes prepared from either untreated or acetone-treated rabbits were subjected to the same fractionation procedures as described for the purification of NMa and NMb. However, proteins corresponding to NMa and NMb were not detected on the basis of SDS–PAGE and Western blot analysis. A polyclonal anti-3a antibody (Koop et al., 1984) which cross-reacts with both NMa and NMb was employed in these studies.

Amino Acid Composition. P-450NMa and P-450NMb were analyzed for their amino acid composition, with the results shown in Table II. The two proteins have significant differences in a number of residues, such as histidine, methionine, phenylalanine, and glycine, and are also different in a number of respects from the known hepatic P-450 isozymes (Koop et al., 1982).

NH_2-Terminal Amino Acid Sequences. The two purified proteins were submitted to automated Edman degradation for determination of the NH_2-terminal sequences. The results are given in Table III, along with the known sequences of eight hepatic P-450 isozymes from the rabbit. The sequences of NMa and NMb are clearly different from each other and from those of all the rabbit hepatic forms shown, as well as those from various other tissues and species published to date, but exhibit the typical highly hydrophobic signal peptide that is retained in microsomal P-450s. The proline-rich segment of NMb (residues 30–41) is also typical of P-450 cytochromes. Optimal alignment of the NH_2-terminal sequence of NMa with the first 23 residues of the rabbit hepatic forms shows 39% pairwise identity with form 2 and much less identity with the other forms. Similar structural comparison of the NH_2-terminal sequence of NMb with the first 50 residues of the hepatic forms shows relatively high pairwise identity: 43% with form 1, 46% with form 2, 36% with form 3a, 43% with form 3b, and about 20% with form 4. In addition, the NH_2-terminal regions of P-450NMa and residues 31–49 of P-450NMb have high structural similarity to P-450a from rat liver, but no nasal P-450s are available from other species for a direct comparison. The present sequence information suggests that NMa and NMb may belong to the P-450II gene family (Nebert et al., 1987), but a definitive classification must await further sequence data.

Peptide Maps. Additional evidence that forms NMa and NMb are unique gene products was obtained by SDS–PAGE analysis of the peptides formed by the action of *S. aureus* V_8 protease (Figure 3). Experiments with P-450LM_2 and LM_{3a} were included for comparison. No peptide fragments were observed in control experiments in the absence of the protease, and the pattern of peptide fragments for each of the nasal forms was unique, indicating major differences in the primary sequences of these cytochromes.

Spectral Properties. The absolute spectra of purified P-450NMa and P-450NMb in the oxidized, reduced, and CO-complexed states are shown in Figure 4. The oxidized spectrum of NMa is characteristic of a low-spin, hexacoordinate hemoprotein, whereas the spectrum of NMb is characteristic of the high-spin, pentacoordinate state. How-

Table III: NH_2-Terminal Amino Acid Sequences of Rabbit Microsomal P-450 Cytochromes

form of P-450	residue identified				
	10	20	30	40	50
nasal mucosa[a]					
a	M L A S G L L L A A L L A S L T V M I L K ? V				
b	M E L G G A F T I F L A L C F S C L L I L I A W K R V Q K P G R L P P G P T P I P F L G N E L Q V R				
liver[b]					
1	M D P V V V L V L G L C C L L L L S I W K Q N S G R G K L P P G P T P F P I I G N I L Q I D A K D I				
2	M E F S L L L L L A F L A G L L L L L F R G H P K A H G R L P P G P S P L P V L G N L L Q M D R K G				
3a	A V L G I T V A L L G W M V I L L F I S V W K Q I H S S W N L P P G P F P L P I I G N L L Q L D L K				
3b	M D L L I I L G I C L S C V V L L S L W K K T H G K G K L P P G P T P L P V V G N L L Q L E T K D I				
3c	M D L I F S L E T W V L L A A S L V L L Y L Y G T				
4	A M S P A A P L S V T E L L L V S A V F C L V F W A V R A S R P K V P K G L K R L P G P S G V P				
5	M L G F L S R L G L W A S G L I L I L G F				
6	M V S D F G L P T F I S A T E L L L A S A V F C L				

[a] The sequences were determined with repetitive yields of 90–95%. [b] The sequence information for hepatic cytochromes is taken from the following sources: form 1 (Tukey et al., 1985), form 2 (Tarr et al., 1983), form 3a (Khani et al., 1987), form 3b (Ozols et al., 1985), form 3c (Black & Coon, 1986), form 4 (Fujita et al., 1984), form 5 (Parandoosh et al., 1987), and form 6 (Coon et al., 1985). In the case of P-450 forms 1 and 3a, the protein sequence was predicted from the corresponding cDNA sequence.

Table IV: Spectral Properties of Purified P-450NMa and -NMb[a]

	oxidized		reduced		CO complex		CO difference spectrum	
cytochrome	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})
NMa	417	113	413	82	450	94	450	68
	534	12.2	545	12.9	552	11.9		
	569	11.6						
NMb	394	92	415	84	451	95	451	70
	532	12.7	546	14.9	552	12.3		
	647	4.5						

[a] The extinction coefficients are based on cytochrome concentrations determined by analysis for heme; the values represent the means of two to three determinations with variation less than 5% of the mean.

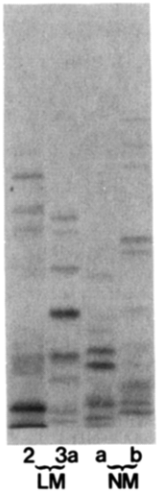

FIGURE 3: Peptide maps generated from purified P-450NMa and P-450NMb by digestion with *S. aureus* V_8 protease, with the peptides from P-450LM2 and P-450LM3a included for comparison. SDS–PAGE was carried out in 12.5% gels after incubation of the proteins at 37 °C for 10 h with the protease, as described under Materials and Methods. The digestion products from 0.6 μg of starting protein were applied to each lane, and the peptides were visualized by silver staining. Migration proceeded from *top* to *bottom*.

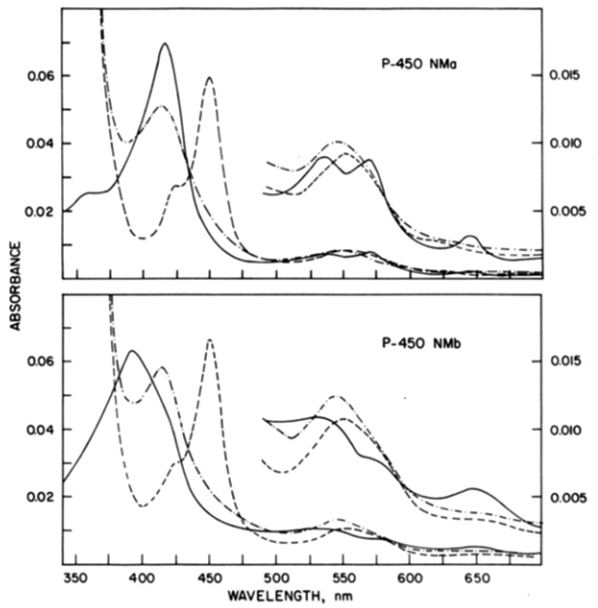

FIGURE 4: Spectra of purified P-450NMa and -NMb from rabbit nasal mucosa determined at 25 °C. The concentrations of the cytochromes, based on heme analysis, were 0.29 nmol of P-450NMa and 0.31 nmol of P-450NMb per milliliter of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.01 mM EDTA. (—) Oxidized; (–·–) dithionite-reduced; (- - -) reduced–CO complex.

ever, as previously seen with hepatic form 4 (Haugen & Coon, 1976) but not hepatic form 3a (Koop et al., 1982), NMb is converted to mixed low- and high-spin states in the presence of detergents such as Tergitol (data not presented). The wavelength maxima and absorption coefficients for the absolute spectra of the oxidized, reduced, and CO-complexed states and for the reduced carbonyl difference spectrum of both

Table V: Catalytic Activity of Purified Rabbit P-450 Cytochromes[a]

	activity [nmol of product min^{-1} (nmol of P-450)$^{-1}$]							
	nasal P-450		hepatic P-450					
substrate	a	b	2	3a	3b	3c	4	6
HMPA (1 mM)	11.0	5.3	14.8	2.1	6.0	0.6	2.7	1.3
PA (2 mM)	28.8	3.1	0.5	1.0	1.6	0.3	3.6	26.8
NDEA (4 mM)	6.6	3.8	2.5	2.7	0.2	<0.1	0.5	1.2
ethanol (80 mM)	8.2	4.9		12.9				

[a] The reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, a reconstituted enzyme system composed of 0.1 nmol of P-450, 0.3 nmol of NADPH–cytochrome P-450 reductase, and 30 μg of dilauroylglyceryl-3-phosphocholine, and a substrate at the indicated concentration in a final volume of 1.0 mL. The reaction was initiated by the addition of 1 μmol of NADPH, and the incubation was at 37 °C. The reaction products were determined as described under Materials and Methods, and the values reported are the average of triplicate determinations.

nasal forms are summarized in Table IV. The spectrum of the pyridine hemochrome of both cytochromes was found to be characteristic of protoheme IX (Furhop & Smith, 1975).

Catalytic Activity of P-450NMa and P-450NMb. The substrate specificity of the nasal forms was studied with the P-450 isozyme as the rate-limiting component in the reconstituted enzyme system containing saturating levels of NADPH, NADPH–cytochrome P-450 reductase, and phosphatidylcholine. Table V shows the activity of NMa and NMb, along with that of six hepatic isozymes for comparison, toward HMPA, PA, and NDEA. These compounds were chosen because of their known metabolism and carcinogenicity in nasal mucosa. Both NMa and NMb are highly active toward the above substrates, with NMa being the most active form among all P-450s studied with PA and NDEA, and almost as active as form 2 and superior to all other hepatic forms toward HMPA. The nasal forms are also active in the oxidation of ethanol, although less active than P-450 3a, the alcohol-inducible form which has been shown to be the most active of the hepatic cytochromes toward several alcohols (Morgan et al., 1982). Similar results were obtained with butanol in data not shown.

The catalytic activity of NMa and NMb toward testosterone was also examined, as shown in Table VI. Both isozymes are active toward this endogenous substrate, but they differ in the positions of oxidation. NMb catalyzes the hydroxylation of testosterone at the 15α-, 16α-, and 19-positions and also gives an unidentified metabolite, whereas NMa oxidizes the substrate in the 17-position to yield androstenedione and other products that have not yet been identified. In contrast to the relative activities toward the exogenous substrates, the total rate of testosterone metabolism catalyzed by NMb is significantly greater than that catalyzed by NMa. Furthermore, the hydroxylation of testosterone in the 15α- and 19-positions is apparently unique among rabbit P-450 isozymes. In experiments not presented, microsomal preparations from the olfactory mucosa, but not from the respiratory mucosa or from liver of untreated rabbits, catalyzed the formation of 19-hydroxytestosterone, indicating that NMb may be expressed specifically in the olfactory mucosa.

Table VI: Oxidation of Testosterone by Purified P-450NMa and -NMb[a]

	act. [nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$] at position of testosterone oxidation				
P-450 isozyme	15α	16α	17	19	total
NMa	0	0	0.26	0	0.83[b]
NMb	0.89	0.48	0	0.66	2.23[c]

[a] The reaction mixtures contained 150 nmol of [^{14}C]testosterone (7.4 μCi/μmol, added in 20 μL of methanol), 50 μmol of phosphate buffer, pH 7.4, and a reconstituted system containing 0.1 nmol of P-450, 0.3 nmol of NADPH–cytochrome P-450 reductase, and 30 μg of phospholipid. The reaction was initiated by addition of 2 μmol of NADPH to make a final volume of 1.0 mL, and the incubation was at 37 °C for 10 min. Testosterone metabolites were analyzed as described under Materials and Methods. The values reported are corrected for zero-time controls and represent the average of duplicate determinations. [b] The total includes several other metabolites, formed at a combined rate of 0.57 nmol min^{-1} (nmol of P-450)$^{-1}$, that do not comigrate with available authentic testosterone derivatives. [c] The total includes an unidentified metabolite [0.20 nmol min^{-1} (nmol of P-450)$^{-1}$].

Discussion

The monooxygenase system of rabbit nasal microsomes contains two unique P-450 enzymes, which we have purified to electrophoretic homogeneity and characterized with regard to molecular weight, amino acid composition, peptide maps, NH_2-terminal amino acid sequence, spectral properties, and substrate specificity. These two forms, NMa and NMb, evidently constitute a major portion of the nasal microsomal P-450 of untreated rabbits and, on the basis of NH_2-terminal sequences, are distinct from all other presently known P-450s. Homologues of several known hepatic P-450 isozymes, including forms 2, 3a, and 4, but not forms 3b and 6, have been identified immunochemically in rabbit nasal microsomes (Ding et al., 1986). A fourth isozyme, P-450NM3c, was identified in the present study and, as judged by the chromatographic behavior, SDS–PAGE analysis, and partial NH_2-terminal sequence of the purified protein, probably corresponds to hepatic form 3c. In addition, form 5 may also be present in the nasal mucosa, based on SDS–PAGE analysis and preliminary purification studies, as well as on the fact that 2-aminoanthracene, a preferred substrate for this cytochrome (Robertson et al., 1981), was activated by rat nasal tissue homogenate to form mutagens in the *Salmonella typhimurium* assay (Bond & Li, 1983).

The nasal microsomes used in the present study as starting material for the purification of NMa and NMb were prepared from the mucosa of the upper, rear portion of the septum and from the ethmoturbinoid, comprising mostly olfactory epithelium. Preliminary experiments with microsomes from the maxilloturbinoid, which is located in the respiratory region of the nasal cavity and does not contain olfactory neuronal cells, indicate that NMa, but not NMb, is also present in the respiratory mucosa. Thus, the high turnover number of P-450NMa toward alcohols and the three nasal carcinogens in the reconstituted system, along with the relatively high level of NADPH–cytochrome P-450 reductase in both olfactory and respiratory mucosa (Ding et al., 1986) (unpublished results), suggests that this isozyme may play a crucial role in the metabolism and toxicity of these compounds in the nasal mucosa. On the other hand, P-450NMb, which is not as active as P-450NMa with the xenobiotic compounds studied, may be important in the metabolism of endogenous substances, of which testosterone is an example. Evidence is presented that NMb is much more active than NMa in the metabolism of testosterone. An intriguing question that remains to be explored is whether the nasal forms of P-450 are involved in

disposing of various odorants that have been deposited on the nasal mucosa, thereby maintaining acuity in the sense of smell.

Registry No. P-450, 9035-51-2; NDEA, 55-18-5; HMPA, 680-31-9; PA, 62-44-2; monooxygenase, 9038-14-6; ethanol, 64-17-5; testosterone, 58-22-0.

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