Purification and characterization of three constitutive cytochrome *P*-450 isoforms from bovine olfactory epithelium

Vincenzo LONGO*, Giada AMATO*, Annalisa SANTUCCI† and Pier Giovanni GERVASI*‡

*Laboratory of Genetic and Biochemical Toxicology, Istituto di Mutagenesi e Differenziamento, CNR, via Svevia 10, 56124 Pisa, Italy, and †Department of Molecular Biology, Siena University, Policlinico Le Scotte, 53100 Siena, Italy

Three constitutive forms of cytochrome *P*-450 (*P*-450s) were isolated from olfactory microsomes of cattle. The purified *P*-450s, designated *P*-450bov1, *P*-450bov2 and *P*-450bov3, were electrophoretically nearly homogeneous by SDS/PAGE and their apparent relative molecular masses were estimated to be 50000, 53000 and 51000 respectively. As indicated by several criteria including the N-terminal sequence and absorption spectra, the three olfactory forms of *P*-450 were distinct from each other and from all the other *P*-450s currently known in cattle. *P*-450bov1 and *P*-450bov2 were purified in the low-spin state, whereas *P*-450bov3 was in the high-spin state. Studies to evaluate, by Western blot analysis, the reactivity of these purified *P*-450s with antibodies raised against rat hepatic *P*-450 2E1, 2B, 1A and 3A and rabbit olfactory *P*-450NMa and *P*-450NMB showed that *P*-450bov3 strongly cross-reacted with anti-*P*-450NMB IgG, and *P*-450bov1 moderately with anti-*P*-450NMa IgG. As determined by immunoblots, *P*-450bov1 and *P*-450bov3 represented a great portion of the total olfactory *P*-450. In a

reconstituted system with NADPH:cytochrome *P*-450 reductase and phospholipids, *P*-450bov1 was more active in the metabolism of xenobiotic compounds (i.e. *O*-de-ethylation of ethoxycoumarin and *N*-demethylation of hexamethylphosphoramide) than towards endogenous substrates (testosterone and progesterone). Conversely, *P*-450bov3 metabolized the xenobiotics at lower rates but exhibited total oxidation rates of the above sex hormones higher than those of *P*-450bov1. From the comparison of the catalytic, immunochemical and structural properties, it was inferred that *P*-450bov1 and *P*-450bov3 are the bovine orthologues of *P*-450NMa (2A) and *P*-450NMB (2G1) respectively, the only two olfactory *P*-450s previously purified from rabbit. *P*-450bov2, which showed low activity toward some exogenous and endogenous compounds, represents a novel purified olfactory hemoprotein possibly belonging to the 3A subfamily. These results are consistent with a specific presence of catalytically and structurally similar *P*-450s, at least for the major ones, in the olfactory mucosa of mammals.

INTRODUCTION

The cytochromes *P*-450 (*P*-450s) are responsible for the metabolism of a wide array of xenobiotics as well as certain endogenous compounds [1,2]. They are predominantly localized in the endoplasmic reticulum of liver cells but are also present in the extrahepatic tissues and particularly in the nasal mucosa [1,3,4]. In fact, nasal mucosa and especially the olfactory epithelium exhibit *P*-450-dependent mono-oxygenase activities that are in many cases comparable to or even much greater than the activities in liver [5,6]. Nasal *P*-450 enzymes seem to differ from those in the liver for catalytic activity, substrate specificity and mechanism of regulation [6–8].

Although several *P*-450 enzymes have been characterized in the liver, at both the protein and the DNA level, relatively little is known about their nasal counterparts [9]. So far only two forms of cytochrome *P*-450 protein, the rabbit olfactory *P*-450NMB and *P*-450NMa, have been isolated from nasal tissues [10]. *P*-450NMB, designated CYP2G1 [9], has been localized by immunochemical studies only in the olfactory epithelium and not in other tissues [11]. A tissue-specific expression of mRNA CYP2G1 in the olfactory mucosa has also been observed in several mammalian species including cattle and rat [12].

A polypeptide, termed *P*-450olf2 or p52, was identified in a detergent-solubilized membrane fraction from bovine olfactory epithelium [13] and suggested to be an orthologue of *P*-450NMa, but the functional haemoprotein was not isolated.

From a screening of a cDNA library from rabbit respiratory

epithelium with antibodies anti-*P*-450NMa, two distinct *P*-450 genes, named CYP2A10 and CYP2A11, were isolated and expressed in *Escherichia coli* [14]. They were highly similar in nucleotide and deduced amino acid sequences (98.4% similarity) sharing the same N-terminal sequence in the first 20 amino acid residues of purified *P*-450NMa. Thus the purified *P*-450NMa contained two proteins.

It is largely accepted that high levels of *P*-450 in the olfactory mucosa are important for maintaining acuity in the sense of smell, but it is not understood why tissue-specific *P*-450s, such as 2G1, are present in this tissue [12,15].

Studies of isolation and characterization of nasal *P*-450 enzymes in other species may assist in an understanding of the role of *P*-450s in the olfaction and in the clearance of xenobiotics including odorants. However, owing to the small amount of nasal tissue it is hard to purify the *P*-450 forms from olfactory epithelium of small animals. The purification of constitutive olfactory *P*-450 enzymes seems more feasible in large animals such as cattle.

Some years ago we reported a study on the drug-metabolizing enzymes in the olfactory and respiratory epithelia of cattle [16]. The results demonstrated that in the bovine olfactory epithelium, *P*-450 content and several mono-oxygenase activities are highly expressed. In the present study, we purified three *P*-450 enzymes, termed *P*-450bov1, *P*-450bov2 and *P*-450bov3, from olfactory epithelium of cattle and characterized some of their catalytic, immunological and structural features.

Abbreviations used: *P*-450s, cytochromes *P*-450; DLPC, dilaurylphosphatidylcholine; DTT, dithiothreitol; 15 α -OH, 16 α -OH, 6 α -OH, 2 α -OH, 7 α -OH, 6 β -OH, 15 β -OH, 16 β -OH and 2 β -OH correspond to 15 α -, 16 α -, 6 α -, 2 α -, 7 α -, 6 β -, 15 β -, 16 β - and 2 β -hydroxytestosterone respectively; 17-OT, androstenedione.

‡ To whom correspondence should be addressed.

EXPERIMENTAL

Chemicals

Sephacrose 4B was purchased from Pharmacia (Uppsala, Sweden), DEAE 52-cellulose, DEAE 53-cellulose and DEAE-Sephacrose CL6B were from Whatman Biosystem (Clifton, NJ, U.S.A.), and hydroxyapatite was obtained from Clarkson Co. (Williamsport, PA, U.S.A.). Emulgen 911 was obtained from KAO Corp. (Tokyo, Japan). Sodium cholate, progesterone, 16 α -, 11 α -, 11 β - and 17 α -hydroxyprogesterone were purchased from Sigma (St. Louis, MO, U.S.A.); 21-, 2 α - and 6 β -hydroxyprogesterone were obtained from Steraloids. (Wilton, NH, U.S.A.). 7-Ethoxycoumarin was obtained from EGA Chemie (Steinheim, Germany). Diethylnitrosamine, hexamethylphosphoramide, haematin, coumarin and acrylamide were obtained from Fluka (Buchs, Switzerland). Ethoxyresorufin was synthesized from resorufin by ethylation with ethyl iodide [17]. Benzphetamine was supplied by the Upjohn Co., (Kalamazoo, MI, U.S.A.). Testosterone and its metabolites were obtained as previously reported [16]. Enzymes and coenzymes were obtained from Boehringer (Mannheim, Germany). Rabbit polyclonal antibodies against rat *P*-450 2B₁, 1A and 2E1 were obtained in our laboratory as previously described [18]. Rabbit polyclonal antibodies against rat *P*-4503A were purchased from Oxygene (Dallas, TX, U.S.A.). Sheep polyclonal antibodies against rabbit *P*-4502G1 (or *P*-450NMb) and rabbit polyclonal antibodies against rabbit *P*-450NMa were kindly donated by Dr. X. Ding (Albany, NY, U.S.A.). Reagents for development by the alkaline phosphatase method were obtained from ICN (Costa Mesa, CA, U.S.A.). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Methods

The protein concentration of the purification fractions was determined by an assay that accounted for the presence of detergents [19], with BSA as standard. *P*-450 was determined as described by Omura and Sato [20]. SDS/PAGE was performed by the method of Laemmli [21]. The immunoblot analysis of microsomal *P*-450 was performed in accordance with previously published procedures [8]. Enzyme assays of purified *P*-450s were conducted under saturating conditions of NADPH and NADPH:cytochrome *P*-450 reductase and at an optimal dilaurylphosphatidylcholine (DLPC) concentration. The system was generally constituted as follows: 0.1 μ M each purified *P*-450, 0.3 μ M NADPH:cytochrome *P*-450 reductase and 30 μ g/ml DLPC, added as freshly sonicated micelles. The individual components of the reconstituted system were mixed and pre-incubated for 1 h at room temperature before adding NADPH (1 mM) or an NADPH-generating system and substrate in a final volume of 1 ml of 100 mM potassium phosphate buffer, pH 7.4. In some reconstitution system experiments, DLPC was replaced with 30 μ g of fresh olfactory phospholipids obtained by the Folch method [22].

Aniline *p*-hydroxylase activity was determined by measuring the amount of *p*-aminophenol formed, as described by Ko et al. [23]. 7-Ethoxycoumarin O-de-ethylase and coumarin 7-hydroxylase activities were assayed by the fluorimetric determination of 7-hydroxycoumarin, as described by Aitio [24]. 7-Ethoxyresorufin O-de-ethylase was measured by the formation of the resorufin in a Perkin-Elmer spectrofluorimeter [25]. Formaldehyde derived from benzphetamine and hexamethylphosphoramide was quantified by the method of Tu and Yang [26]. Testosterone and progesterone hydroxylase activities were assayed by a HPLC method as described by Platt et al. [27] and

Swinney et al. [28] respectively. Sequence analysis and amino acid composition analysis were performed as previously described [29].

Isolation of microsomes and purification of *P*-450s

The olfactory epithelium (yellow-brown), covering the nasal ethmoturbinoids and septum, was removed by dissection from 20 bovine heads (male Limousine 14–24 months old, supplied by the municipal slaughterhouse of the Pisa area). Within 2 h of animal death, the tissue was collected and frozen in liquid nitrogen, followed by storage at -80°C until use. The tissue was homogenized with an Ultra-Turrax homogenizer in 0.1 M Tris/HCl buffer, pH 7.4, containing 1.15% KCl, 0.1 mM PMSF, 1 mM dithiothreitol (DTT) and 1 mM EDTA. The microsomes were prepared by differential centrifugation [29] and solubilized (2.5 mg/ml protein) with 0.6% ethanol-recrystallized sodium cholate in 0.1 M potassium phosphate buffer, pH 7.25, containing 1 mM DTT, 0.1 mM PMSF, 1 mM EDTA and 20% (v/v) glycerol, for 30 min, after which the mixture was centrifuged for 40 min at 100 000 *g*. All the microsomal preparations and *P*-450 purification steps were performed at 4°C .

The 100 000 *g* supernatant, containing 275 nmol of *P*-450 and 108 nmol of cytochrome *P*-420, was applied to an n-octylamine-Sepharose 4B column (2.5 cm $\times$ 21 cm) equilibrated with the above buffer. After sample application, the column was washed with 500 ml of 0.1 M potassium phosphate buffer, pH 7.25, containing 0.42% sodium cholate, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol and 0.1 mM PMSF. Most of the cytochrome *P*-420 and other non-binding proteins were washed away.

The column was then eluted with the same buffer containing 0.33% sodium cholate and 0.06% Emulgen 911. This fraction (fraction I) contained 95 nmol of *P*-450. Successively another fraction (fraction II), containing 78 nmol of *P*-450, was eluted with 0.6% Emulgen 911. *P*-450bov1 was obtained from fraction I, and *P*-450bov2 and *P*-450bov3 from fraction II (see Table 1). Fraction I was concentrated by ultrafiltration and dialysed against 10 mM potassium phosphate buffer, pH 7.25, containing 1 mM DTT, 1 mM EDTA, 0.01 mM PMSF, 0.3% sodium cholate and 20% (w/v) glycerol and loaded on a hydroxyapatite column (1.5 cm $\times$ 8 cm) equilibrated with the same buffer. The column, after washing, was eluted stepwise with 40, 100 and 500 mM potassium phosphate, pH 7.25, containing the same remaining components as the dialysis buffer. The eluate at 40 mM phosphate, enriched in *P*-450s, was brought to 0.6% in Emulgen 911 and dialysed against 5 mM potassium phosphate buffer, pH 7.7, containing 1 mM DTT, 1 mM EDTA, 0.01 mM PMSF and 20% (w/v) glycerol, and applied to a Whatman DEAE 53-cellulose anion-exchange column (1.5 cm $\times$ 10 cm) equilibrated with dialysis buffer containing 0.6% Emulgen 911. A fraction, enriched in *P*-450bov1, was eluted in the flow-through. A second fraction, containing the other two proteins, was eluted from the column by extensive washing with equilibration buffer. The fraction containing *P*-450bov1 was concentrated and applied to a DEAE-Sepharose CL column (1.5 cm $\times$ 8 cm) equilibrated with the same buffer as used in the previous step. *P*-450bov1, purified to near-homogeneity, as judged by electrophoretic analysis, was recovered again in the flow-through. To separate the two proteins obtained from the DEAE 53-cellulose column by extensive washing, we tried a DEAE-Sepharose CL6B and successively a CM-cellulose cation-exchange column. However, the two proteins did not bind to either column and at the end we again obtained two proteins with one predominating. Successively, the two proteins were electrotransferred to a poly(vinylidene difluoride) membrane and

the predominant one, named olfprot I, was analysed for the N-terminal sequence (Met-Ser-Arg-Ala-His-Ile-Asn-Tyr-Gly-Val-Arg-Gly-Asp-Val-Ala-Val-Ile-Arg-Ile-Asn) and was found not to be a *P*-450 protein. An analysis of the protein sequences reported in the GenBank database did not allow us to recognize olfprot I as a previously known protein.

Fraction II was concentrated and dialysed against 5 mM potassium phosphate buffer, pH 7.7, containing 1 mM DTT, 1 mM EDTA, 0.01 mM PMSF and 20 % (w/v) glycerol and loaded on a Whatman DEAE 52-cellulose anion-exchange column (1.5 cm × 10 cm) equilibrated with dialysis buffer containing 0.6 % Emulgen 911.

The column was washed with equilibration buffer; approx. 19 nmol of *P*-450 was eluted in the flow-through. The remaining portion of the proteins, containing only traces of *P*-450, was eluted with a linear gradient of KCl and discarded. The fraction eluted in the flow-through, enriched in the forms that we designated *P*-450bov2 and *P*-450bov3, was dialysed against 3 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTT, 1 mM EDTA, 0.01 mM PMSF and 20 % (w/v) glycerol, and applied to a DEAE-Sepharose CL6B column (1.5 cm × 8 cm) equilibrated with dialysis buffer containing 0.6 % Emulgen 911. The column was washed with equilibration buffer; a fraction containing *P*-450bov2 was recovered in the flow-through, whereas a fraction containing *P*-450bov3 was eluted with the same buffer containing 50 mM potassium phosphate.

At this stage *P*-450bov2 and *P*-450bov3 had been purified to near-homogeneity as judged by electrophoretic analysis. The Emulgen 911, which absorbed at 280 nm, was removed from the purified isoenzymes by chromatography on hydroxyapatite. After this treatment only traces of Emulgen 911 were still present [30].

RESULTS

Purification of *P*-450s

The procedure used for the isolation of olfactory *P*-450s is an application of the classical method used for the purification of *P*-450 isoenzymes from liver of various animals [29]. A summary of a typical purification of *P*-450bov1, *P*-450bov2 and *P*-450bov3 from olfactory epithelium microsomes of cattle is given in Table 1 and described in detail in the Experimental section. *P*-450bov1, *P*-450bov2 and *P*-450bov3 were purified 31.6-fold, 21-fold and 34.2-fold respectively. Their specific *P*-450 content, however, was low, possibly owing to a loss of haem during the purification. The

Figure 1 Gel electrophoresis of microsomal proteins and of *P*-450bov1, *P*-450bov2 and *P*-450bov3 at some stages of purification

Lane 1, bovine olfactory microsomes; lane 2, fraction I eluted from n-octylamine-Sepharose 4B column; lane 3, fraction II eluted from n-octylamine-Sepharose 4B column; lane 4, *P*-450bov1; lane 5, *P*-450bov2; lane 6, *P*-450bov3; lane 7, M_r standards: BSA (M_r 68 000), catalase (M_r 60 000), glutamate dehydrogenase (M_r 53 000) and fumarase (M_r 49 000).

addition of haematin to the purified proteins, was unable to increase their specific *P*-450 content.

The SDS/PAGE profile of some steps of the purification is shown in Figure 1. A comparison of the relative mobilities of purified haemoproteins with the M_r values of the protein standards under different electrophoretic conditions indicated the following apparent minimal M_r values for the purified enzymes: 50 000 for *P*-450bov1, 53 000 for *P*-450bov2 and 51 000 for *P*-450bov3.

Spectral properties and N-terminal sequences

The spectrum of oxidized *P*-450bov1 and *P*-450bov2 showed absorbance maxima at 416, 535 and 570 nm. These peaks are indicative of low-spin ferric haemoproteins. In contrast, the oxidized spectrum of *P*-450bov3 had absorbance maxima at 392, 545 and 650 nm, characteristic of a high-spin, pentaco-ordinate state. When reduced in the presence of CO, all the purified cytochromes had a spectral maximum at 450–451 nm. On re-

Table 1 Purification of *P*-450bov1, *P*-450bov2 and *P*-450bov3 from olfactory microsomes of cattle

Abbreviation: KP, potassium phosphate.

Fraction	Total protein (mg)	Total <i>P</i> -450 (nmol)	Specific <i>P</i> -450 content (nmol/mg)	Purification (fold)	Yield (%)
Solubilized microsomes	1450	275	0.19	1	100
<i>P</i> -450bov1					
n-Octylamine-Sepharose 4B fraction I eluate	136	95	0.7	3.7	34.5
Hydroxyapatite 40 mM KP eluate	60	58	0.97	5.1	21
DEAE 52-cellulose flow-through eluate	10	25	2.5	13.1	9
DEAE-Sepharose CL6B flow-through eluate	0.67	4	6	31.6	1.4
<i>P</i> -450bov2 and <i>P</i> -450bov3					
n-Octylamine-Sepharose 4B fraction II eluate	100	78	0.78	4.1	28.4
DEAE 52-cellulose flow-through eluate	10.7	18.9	1.76	9.3	6.9
DEAE-Sepharose CL6B					
Flow-through eluate (<i>P</i> -450bov2)	0.33	1.3	4	21	0.47
50 mM KP eluate (<i>P</i> -450bov3)	0.67	4.4	6.5	34.2	1.6

Table 2 N-terminal amino acid sequences of *P*-450bov1, *P*-450bov2, *P*-450bov3 and, for comparison, rabbit olfactory *P*-450NMa and *P*-450NMb [10]

No.	<i>P</i> 450bov1	<i>P</i> 450NMa	<i>P</i> 450bov2	<i>P</i> 450bov3	<i>P</i> 450NMb
1	Met	Met	Met	Met	Met
2	Glu	Leu	Asp	Glu	Glu
3	Ala	Ala	Pro	Leu	Leu
4	Ser	Ser	Val	Phe	Gly
5	Gly	Gly	Ile	Ile	Gly
6	Leu	Leu	Asn	Ala	Ala
7	Leu	Leu	Asp	Phe	Phe
8	Leu	Leu	Ala	Thr	Thr
9	Val	Ala	Val	Ile	Ile
10	Ala	Ala		Phe	Phe
11	Leu	Leu		Leu	Leu
12	Leu	Leu		Ala	Ala
13	Ala	Ala		Leu	Leu
14	Leu	Ser			
15	Leu	Leu			
16	Leu	Thr			
17	Ile	Val			

duction with dithionite all the *P*-450s exhibited a Soret peak at 412–413 nm. The absolute oxidized spectra of *P*-450bov1, *P*-450bov2 and *P*-450bov3 did not change in the presence of a detergent such as Emulgen 911.

The N-terminal amino acid sequences of cytochromes *P*-450bov1, *P*-450bov2 and *P*-450bov3 as determined by Edman degradation are listed in Table 2. Only one N-terminal sequence was obtained for each sample, indicating that the proteins were not contaminated with other proteins except, perhaps, with *P*-450s with the same M_r and the same N-terminal sequences. The composition of the N-terminal sequences of the three purified *P*-450s consisted largely of hydrophobic amino acid residues typical of *P*-450s. When the N-terminal sequences of *P*-450bov1 and *P*-450bov3 were compared with those of olfactory rabbit NMa and NMb [10] a high degree of similarity was observed: 12 out of 17 (70 % identity) and 11 out of 13 (85 % identity) respectively. This suggests that bov1 and bov3 represent the bovine homologues of rabbit NMa and NMb respectively.

In contrast, the composition of *P*-450bov2 was different. The presence of two Asp residues with a negative charge in the N-terminal sequence in this haemoprotein could indicate that it belongs to the 3A family, known to have this protein motif [31].

Immunological studies

In further characterization of *P*-450bov1, *P*-450bov2 and *P*-450bov3, we evaluated the reactivity of these three *P*-450 forms by Western blot analysis (results not shown) with polyclonal antibodies raised against rat hepatic *P*-450 2E1, 2B1, 1A1, 3A1 and rabbit olfactory *P*-450 NMb and NMa (Table 3). *P*-450bov1 cross-reacted weakly, as observed by immunochemical stain, with various rat hepatic antibodies to *P*-450s. *P*-450bov2 stained moderately with anti-*P*-4503A1 and weakly with anti-*P*-4501A. The anti-*P*-450NMb (or anti-*P*-4502G1) showed no reactivity towards these two *P*-450s but reacted strongly with the purified *P*-450bov3. Moreover, *P*-450bov3 was also recognized weakly by anti-*P*-450 2E1 and 2B1. Anti-NMa recognized *P*-450bov1, but also cross-reacted with a similar intensity with *P*-450bov3.

With the purified *P*-450bov1 and *P*-450bov3 as standards for the titration of olfactory *P*-450s with anti-NMb and anti-NMa, a provisional and cautious estimate of their microsomal content was made. It was found that *P*-450 bov1 and bov3 accounted for

Table 3 Western blot analysis of purified bovine *P*-450s and olfactory microsomes with antibodies raised against hepatic *P*-450 2E1, 2B, 1A and 3A of rat, and olfactory *P*-450 NMb and NMa of rabbit

P-450bov1, *P*-450bov2 and *P*-450bov3 and bovine olfactory microsomes were subjected to SDS/PAGE on 7.5 % (w/v) polyacrylamide gel, transferred to nitrocellulose and probed with the antibodies described above. Staining indicated as follows: +, weak staining; ++, moderate staining; +++, intense staining; n.d., no stain detected.

Antibodies directed against	<i>P</i> 450bov1	<i>P</i> 450bov2	<i>P</i> 450bov3	Olfactory microsomes
<i>P</i> 4502E1	+	n.d.	+	+
<i>P</i> 4502B1	+	n.d.	+	+
<i>P</i> 4501A1	+	+	n.d.	++
<i>P</i> 4503A1	+	++	n.d.	+
<i>P</i> 450NMb	n.d.	n.d.	+++	+++
<i>P</i> 450NMa	++	n.d.	++	++

approx. 16 % and 11 % respectively (on the basis of their protein content) of the total *P*-450 in olfactory microsomes of cattle.

Anti-1A1 cross-reacted with a olfactory protein with a M_r of 52000. The nature of this protein remains to be established. However, it should be remembered that an orthologue *P*-4501A2 and not 1A1 was found in the olfactory epithelium of rabbit and rat [4]. In contrast, each of the antibodies raised against rat *P*-450 2E1, 2B1 and 3A1 weakly stained, in the olfactory microsomes, a single protein, suggesting that the recognized *P*-450s are poorly expressed in olfactory mucosa or alternatively they have different epitopes from their rat counterparts. Without the availability of these olfactory bovine proteins it is not possible to discriminate between these two possibilities.

Catalytic activity of purified *P*-450s

The results of catalytic studies conducted with each purified *P*-450 toward some substrates in a reconstituted mono-oxygenase system are presented in Table 4; the activities with olfactory and hepatic microsomes toward the same substrates are also reported. The limited amount of *P*-450bov2 obtained during purification did not permit us to assay all the substrates used with *P*-450bov1 and *P*-450bov3. *P*-450bov1 showed a good activity in the N-demethylation of hexamethylphosphoramide, the O-de-ethylation of 7-ethoxycoumarin and the oxidation of coumarin. In particular, it metabolized 7-ethoxycoumarin with a rate that was 57-fold and 150-fold that of *P*-450bov3 and *P*-450bov2 respectively. Both benzphetamine and diethylnitrosamine were efficiently catalysed by *P*-450bov1 and *P*-450bov3, but only at low level by *P*-450bov2. Oxidation of aniline was observed with *P*-450bov3 but not with *P*-450bov1. Furthermore low activities towards ethoxyresorufin were measured with *P*-450bov1 and *P*-450bov3. As *P*-450bov2 cross-reacted with anti-*P*-4503A1, we also determined its ability to catalyse the erythromycin demethylation considered to be selective for the *P*-4503A family [32]: no activity was observed. When the olfactory phospholipids were used in place of DLPC in a reconstituted system, the 7-ethoxycoumarin de-ethylase activity with all the three purified *P*-450s was not significantly enhanced. The catalytic activities of *P*-450 bov1, bov2 and bov3 toward steroid sex hormones such as testosterone and progesterone were also examined and compared with those of olfactory microsomes (Table 5). The testosterone or progesterone metabolites were identified on the basis of co-migration with standards in HPLC analysis. Regarding testosterone, 10 different metabolites were formed by olfactory microsomes, as we have shown previously [16].

Table 4 Catalytic activity of purified *P*-450s, and olfactory and hepatic microsomes

Abbreviations: HMPA, hexamethylphosphoramide; DEN, diethylnitrosamine; n.d., not determined.

Substrate	Activity (nmol/min per nmol of <i>P</i> -450)				
	<i>P</i> -450bov1	<i>P</i> -450bov2	<i>P</i> -450bov3	Olfactory microsomes	Hepatic microsomes
Aniline hydroxylase	< 0.05	n.d.	1.2	4	0.29
7-Ethoxycoumarin de-ethylase	23	0.15	0.4	30	1.1
Coumarin hydroxylase	0.6	n.d.	0.03	3.2	1.5
Benzphetamine demethylase	3.4	0.2	2.6	14.8	3.6
7-Ethoxyresorufin de-ethylase	0.06	n.d.	0.01	0.3	0.29
HMPA demethylase	13.8	n.d.	5.8	22	0.38
DEN de-ethylase	2	0.5	2.8	10	n.d.

Table 5 Oxidation of testosterone and progesterone by purified *P*-450s and olfactory microsomes

The reaction mixtures consisted of a reconstituted system, as described in the Experimental section, containing 0.5 mM testosterone or 0.05 mM progesterone as substrate. Values are averages of duplicate determinations. The metabolites X and Y might be 15-ketotestosterone and 16-ketoprogesterone respectively. Abbreviation: n.d., not determined.

Substrate	Position of substrate oxidation	Activity (nmol product/min per nmol of <i>P</i> -450)			Olfactory microsomes
		<i>P</i> -450bov1	<i>P</i> -450bov2	<i>P</i> -450bov3	
Testosterone	15 α	0.28	< 0.01	0.48	2.3
	6 β	< 0.01	0.13	< 0.01	2
	X	< 0.01	< 0.01	0.31	0.63
	16 α	< 0.01	< 0.01	0.16	0.96
	17	< 0.01	0.07	0.12	0.63
Progesterone	16 α	0.26	n.d.	2.9	1.8
	11 α	0.03	n.d.	< 0.01	0.13
	Y	< 0.01	n.d.	0.47	0.17
	21	0.04	n.d.	0.04	0.8
	17 α	< 0.01	n.d.	0.1	0.17

All the purified isoenzymes oxidized testosterone, although at various rates and in different positions. *P*-450bov1 catalysed the hydroxylation of testosterone mainly at the 15 α position, but an unidentified metabolite that ran close to 6 α -hydroxytestosterone (6 α -OH) with a rate of 0.2 nmol/min per nmol of *P*-450 was also observed. *P*-450bov2 oxidized testosterone at 6 β , 17 and a third unknown position with a rate of 0.22 nmol/min per nmol of *P*-450. *P*-450bov3 exhibited the capacity to oxidize testosterone to produce 15 α -hydroxytestosterone (15 α -OH), 16 α -hydroxytestosterone (16 α -OH) and androstenedione (17-OT) and a fourth unidentified X metabolite with a total rate significantly greater than that of *P*-450bov1 and *P*-450bov2. The unidentified metabolite X, formed specifically by bov3 and running very close to 6 β -hydroxytestosterone (6 β -OH), might be, by analogy with that reported for NMb [33], 15-ketotestosterone.

The catalytic-centre activities for the total testosterone oxidations were 0.48, 0.41 and 1.07 for *P*-450bov1, *P*-450bov2 and *P*-450bov3 respectively.

Besides the 15 α -OH, 16 α -OH and 17-OT metabolites formed by the three purified *P*-450s, the oxidation of testosterone by the

olfactory microsomes also yielded 6 α -OH, 15 β -OH, 6 β -OH, 16 β -OH, 2 α -OH, 2 β -OH and an unknown metabolite as previously reported [16]. In that study, we confused the 15 α -OH and 7 α -OH because they co-migrated under our previous HPLC conditions. Now it has been possible to separate them by using a Supercosil L-C₁₈ column under isocratic conditions with methanol/ acetonitrile/water (47.5:42.5:10, by vol.) and to identify 15 α -OH as the testosterone metabolite formed. When progesterone was studied, it was found that *P*-450bov1 metabolized this substrate poorly: predominantly (more than 70%) at the 16 α position but also at the 11 α and 21 positions.

P-450bov3 also formed primarily the 16 α -hydroxyprogesterone and in smaller amount an unidentified Y metabolite that might be 16-ketoprogesterone, by analogy with what was found with rabbit NMb [33]. Further, *P*-450bov3, at variance with NMb [33], resulted in trace amounts of 21-hydroxyprogesterone and 17 α -hydroxyprogesterone. This might have been due to contamination of *P*-450bov3, or alternatively this unique *P*-450 isoform expressed only in olfactory epithelium differs from NMb in some catalytic aspect, possibly reflecting a species-specific role in bovine olfactory chemoreception. The comparison of the progesterone total oxidation rates between these two isoenzymes showed that *P*-450bov3 was 10-fold more active than *P*-450bov1.

It is worth noting that *P*-450bov3 formed 16 α -hydroxyprogesterone more expeditiously than microsomes, suggesting that this enzyme might have a major responsibility for this reaction in the olfactory tissue. Other than the positions indicated in Table 5, olfactory microsomes catalysed the progesterone at the 6 β and 11 β positions with rates of 0.03 and 0.02 nmol/min per nmol of *P*-450 respectively. Furthermore the three purified *P*-450s did not produce all the testosterone or progesterone metabolites obtained with the olfactory microsomes, indicating that other important constitutive *P*-450 isoforms active towards sex hormones are present in the bovine olfactory mucosa. Almost all the activities examined by purified *P*-450 forms were lower than those in microsomes. This might be due to several causes, including: (1) the presence in our purified *P*-450 of a large amount of inactive *P*-450 apoprotein that might facilitate in the reconstituted system an aggregation of the olo-*P*-450; (2) the lack of some important components (i.e. cytochrome *b*₅, specific lipids, unknown cofactors); or (3) the inhibitory action of a very low concentration of Emulgen 911 on olfactory *P*-450s.

DISCUSSION

In the present study we purified to near-homogeneity three *P*-450 forms, designated *P*-450bov1, *P*-450bov2 and *P*-450bov3, from the olfactory epithelium of cattle and characterized some of their biochemical properties. The purified proteins had low specific *P*-450 content, probably due to a large presence of the corresponding *P*-450 apoprotein. A fourth protein, named olfprot I, was also isolated, but from its N-terminal sequence and its spectral properties it was found not to be a *P*-450 protein and was not further characterized.

The three *P*-450s, on the basis of their structural and biochemical features, are distinct from the few microsomal *P*-450s, a *P*-450 belonging to 2D subfamily [34] and the trivially named *P*-450A and *P*-450B [35], purified from any bovine tissue so far. However, the comparison of the N-terminal sequences of *P*-450bov1, *P*-450bov2 and *P*-450bov3 with published *P*-450 sequences revealed that *P*-450bov1 and *P*-450bov3 showed a similarity to *P*-450NMb and *P*-450NMb respectively, both purified from rabbit olfactory microsomes [10].

P-450bov1, as *P-450NMa*, was purified in the low-spin state and had an M_r of 50 000, whereas *P-450bov3*, as *P-450NMb*, was purified in the high-spin state and had an M_r of 51 000 [10]. The peptide map of *P-450 bov1* and *bov3* showed a pattern of fragments like that reported for *P-450 NMa* and *NMb* respectively [10] (results not shown).

The similarity between *P-450bov1* and *P-450NMa* and between *P-450bov3* and *P-450NMb* was further supported by immunoblot analysis with monospecific anti-*P-450NMa* and *P-450NMb* IgG (Table 3). In addition, *P-450bov1*, as *P-450NMa* [10], was found to be more active in the metabolism of xenobiotic compounds, such as 7-ethoxycoumarin and the nasal carcinogen hexamethylphosphoramide, rather than endogenous substances. It should be noted that the purified *P-450bov1* might contain more than one *P-450* protein, as *NMa*, expressed in the nasal tissue of rabbit, has been found to be composed of two highly similar *P-450s* (2A10 and 2A11) [14].

In contrast, *P-450 bov3*, as *NMb* compared with *NMa* [10,11], was more active than *P-450bov1* towards important endogenous compounds, such as the sex hormones testosterone and progesterone, known to influence olfactory sensitivity [36]. Of course, the definitive assignment of *P-450 bov1* and *bov3* awaits the cloning of related bovine genes.

With regard to *P-450bov2*, the analysis of its sequence alignment with the sequence of *P-450s* available in the GenBank database did not reveal any important similarity. However, the presence of a protein motif such as two negative charges in the N-terminal protein might indicate that *P-450bov2* is a member of the 3A family. In addition the immunological analysis suggests that *P-450bov2* might be the bovine homologue of the rabbit *P-450NM3c* identified by Ding and Coon [10] or the rat *P-450olf2*, reported by Nef et al. [37] and Nelson et al. [9], both belonging to the 3A subfamily.

P-450bov1 and *P-450bov3*, as determined by quantitative immunoblot analysis with anti-*NMa* and anti-*NMb*, represent a great portion of the *P-450* isoenzymes in the olfactory microsomes of cattle (at least 27% of total *P-450*). *P-450NMa* and *P-450NMb* were also reported to be the *P-450* forms more representative in rabbit olfactory mucosa [11]. Thus we found that two homologous olfactory *P-450* isoforms, probably belonging to the 2G and 2A subfamily, seem to be highly expressed and functionally similar in cattle and in rabbit. These findings strengthen the opinion that a specific subset of *P-450* isoenzymes might occur in the olfactory epithelium of mammals and that they might be important to maintain the homeostasis of the cells and for the physiological function of the sense of smell [33].

The high level and the broad substrate specificity of *P-450bov1* in olfactory mucosa might reflect its primary involvement in the biotransformation of inhaled odorants, toxic airborne chemicals and environmental pollutants. Aflatoxin B_1 , for example, has been reported to be metabolized at a much higher rate in nasal mucosa than in the liver of both cattle [38] and rabbit [39]. In rabbit, the nasal *P-450*2A10 and 2A11 were found to be the major catalysts of the metabolic activation of aflatoxin B_1 [39]. Therefore the bovine *P-450bov1* might be responsible for the bioactivation of aflatoxin B_1 , which is suspected to be the agent for nasal tumours of cattle in developing countries [38].

In summary, the results of the present study suggest that *P-450bov1* and *P-450bov3* are, on several criteria, the nasal bovine homologues of *P-450NMa* and *P-450NMb* respectively, and *P-450bov2* might represent another important haemoprotein identified in the nasal tissue of cattle that remains to be further characterized. Additional studies need to demonstrate: (1)

whether the pattern of the major *P-450s* is the same in the olfactory mucosa of mammals; (2) whether other *P-450s* are specifically expressed in the olfactory tissue; and (3) what distinct role the olfactory *P-450* isoforms play in the xenobiotic, odorant and pheromone clearance mechanism and in the odour signal.

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