Identification and Biochemical Analysis of Novel Olfactory-Specific Cytochrome P-450IIA and UDP-Glucuronosyl Transferase†

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Received October 13, 1989; Revised Manuscript Received February 5, 1990

ABSTRACT: Two major transmembranal polypeptides of bovine olfactory epithelium were identified by SDS electrophoretic analysis of Triton X-114 solubilized membranes. Both polypeptides were present in large amounts in membranes of the olfactory epithelium but were barely detectable in membranes of the nasal respiratory epithelium. Both polypeptides are enriched in the deciliated epithelium as compared with isolated cilia. One of them is a glycoprotein with an apparent molecular mass of 56 kDa (gp56); the other is an unglycosylated protein with an apparent molecular mass of 52 kDa (p52). Sequence analysis of peptides obtained by CNBr cleavage of purified gp56 indicates that it is highly homologous to UDP-glucuronosyl transferase (UDPGT). Parallel analysis shows that p52 is highly homologous to cytochrome P-450 sequences of the IIA subfamily. This protein is assigned the name P-450olf2. Polyclonal antibodies were raised against synthetic peptides corresponding to gp56 and p52 peptide sequences. Immunoblots with these antibodies reveal the following properties of gp56 and p52: (1) they are enriched in the microsomal fraction of the bovine olfactory epithelium; (2) they are possibly specific to the olfactory epithelium, as we could not detect reactivity in microsomes derived from respiratory epithelium or lung, and only a very small amount of basal reactivity was seen with liver microsomes; (3) cross-reacting proteins exist in microsomes derived from the rat olfactory epithelium. These results are consistent with a mechanism whereby the microsomal enzymes are involved in odorant modification and clearance from the nasal tissue.

Cytochrome P-450 and UDP-glucuronosyl transferase (UDPGT)[1] belong to a broad class of enzymes, collectively referred to as biotransformation enzymes. They are predominantly found in the endoplasmic reticulum of liver cells but are also present in other tissues such as lung and kidney. They are involved in metabolizing and detoxifying small lipophilic molecules, including xenobiotics, pharmaceutical drugs, steroid hormones, and prostaglandins (Black & Coon, 1987; Nebert & Gonzalez, 1987; Tephly et al., 1988; Siest et al., 1987). Several of the substrates of cytochrome P-450 and UDPGT, such as coumarin, anisole, eugenol, and monoterpenoid alcohols, are odorants (Boutin et al., 1985; Boutin, 1987). Cytochrome P-450 (phase I biotransformation enzyme) catalyzes the hydroxylation of a vast number of substrates, while UDPGT (phase II biotransformation enzymes) catalyzes the transfer of glucuronic acid from UDP-glucuronate to hydroxyl moieties. The combined action of cytochrome P-450 and UDPGT transforms a hydrophobic molecule into a hydrophilic, membrane-impenetrable one, which can be readily excreted (Phillips, 1986; Burchell & Coughtrie, 1989). Certain subsets of UDPGT and cytochrome P-450 enzymes are induced to high levels by several prototypic inducers such as phenobarbital, 3-methylcholantrene, tetrachlorodibenzodioxin, and ethanol [reviewed by Adesnik and Atchison (1986), Black and Coon (1987), Nebert and Gonzalez (1987), and Burchell and Coughtrie (1989)].

More than 70 subtypes of cytochrome P-450 have been characterized in 11 species. The cytochrome P-450 gene superfamily has been divided into 12 families and each family further divided into subfamilies. Any protein belonging to a given family is less than 40% homologous to proteins of the other families, and proteins exhibiting more than 60% identity are grouped in the same subfamily (Nebert et al., 1989). Likewise, multiple forms of UDPGT exist, although their heterogeneity to date is less pronounced than that for cytochrome P-450 (Burchell & Coughtrie, 1989). cDNAs corresponding to eight different UDPGT's have been sequenced and exhibit 44–97% mutual identity at the amino acid level.

It has been previously reported that cytochrome P-450's are present in the nasal mucosa and that their substrate specificity and kinetic parameters are different from those of the liver enzymes (Dahl, 1988; Jenner & Dodd, 1988; Reed et al., 1986; Dahl et al., 1982; Hadley & Dahl, 1982). While considerable information is available on drug-metabolizing enzymes in the liver, including substrate specificities and cDNA and genomic sequences, relatively little is known about their nasal counterparts. Two forms of cytochrome P-450 proteins, designated NMa and NMb, have recently been isolated from rabbit nasal mucosa and their N-terminal sequences found to be different from those of known liver subtypes (Ding & Coon, 1988). We have recently reported the molecular cloning of a rat olfactory cytochrome P-450 cDNA (cytochrome P-450olf1, P-450IIG1) obtained by screening a rat olfactory epithelial cDNA library with a cDNA probe derived from rat liver cytochrome P-450b

† This research was supported by grants from the National Institutes of Health, the United States Army Research Office, the Fund for Basic Research administered by the Israeli Academy of Sciences and Humanities, and the Minna-James-Heineman Foundation, München, FRG.

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[1] Abbreviations: SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UDPGT, UDP-glucuronosyl transferase; DTT, dithiothreitol; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; EDCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BSA, bovine serum albumin; Con A, concanavalin A.

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(P-450IIB1) (Nef et al., 1989). Cytochrome P-450olf1 defines a new subfamily (cytochrome P-450IIG) and is specifically expressed in the olfactory epithelium.

Olfactory UDPGT has not been previously characterized, although the existence of UDPGT activity toward umbelliferone and 1-naphthol in rat and dog nasal tissue has been described (Bond, 1983; Bond et al., 1988; Longo et al., 1988). We report here the biochemical and immunochemical characterization of the olfactory-enriched transmembrane glycoprotein gp56, which is identified as a novel form of UDPGT highly expressed in olfactory epithelium. We have also carried out the molecular cloning of its corresponding cDNA (Lancet et al., 1989; Lazard et al., unpublished results). In parallel, we show that a second olfactory-specific major transmembranal protein, p52, is a form of cytochrome P-450 (P-450olf2), which belongs to the IIA3 subfamily. The identity and tissue specificity of both proteins are established by immunoblotting analysis.

Materials and Methods

Materials. Reagents for SDS–PAGE and prestained molecular mass standards were from Bio-Rad. Protein A was from Bio-Makor, Israel. Complete and incomplete Freund's adjuvant was from DIFCO. Hybond C nitrocellulose paper was from Amersham. All other reagents were from Sigma.

Animals and Tissue. Bovine olfactory ethmoturbinates, comprising olfactory epithelium and the underlying bone, and respiratory nasoturbinates and maxilloturbinates, comprising respiratory epithelium and the underlying bone, were removed 30–60 min after death at the Jerusalem Municipal Slaughterhouse following sagittal section of the head. The ethmoturbinates from 3-month-old calves were dissected into 1–2 cm^2 pieces and collected in ice-cold buffer A (30 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride, pH 8.0) and kept at 0 °C for 2–4 h until further processed. Respiratory turbinates were similarly treated. For microsome preparation, pieces of olfactory turbinates, respiratory turbinates, liver, and lung were cut, immediately frozen in liquid nitrogen, and stored at −70 °C until further processed.

Rats (Wistar, 3 months old) and rabbits (NZB, 3 months old) were from the animal breeding facility of the Weizmann Institute of Science.

Cilia and Membrane Preparations. Cilia from both olfactory and respiratory epithelium and membranes from deciliated olfactory epithelium were prepared by the calcium shock procedure as previously described (Chen et al., 1986a; Lazard et al., 1989), with the addition of 10% ethanol. Microsomes from bovine olfactory epithelium, respiratory epithelium, liver, and lung, and from rat olfactory epithelium, liver, lung, kidney, and small intestine, were prepared as described by Hadley and Dahl (1982). The frozen tissues were thawed in buffer C (0.1 M Tris-HCl/0.2 M KCl, pH 7.4), washed, and homogenized in 10 volumes of the same buffer using a Polytron homogenizer at maximal speed for 1 min. The homogenate was centrifuged at 10000*g* for 15 min at 4 °C and the pellet (10k pellet) set aside. The supernatant was centrifuged at 100000*g* for 90 min. The pellet (100k pellet) was resuspended in 0.1 M Tris-HCl, pH 7.4, at a concentration of 5–10 mg of protein/mL and stored frozen at −70 °C.

Triton X-114 Extraction and Phase Separation. Cilia or membranes were fractionated by the Triton X-114 method as described (Bordier, 1981; Chen et al., 1986b). First, membranes were incubated with a 1% solution of Triton X-114 at 0 °C and separated into a Triton X-114 insoluble fraction, which comprises mainly the cytoskeleton and its associated proteins, and a detergent-soluble extract. The detergent-soluble extract was then phase-separated at 35 °C, whereby it fractionated into a detergent phase, enriched in transmembranal proteins, and a buffer phase, containing soluble proteins. After phase separation, the detergent phase was diluted 10-fold with buffer B (50 mM Tris-HCl, 150 mM NaCl, and 0.1% NaN_3, pH 7.5). Both phases were collected and stored at −20 °C.

Electrophoresis. Samples were electrophoresed in 7% or 10% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Gels were either 10 cm long, or 20 cm long for high resolution. Gels were then fixed and stained with Coomassie Brilliant Blue or by silver staining (Merril et al., 1981). Glycoproteins were identified directly on the electrophoresis gels by the lectin overlay method (Burridge, 1978) using ^{125}I-labeled concanavalin A (Con A) (4 $\mu Ci/\mu g$, 10^6 cpm/mL) as described (Chen et al., 1986b).

Reduction and Alkylation. Samples were first brought to pH 8.0 by addition of Tris-HCl, pH 8.0, to a final concentration of 0.2 M. Dithiothreitol (DTT) was then added to a final concentration of 10 mM, and the sample was incubated in the dark for 1 h at 35 °C. Iodoacetamide was then added to a final concentration of 22 mM and the sample incubated for 1 h at 4 °C. At the end of the procedure, sample buffer containing no DTT was added, and the sample was electrophoresed as described above.

Purification of Proteins by Electroelution and Cyanogen Bromide Cleavage. In a first experiment, the detergent phase of a Triton X-114 extract of membranes from deciliated bovine olfactory epithelium containing 25–50 mg of protein was electrophoresed on 10 cm long 10% SDS–PAGE gels. This preparation had not been previously reduced and alkylated. Immediately after electrophoresis (i.e., without fixation and staining), gel slices corresponding roughly to the 56-kDa region were excised (using prestained markers as estimates of the molecular mass). The gel pieces were then equilibrated in electroelution buffer (25 mM Tris-HCl, 20 mM glycine, 0.1% SDS, and 0.07% thioglycolic acid, pH 8.3) and electroeluted in a Schleicher & Schuell Biotrap BT 1000 electroelution chamber at 100 V at 4 °C for approximately 60 h. The electroeluted material was collected after the first 12 h and every 24 h thereafter. The buffer of the pooled electroeluted material was exchanged to 0.1% SDS in PBS by several rounds of ultrafiltration using a Centricon concentrator (Amicon). Protein was then precipitated by addition of trichloroacetic acid to a final concentration of 15%, incubating 20 min on ice, and centrifuging at 10000*g* for 10 min. The protein pellet (1.8 mg) was washed twice with cold acetone, solubilized in 70% formic acid containing 20 mg/mL CNBr, and cleaved at methionine residues during a 24-h incubation at room temperature.

In a second experiment designed to augment the resolution power of electrophoresis in the 56-kDa range, a similar sample of detergent phase containing the same amount of protein was reduced and alkylated, and then protein was precipitated with trichloroacetic acid and washed with cold acetone as described above. The protein pellet was then dissolved in 3 mL of 8 M urea/1% SDS. The solution was dialyzed against two changes of 0.1% SDS in PBS in order to eliminate most of the urea. Electrophoresis sample buffer was then added and the sample electrophoresed in 20 cm long SDS–PAGE gels for 16 h at 80 V at 4 °C. After being stained, the gels with Coomassie Brillant Blue stained bands corresponding to the individually migrating polypeptides in the 56-kDa region (see Results) were excised, and the gel pieces were electroeluted as described

above; 400 μg of one of the polypeptides, gp56.1, was subjected to CNBr cleavage as described above.

Separation of CNBr Cleavage Peptides and Protein Microsequencing. The peptide mixture resulting from CNBr cleavage of proteins was loaded on an Aquapore RP-300 C03GU reverse-phase column (Applied Biosystems, Santa Clara, CA) and separated by HPLC using a linear gradient of acetonitrile in 0.3% trifluoroacetic acid as described in Figure 4. The flow rate was 0.5 mL/min, and 0.5-mL fractions were collected. The separated peptides were detected by the fluorescence of their fluorescamine reaction products (Böhlen et al., 1975). Fractions containing amounts of peptides sufficient for sequencing were concentrated in a Speedvac vacuum evaporator and applied to a polybrene-pretreated filter. Sequence analysis was performed on an automatic-pulse liquid–gas-phase protein microsequencer (Model 475) equipped with an on-line HPLC PTH-amino acid analyzer (Model 120) and a data acquisition and processing unit (Model 900) (all from Applied Biosystems Inc., Foster City, CA).

Preparation of Antisera. Synthetic peptides were prepared by Dr. Ora Goldberg of Chemical Services, Weizmann Institute of Science. Peptides were coupled to bovine serum albumin (BSA) as described by Kris et al. (1985). Twenty milligrams of peptide dissolved in 5 mL of PBS was mixed with 40 mg of BSA and the mixture stirred 30 min at room temperature. Twenty milligrams of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDCI) was added and the mixture was stirred overnight at room temperature. Coupled peptide was dialyzed against PBS, and any precipitate was removed by 15-min centrifugation at 27000*g*. Samples were aliquoted and stored at −20 °C.

Rabbits were injected subdermally at four to six sites each with 1 mg of coupled peptide in complete Freund's adjuvant (total volume infected was 2 mL). Two weeks later, the rabbits were boosted with 1 mg of coupled peptide in incomplete Freund's adjuvant, and after 2 additional weeks, they received a second boost of 1 mg of coupled peptide in incomplete Freund's adjuvant. Rabbits were bled 2 weeks after the last boost and every 2 weeks thereafter. Blood was allowed to clot overnight at 4 °C, and the sera were centrifuged for 15 min at 47*g* followed by a 20-min centrifugation at 750*g*. Sera were then heat-inactivated at 56 °C for 30 min, aliquoted, and stored at −20 °C.

Positive antisera were identified by their reactivity with peptides and with a Triton X-114 detergent-phase preparation of bovine olfactory cilia using standard enzyme-linked immunoabsorbent assay (ELISA) technique (Eshhar, 1985).

Immunoblot Analysis. SDS–PAGE of samples was as described above on a minigel apparatus (Hoeffer, FRG). Prior to transfer, gels were equilibrated in transfer buffer (0.192 M glycine, 25 mM Tris, and 20% methanol). Proteins were transferred to nitrocellulose for 1 h at 100 V in transfer buffer on a Bio-Rad mini-blot apparatus, or for 16 h at 30 V on a locally produced transblot apparatus with a gradient electric field. Efficiency of transfer was assessed by staining the gels with Coomassie Blue after transfer.

For immunoblot analysis, the nitrocellulose was first washed 2 times in PBS for 5 min/wash at room temperature. Nonspecific binding sites were then blocked by incubating the nitrocellulose for 1 h at room temperature in Tris-buffered saline (200 mM Tris/500 mM NaCl, pH 7.5) containing 3% gelatin. The nitrocellulose was then incubated in antibody solution, a 1:30 dilution of serum in PBS. Incubation was continued overnight at 4 °C, and the nitrocellulose was washed 4 times with PBS and then incubated with ^{125}I protein A (10^5 cpm/mL) in PBS containing 1% gelatin for 2 h at room temperature, washed 5 times with PBS, air-dried, and autoradiographed for 5–16 h at −70 °C with an intensifying screen.

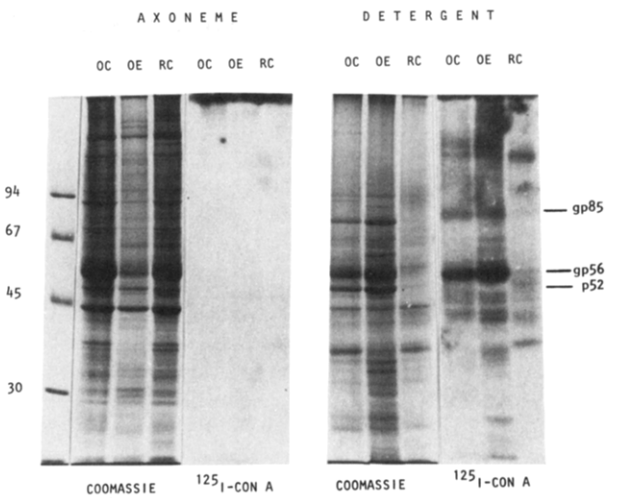

FIGURE 1: SDS–PAGE polypeptide pattern of bovine nasal membranes. 10% SDS–PAGE gel of the Triton X-114 insoluble fraction (AXONEME) and detergent-phase fraction (DETERGENT) of olfactory cilia (OC), membranes from deciliated olfactory epithelium (OE), and respiratory cilia (RC). Visualization is by Coomassie Brilliant Blue staining (COOMASSIE) and ^{125}I-Con A overlay autoradiography of the same gel (^{125}I-CON A). Molecular mass markers (in kilodaltons) appear on the left.

Iodination of Proteins. Con A was radiolabeled with ^{125}I by the chloramine-T method. Protein A was radiolabeled with ^{125}I by using Iodo-Beads (Pierce Chemical Co.) according to the manufacturer's instruction.

Protein Determination. Protein concentration of samples was determined by the method of Bradford (1976), using BSA as standard.

Computer Sequence Data Analysis. This was performed on a VAX 3600 computer at the Biological Computing Division of the Weizmann Institute, using a University of Wisconsin software package and the Genbank and EMBL databases.

Results

Identification of Transmembrane Proteins in Olfactory Cilia. In order to identify major transmembranal proteins specific to the olfactory epithelium, we studied the SDS–PAGE polypeptide patterns of the different nasal tissues. The three preparations studied were isolated olfactory cilia, membranes from deciliated olfactory epithelium, and isolated respiratory cilia. Each of the preparations was subjected to the Triton X-114 fractionation technique that separates integral transmembrane proteins from those that are not lipid-attached (Bordier, 1981). The polypeptide patterns in the detergent phase of the three membrane preparations are shown in Figure 1 (right).

Since transmembrane proteins are often glycosylated, we compared the glycoprotein patterns of the detergent phase of the three preparations, as identified by ^{125}I-labeled Con A overlay of the Coomassie Brilliant Blue stained gels (Figure 1). The cytoskeletal fraction was included in this analysis for comparison. When starting with an equal amount of protein in the three preparations, olfactory cilia and respiratory cilia yield a large and comparable amount of polypeptides in the Triton X-114 insoluble fraction, with a practically indistinguishable protein pattern. The deciliated olfactory membrane preparation has a smaller amount of protein in this cytoskeletal fraction. Significantly, the cytoskeletal fraction of all three

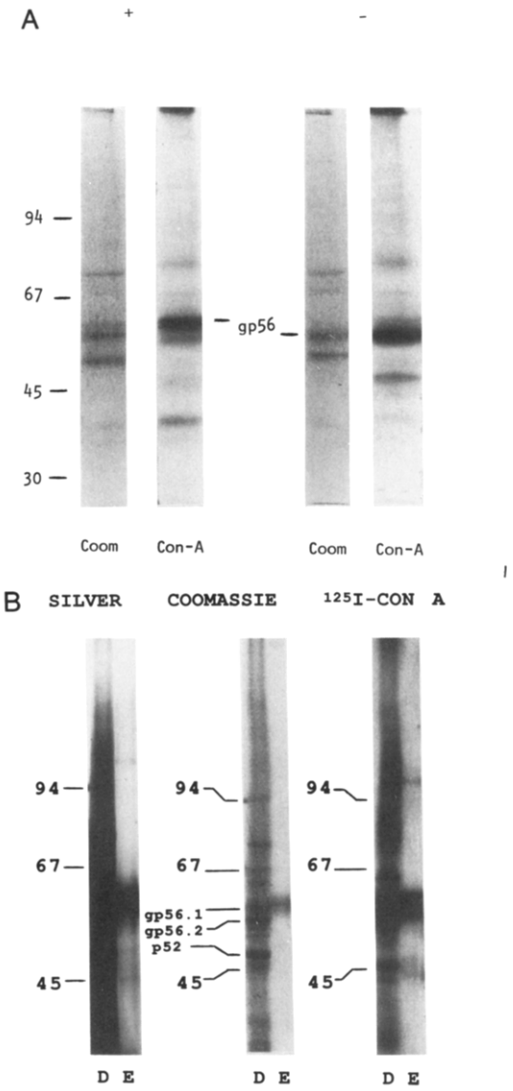

FIGURE 2: Reduction/alkylation and electroelution of olfactory membrane proteins. (A) Effect of reduction and alkylation on proteins in the Triton X-114 detergent phase. Coom, Coomassie Brilliant Blue staining of 8% SDS–PAGE gels; Con A, autoradiography of the same gels overlayed with ^{125}I-Con A; (+), reduction and alkylation treatment; (−), no treatment. (B) Lanes marked D show samples of the Triton X-114 extract detergent phase of deciliated olfactory membranes, reduced and alkylated, electrophoresed under the conditions used for electroelution (20 cm long 7% SDS–PAGE gels for 16 h at 80 V and 4 °C). Lanes marked E show parallel runs of electroeluted purified gp56.1. Visualization (as marked) is by silver staining, Coomassie Brilliant Blue staining, and ^{125}I-Con A autoradiography of the Coomassie Brilliant Blue. The faint band in the ^{125}I-Con A labeled lane of electroeluted material, which migrates at roughly 100 kDa, is probably a dimer of gp56.1. Protein quantities loaded: silver, 40 μg in lane D, 5 μg in lane E; Coomassie Brilliant Blue and ^{125}I-Con A, 50 μg in lane D, 5 μg in lane E.

tissues is devoid of glycoproteins recognized by Con A, as expected.

The detergent-phase fractions of olfactory cilia and deciliated olfactory membranes have a similar polypeptide pattern, while respiratory cilia show much less protein and a different polypeptide pattern. Two major proteins are notable in this fraction. The first is clearly visible in the ^{125}I-Con A overlay, which reveals a glycoprotein of 56 kDa (gp56), present in both olfactory cilia and deciliated olfactory membranes but practically absent from respiratory cilia (Figure 1), or from respiratory epithelial membranes (not shown). A polypeptide comigrating with gp56 appears to be a major Coomassie Brilliant Blue stained specific transmembranal glycoprotein

Table I: Peptides Synthesized According to the Sequence of Peptides 19 and 21[a]

DP19	M G Y L P G P Q Q Q A F K E L Q D E	(mix of 4 peptides)
DP21	M G K T L T E E K A N R I A S A Y C F	(mix of 4 peptides)

[a] The mixed peptides shown were synthesized according to the sequence of peptides 19 and 21, to be coupled to a carrier and used for immunizations. Due to sequence determination ambiguities, each peptide was synthesized as a mixture of four. Unassigned amino acids have been replaced by a glycine. A tyrosine residue has been added to the C-terminal of DP21 to enable iodination.

of olfactory cilia. It is found in even higher amounts in the deciliated olfactory membranes. The second protein is unglycosylated and has an apparent molecular mass of 52 kDa (p52). It is also identified in our gels as a major transmembrane protein specific to the olfactory epithelium. Both p52 and gp56 are present in high amounts in the olfactory Triton X-114 detergent phase (Figure 1) but practically absent in the corresponding buffer phase (not shown), suggesting that these proteins are transmembranal. An additional ^{125}I-Con A labeled glycoprotein, gp85, seemingly specific to olfactory cilia, was not studied further because it is a minor polypeptide undetectable by silver staining, and because it appeared in some of the respiratory cilia preparations examined (not shown).

A singular property of gp56 is its change of electrophoretic mobility upon reduction and alkylation. When reduced with DTT and alkylated with iodoacetamide, gp56 has a markedly slower mobility in SDS–PAGE gels than when it is not treated (Figure 2A). This behavior is generally taken as an indication for the existence of internal disulfide bonds, which keep the unreduced protein in a more "folded" and compact conformation even in SDS–PAGE.

The reduction and alkylation study also shows that gp56 can be resolved into two discrete protein bands. This can be seen in Figure 2A, and more clearly in Figure 2B. The heavier polypeptide band, termed gp56.1, migrates more slowly following reduction and alkylation and is more reactive with Con A. The apparent molecular mass of this band is changed from 56 to 58 kDa by the reduction and alkylation treatment. The second band, termed gp56.2, is less affected by the reduction and alkylation, and shows only minimal reactivity with Con A. The other olfactory-specific transmembranal protein, p52, is largely unaffected by reduction and alkylation.

Purification and Sequencing of Fragments Derived from gp56 and p52. In a preliminary experiment, protein obtained by electroelution at around 56 kDa from a low-resolution electrophoretogram of nonreduced Triton X-114 detergent phase yielded two CNBr peptides (numbers 19 and 21). Sequence analysis showed that peptide 19 has a high degree of homology to cytochrome P-450IIA (Figure 3A), while peptide 21 is highly homologous to UDP-glucuronosyl transferase (Figure 3B). As the two peptides were homologous to different proteins, it was realized that this particular electroelution preparation was not homogeneous. Subsequent high-resolution gel electrophoretic analysis confirmed the presence of gp56.1 and gp56.2 as well as p52 in this preparation (data not shown).

We subsequently purified gp56.1 to apparent homogeneity by high-resolution gel electrophoresis, followed by electroelution of the Coomassie Brilliant Blue stained band (Figure 2B). The two other proteins, gp56.2 and p52, were also purified to apparent homogeneity by the same procedure. Pu-

A	peptide 19	MXYLPGPQQQAFKELQGL
		D E
	P450 IIA3	MK E LPGPQQQAFKELQGL
	P450 (1)	MK Q LPGPQQQAFQ L LQGL
	P450 a	MK YLPGPQQQI I KV T QK L
B	peptide 21	MXKTLTEEKANRIASALAQI
		CF
	rat udpgt1	MV KN LTEEKANV V ASALAQI
	rat udpgt2	MV S N M TEEKANA IAW ALAQI
	rat udpgt3	MV S S M TEEKANA IAW ALAQI
	hum udpgt	MV S N T S EER ANV IASALAK I
C	peptide 201	MAKVIKDFHTW
	peptide 202	MVKTLTEEKANRIASALAQI
	peptide 204	MVGVPIFADQLXN
		D
	peptide 206	MFADQPDN
	hum udpgt	MVGVPL FADQPDN
	rat udpgt1	I VGI PL FADQPDN
	rat udpgt2	MI GI PL FG DQPDN
	rat udpgt3	MVGI PMFG E QH DN

FIGURE 3: Sequences of the p52- and gp56-derived CNBr peptides and their homology to known proteins. (A) Sequence of peptide 19 and comparison with the sequence of selected members of the cytochrome P-450IIA subfamily. P-450IIA3, rat lung cytochrome P-450IIA3 (amino acid, 227–244, Kimura et al., 1989). P-450 (1), human cytochrome P-450IIA3 (amino acids 65–82, partial sequence; Phillips et al., 1985). P-450a, rat cytochrome P-450IIA1 (amino acids 226–243; Nagata et al., 1987). (B) Sequence of peptide 21 and comparison to UDPGT sequences. References and amino acid positions for the UDPGT sequences are as follows: udpgt1, amino acids 313–332; Mackenzie, 1986a; udpgt2, amino acids 313–332; Mackenzie, 1986b; udpgt3, amino acids 313–332; Mackenzie, 1987; human udpgt, amino acids 312–331; Jackson et al., 1987. (C) Sequence for the four gp56.1 peptides separated by HPLC. Peptide 201 has no significant homology to any known sequence. Peptide 202 is identical with peptide 21 shown in (B), with fewer sequence ambiguities. Peptides 204 and 206 are probably related by partial CNBr cleavage and are homologous to sequences found in the UDPGT proteins cited in (B) (human udpgt, amino acids 390–402; udpgt1, amino acids 391–403; udpgt2, amino acids 391–403; udpgt3, amino acids 391–403). X denotes an unassigned amino acid. Two amino acids at the same position in the peptide's sequences denote two possible assignments, with a lower probability for the bottom assignment. Amino acid identities appear in large boldface letters, while nonidentities are marked with smaller type.

rified gp56.1 (Figure 2B), but not purified gp56.2 or p52 (not shown), reacted with ^{125}I-Con A. Four of the gp56.1 CNBr cleavage peptides, as marked in Figure 4, gave a readable sequence long enough to be useful (Figure 3C).

Antibodies to Synthetic Peptides. Two synthetic peptides were produced based on the sequences of peptide 19 (DP19) and peptide 21 (DP21) (Table I). The peptides were coupled to BSA and injected into rabbits. Two antisera were obtained, which in immunoblot analysis of the detergent phase of deciliated olfactory epithelial membranes respectively react with a 52-kDa protein and a 56-kDa protein (Figure 5A). Western blots of the purified gp56.1, gp56.2, and p52 show that the DP19 antiserum recognizes p52 but not gp56.1 or gp56.2, while the DP21 antiserum recognizes both gp56.1 and gp56.2, but not p52 (Figure 5B). The results suggest that gp56.1 and gp56.2 may be isozymes, commonly recognized by an antibody which is directed against a sequence conserved among UDPGT

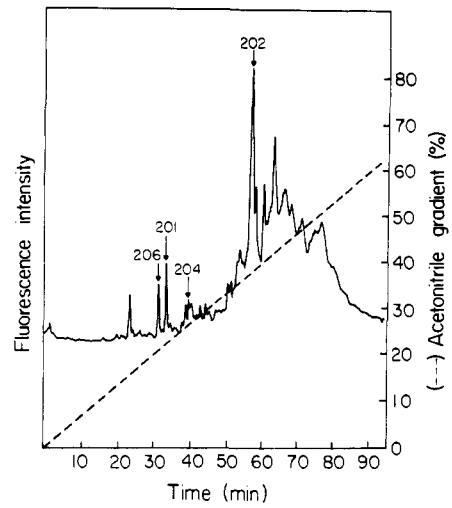

FIGURE 4: Separation of CNBr cleavage peptides of gp56.1. The CNBr cleavage mixture of gp56.1 was separated by HPLC on a RP-300 reverse-phase column with a gradient of acetonitrile in 0.3% trifluoroacetic acid. Peptides were detected by the fluorescence of their fluorescamine reaction products. Four peptides, indicated by numbers, were subjected to microsequencing. The dashed line indicates the percent acetonitrile along the elution profile, as marked on the right vertical axis.

enzymes, including olfactory UDPGT (Lazard et al., unpublished results). Alternatively, the two species may be related by proteolysis or glycosylation differences. The latter possibility is consistent with the apparent heavier labeling of gp56.1 with ^{125}I-Con A.

Next, we examined the tissue and membrane fraction distribution of these immunoreactivities by Western blot analysis (Figure 6). Both p52 and gp56 are found mainly in the microsomal fraction of bovine olfactory epithelium, with only a small residual reactivity appearing in the plasma membrane fraction. Other tissues known to be rich in biotransformation enzymes (respiratory epithelium, liver, and lung) were practically devoid of immunoreactive proteins (Figure 6). When liver membranes were loaded in a 3-fold excess, a weak reactivity was seen, the gp56 antisera labeling at a slightly different molecular mass (not shown). since bovine serum albumin was used as an immunogen carrier, a labeled band at 67 kDa appears here (and in Figure 5C below), which serves as a useful internal marker, and does not obscure the results in the 50–60-kDa region.

A parallel experiment was conducted in another mammalian species, i.e., rat (Figure 5C). Clearly labeled polypeptides appeared in olfactory microsomes, while liver microsomes had residual immunoreactivity (with anti-p52) or practically no reactivity (with anti-gp56). No reactivity to either antisera was detected in microsomal preparations of rat lung, rat kidney, and rat small intestine (not shown).

Discussion

We report here the identification of two major transmembrane proteins, p52 and gp56, which are enriched in olfactory epithelial membranes compared with control nonsensory epithelial membranes. Such olfactory-enriched proteins were considered likely to be related to olfactory function and were therefore studied in detail. The use of the recently reported bovine olfactory epithelial preparation (Lazard et al., 1989), which provides large quantities of membranes, made it possible to purify both proteins to homogeneity and to subject them to amino acid microsequence analysis.

This study was originally motivated by the search for potential odorant receptor proteins, likely to be major trans-

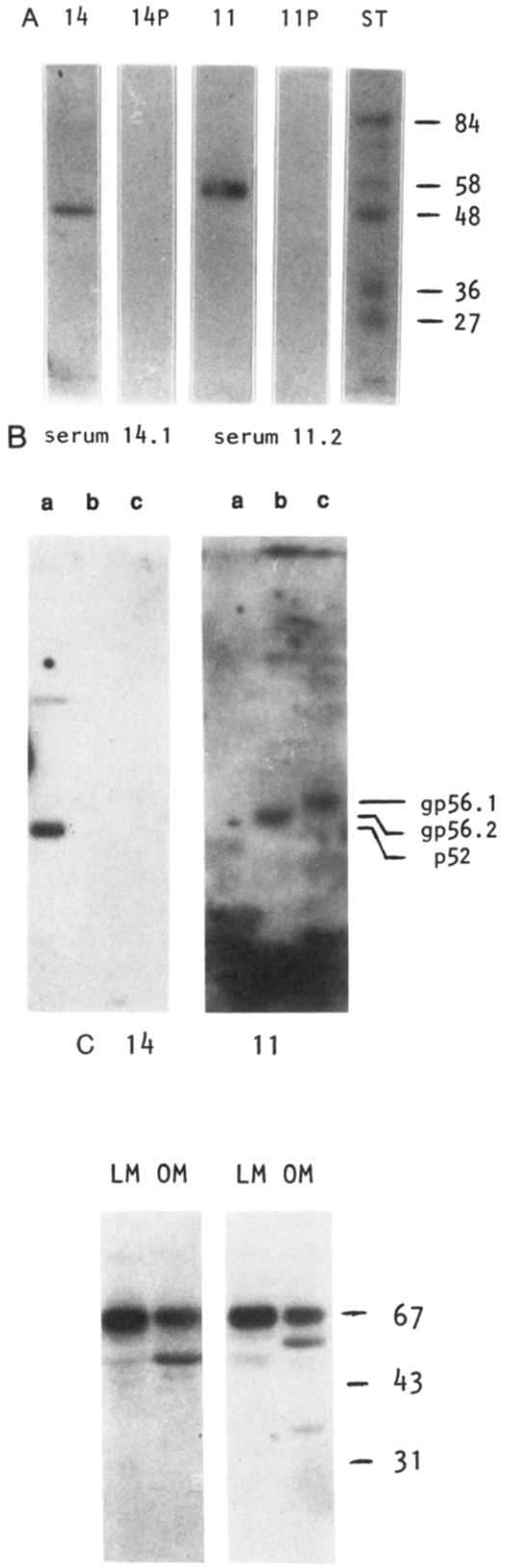

FIGURE 5: Immunoblotting analysis with antipeptide antisera. (A) Western blot of the detergent phase of a Triton X-114 extract of membranes from deciliated bovine olfactory epithelium, reacted with the following rabbit antisera: 14, serum 14.1 of a rabbit immunized with peptide DP19 (derived from p52); 14P, its corresponding preimmune serum; 11, serum of rabbit immunized with peptide DP21 (derived from gp56); 11P, its corresponding preimmune serum; ST, prestained blotted molecular mass standards. (B) Western blot of purified p52 (a), gp56.2 (b), and gp56.1 (c) with rabbit antisera 14.1 and 11.2 as marked [see (A)]. (C) Western blot of rat liver microsomes (LM) and rat olfactory microsomes (OM), reacted with rabbit sera 14.1 and 11.2, as marked [see (A)].

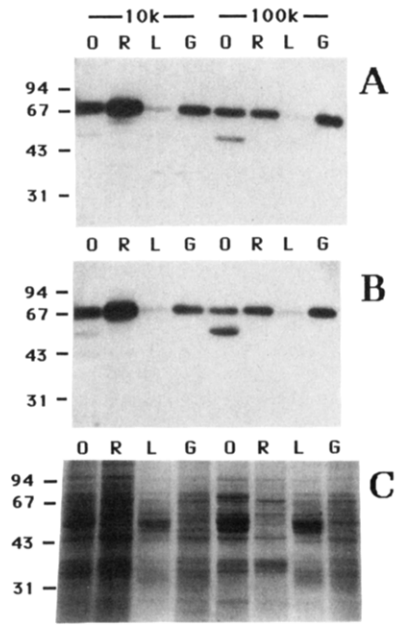

FIGURE 6: Tissue specificity of the p52 and gp56 antisera. Western blots of SDS–PAGE-separated proteins from membrane preparations from various bovine tissues probed with (A) serum 14.1 to peptide DP19 derived from p52, the cytochrome P-450 polypeptide, and (B) serum 11.2 to peptide DP21 derived from gp56, the UDPGT polypeptide. The Triton X-114 extract detergent phases of the plasma membrane fraction (10000*g* pellet, 10k) and of a microsomal fraction (100000*g* pellet, 100k) are shown for all tissues. (C) Coomassie Brilliant Blue staining of corresponding lanes in an identical unblotted gel. O, olfactory epithelium; R, respiratory epithelium; L, liver; G, lung. The band at 67 kDa seen in all tissues except liver is most probably serum albumin, strongly reacting with the antisera because BSA has been used as a carrier in the immunization.

membranal glycoproteins specific to olfactory epithelium (Lancet, 1986; Lancet & Pace, 1987; Lancet et al., 1988). The presently reported amino acid sequence results allowed us to identify p52 and gp56 as cytochrome P-450 and UDP-glucuronosyl transferase, respectively. Thus, while these two proteins represent the first examples of membrane-associated olfactory-specific proteins identified by sequence analysis, they do not seem to bear a relation to the long-sought olfactory receptor proteins. It should be noted, however, that cytochrome P-450 enzymes may share some hypothetical properties with odorant receptors (Margolis, 1987; Lancet et al., 1988).

One of the sequenced peptides reported here, peptide 19, was obtained from a preparation containing a mixture of three different proteins, namely, gp56.1, gp56.2, and p52. This peptide is highly homologous to sequences of proteins belonging to the cytochrome P-450IIA subfamily. This suggests that peptide 19 is derived from a cytochrome P-450IIA protein. Using antibodies directed against a synthetic peptide corresponding to peptide 19, we show that this peptide is most probably derived from protein p52. The apparent molecular mass of p52, its lack of glycosylation, its transmembranal disposition, and its microsomal fraction enrichment are consistent with the hypothesis that it is a member of the cytochrome P-450 superfamily [cf. Black and Coon (1987)].

Our electrophoretic and immunoblotting analyses show that p52 is abundantly present in deciliated olfactory epithelial membranes and in olfactory epithelial microsomes, but not in membranes prepared from other tissues, including respiratory epithelium, lung, and liver. Another type of olfactory-specific cytochrome P-450 recently reported is cytochrome P-450olf1, which defines a new subfamily, cytochrome P-450IIG. This species was identified by us through cDNA cloning, and its specificity to olfactory tissue was determined by RNA hy-

bridization analysis (Nef et al., 1989). We propose the trivial name cytochrome P-450olf2 for the cytochrome P-450IIA protein (p52) reported here.

Ding and Coon (1988) have reported the purification and partial amino acid sequencing of two species of cytochrome P-450 in rabbit nasal epithelium (NMa and NMb). A comparison of the reported N-terminal sequences shows that NMa is more similar (61%) to rat lung cytochrome P-450IIA3 (Kimura et al., 1989) than to any other rabbit or rat cytochrome P-450 sequence (<40%). As peptide 19, derived from p52, is nearly identical with a sequence found in cytochrome P-450IIA3, it is likely that cytochrome P-450olf2 (p52) is the bovine ortholog of rabbit NMa and of rat cytochrome P-450IIA3. Parallel sequence comparison shows that the amino-terminal segment of rat P-450olf1 (P-450IIG; Nef et al., 1989) is 76% identical with rabbit NMb (Ding & Coon, 1988). Thus, our results agree with those of Ding and Coon (1988), suggesting that nasal epithelia contain at least two types of cytochrome P-450, which respectively belong to subfamilies IIA and IIG.

It should be noted, however, that while both Ding and Coon (1988) and Nef et al. (1989) agree on the specificity of the cytochrome P-450IIG protein to olfactory tissue, the tissue distribution of the cytochrome P-450IIA protein is not straightforward. NMa is reported to be present in both olfactory and respiratory nasal epithelia; cytochrome P-450IIA3 was specifically localized in lung, but its presence in nasal tissue was not examined. Cytochrome P-450olf2 is reported here to be highly enriched in olfactory epithelium, but could not be detected in lung or respiratory nasal epithelium. Such species differences in the tissue distribution of cytochrome P-450IIA3 orthologs were previously reported (Kimura et al., 1989), and should be further investigated.

The present report suggests that glycoprotein gp56 is a member of the UDP-glucuronosyl transferase (UDPGT) superfamily. This is based on the high degree of sequence identity between three of the four gp56 peptides and known UDPGT sequences. Further support is provided by the fact that gp56 is a transmembranal glycoprotein with an electrophoretic molecular mass in the range of 50–60 kDa, features shared by all reported UDPGT enzymes (Siest et al., 1987). The localization of gp56 to the microsomal fraction of olfactory epithelial membranes is also in agreement with this conclusion.

The exclusive localization of gp56 to the olfactory epithelium, as well as the fact that one of the four gp56 peptides shows no significant homology to UDPGT or to any other known sequence, suggests a novel, olfactory-specific UDPGT subtype. The sequence of the nonhomologous peptide (as well as of all three other gp56 peptides) is found within a bovine λgt11 full-length cDNA clone isolated by us (Lazard et al., unpublished results), which has an unequivocal homology to published UDPGT sequences, and truly represents a new member of this superfamily.

Cytochrome P-450 and UDPGT in the liver act upon small hydrophobic compounds, some of which are odorants. This suggests a possible role for their olfactory counterparts in metabolizing odorous compounds. Such biotransformation, which consists of hydroxylation by cytochrome P-450 followed by glucuronidation by UDPGT, could render odorants much more hydrophilic, and facilitate their clearance by mucus flow or through the bloodstream. Thus, the presently described olfactory-specific forms of cytochrome P-450 and UDPGT could play a role in rapid odor signal termination or in the long-term protection of the sensory neuroepithelium from toxic air-borne compounds.

Registry No. UDPGT, 9030-08-4; cytochrome P-450, 9035-51-2.

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Fluorescence and NMR Investigations on the Ligand Binding Properties of Adenylate Kinases

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Received August 16, 1989; Revised Manuscript Received April 2, 1990

ABSTRACT: A new system for measurement of affinities of adenylate kinases (AK) for substrates and inhibitors is presented. This system is based on the use of the fluorescent ligand α,ω-di[(3′ or 2′)-*O*-(*N*-methylanthraniloyl)adenosine-5′] pentaphosphate (mAP5Am), which is an analogue of the bisubstrate inhibitor diadenosine pentaphosphate (AP5A). It allows the determination of dissociation constants for any ligand in the range of 1×10^{-9} to 5×10^{-2} M. Affinities for different bisubstrate inhibitors (AP4A, AP5A, AP6A) and substrates (AMP, ADP, ATP, GTP) were determined in the presence and absence of magnesium. An analysis of the binding of bisubstrate inhibitors is proposed and applied to these data. The techniques are used to describe the properties of a mutant enzyme with Gln-28 → His (Q28H) prepared by site-directed mutagenesis in comparison to those of wild-type AK from *Escherichia coli*. This newly introduced histidine is already present in most other adenylate kinases and was regarded to be important or even essential for the catalytic reaction of AK. Temperature denaturation experiments indicate that the mutant enzyme has the same thermal stability as the wild-type enzyme and, as NMR studies indicate, also a very similar structure. However, steady-state catalytic studies and binding experiments showed that the affinities for substrates and inhibitors are elevated from 3-fold (AMP) to 5-fold (ATP) to 15-fold (AP5A) compared to those of the wild-type enzyme. Together with the results obtained by Tian et al. [Tian, G., Sanders, C. R., Kishi, F., Nakazawa, A., & Tsai, M.-D. (1988) *Biochemistry 27*, 5544-5552] on the effect of replacement of the conserved His-36 in the cytosolic AK (AK1) from chicken by glutamine and asparagine, this shows that residues 28 of AK from *E. coli* (AKec) and 36 of AK1 are situated in a comparable environment and are not essential for catalytic activity.

The enzyme adenylate kinase (AK)[1] catalyzes the transfer of the γ-phosphate group of the "phosphate donor" (Mg·ATP) to the "phosphate acceptor" (AMP). It is one of the smallest phosphotransferases yet known. The ubiquitous presence of highly homologous forms of this enzyme in many different organisms reflects its important role in maintaining the energy charge of cells (Noda, 1973; Schulz et al., 1986). It was shown by Konrad (1988) that disruption of the yeast adk1 gene did not affect viability of the cells but that it is needed for normal cell proliferation. The fact that these cells still have about 10% of adenylate kinase activity compared to wild-type cells leads to the question of whether AK is absolutely essential for cell metabolism.

The mammalian cytosolic forms of pig and rabbit adenylate kinases (AK1) obey a random bi-bi rapid equilibrium kinetic mechanism, where the rate-limiting steps in catalysis were assigned to interconversion of the ternary complex between protein and substrates (Su & Russell, 1968) or to the dissociation of products (Rhoads & Lowenstein, 1968) from the protein. The stereochemical course of the catalyzed reaction was shown to follow an S_N2 mechanism, which suggests a direct transfer of the phosphoryl group without covalent enzymatic intermediates (Richard & Frey, 1978).

Despite the fact that numerous NMR experiments have been performed [Rösch et al., 1989; McDonald & Cohn, 1975; Nageswara Rao & Cohn, 1978; for a review, see Mildvan

[1] Abbreviations: AK, adenylate kinase (EC 2.7.4.3); AKec, adenylate kinase from *E. coli*; AKy, adenylate kinase from yeast; AK1, mammalian cytosolic enzyme; AMP, ADP, and ATP, adenosine 5′-mono-, 5′-di-, and 5′-triphosphate; AP*n*A, P^1,P^n-di(adenosine-5′) *n*-phosphate; mAP5Am, α,ω-di[(3′ or 2′)-*O*-(*N*-methylanthraniloyl)adenosine-5′] pentaphosphate; mAP5A, α-[(3′ or 2′)-*O*-(*N*-methylanthraniloyl)adenosine-5′] ω-(adenosine-5′) pentaphosphate; mdATP, 3′-*O*-(*N*-methylanthraniloyl)-2′-deoxyadenosine 5′-triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; CD, circular dichroism; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; Q28H, mutant adenylate kinase from *E. coli* with a Gln-28 → His substitution.

0006-2960/90/0429-7440$02.50/0