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PURIFICATION OF A SHEEP LIVER CYTOCHROME P-450 FROM THE P450IIIA GENE SUBFAMILY

ITS CONTRIBUTION TO THE N-DEALKYLATION OF VETERINARY DRUGS

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Abstract—Oral administration of troleandomycin at a dose of 100 mg/kg/day for 6 days to three adult male Lacaune sheep produced a 1.6-fold increase in specific content of liver microsomal cytochrome P-450. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis, microsomal preparations from treated animals exhibited a strong band in the zone of electrophoretic mobility of cytochromes P-450. This band corresponded to a cytochrome P-450 which cross-reacted with rabbit P450IIIA6 antibodies, as demonstrated by immunoblotting. The ovine isozyme was purified to electrophoretic homogeneity by means of successive DEAE cellulose, CM cellulose and hydroxylapatite chromatographic separations. This hemoprotein had an apparent molecular weight of 52 kD as determined by calibrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was characterized in terms of spectral data, NH_2-terminal amino acid sequence, immunologic and catalytic properties. This study revealed some interspecies differences with the orthologous rabbit isozyme. The contribution of this form to the N-demethylation of erythromycin and of three veterinary drugs: chlorpromazine, chlorpheniramine and bromhexine was demonstrated from inhibition by TAO, from immunoinhibition studies, using polyclonal antibodies raised in rabbit and from the existence of significant correlations between its microsomal level and these *N*-demethylase activities. In contrast, the results suggest that ovine P450IIIA could not be predominantly involved in the N-dealkylation of benzphetamine, ephedrine, ivermectine or spiramycin.

Cytochrome P-450 (P-450‖) is the major catalytic component of the liver microsomal mixed-function oxidase system that catalyses the biotransformation of endogenous substrates including steroids and lipids and exogenous substrates such as drugs and chemical carcinogens. It is generally accepted that the broad substrate specificity of this system is due to the existence of numerous isozymes of cytochrome P-450 exhibiting distinct but overlapping specificities. Much of the informations on P-450 systems has been derived from the characterization of constitutive or inducible isozymes purified from livers of several animals species [1–3]. There is a lack of information about the physicochemical and catalytic properties of hepatic P-450 in ruminant animals except for two constitutive forms isolated from bovine liver [4]. However, in these breeding species, the risk of retention of veterinary drugs and xenobiotics in the animal's body represents a major concern for the quality of both meat and dairy products. It is therefore evident that a better knowledge on the liver detoxication enzymes from these species is greatly needed.

The objective of the present study was to investigate the structural and enzymatic properties of an ovine liver cytochrome P-450 isozyme inducible by macrolide antibiotics. Because of the previous isolation and characterization of the corresponding form in TAO-treated rabbit [5], the same inducer was used and the two proteins were structurally compared. In order to assess the contribution of the ovine isoenzyme to the metabolism of some therapeutic agents, we selected the N-dealkylation of six widespread used alkylamines in veterinary practice, namely spiramycine, ephedrine, chlorpromazine, chlorpheniramine, bromhexine and ivermectine.

MATERIALS AND METHODS

Chemicals. Aminopyrine, chlorpheniramine maleate, erythromycin, dilaurohylphosphatidylcholine, tergitol NP 10 as well as complete and incomplete Freund's adjuvant, were purchased from the Sigma Chemical Co. (St Louis, MO). Benzphetamine, bromhexine, ivermectine, troleandomycine and chlorpromazine, ephedrine, spiramycine were kindly provided by Upjohn Laboratories (Le Vaudreuil, France), Boehringer Ingelheim (Paris, France), Merck Sharp Dohme (Paris, France), Pfizer (Orsay, France) and Rhône-Mérieux (Toulouse, France), respectively. DE52 or CM52 celluloses and hydroxy-

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‖ Abbreviations: P-450, cytochrome P-450; CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediamine-tetraaecetic acid; SDS, sodium dodecylsulfate; TAO, triacetyl-oleandomycin; in the text, the recommended P-450 nomenclature was adopted, IA1 (c, BNF-B, 6), IA2 (d, ISF-G, 4), IIB1 (b, PB-B, 2), IIIA4 (3c).

lapatite were respectively obtained from Whatman (Maidstone, U.K.) and IBF Biotechnics (Villeneuve-la-Garenne, France). CHAPS and NADPH were from Boehringer-Mannheim (Meylan, France). All other chemicals and biochemicals were of the highest quality available and distilled deionized water was used in all studies.

Enzyme purification. Three male adult Lacaune sheep weighing 35.3 ± 2.6 kg were selected from a flock which had been reared parasite-free from birth. The animals received an oral daily administration of 100 mg/kg body weight of troleandomycin for six consecutive days as pounded tablets suspended in a gummi solution. Sheep were killed by bleeding 24 hr after the last administration. Ovine liver microsomes were prepared according to the previously published procedure [6]. Microsomal pellets were finally suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol.

The purification procedure was carried out at 4°. Microsomal protein was resuspended in buffer to a final concentration of 15 mg/mL, solubilized with 0.5% (w/v) of both tergitol NP 10, sodium cholate and (cholamidopropyl) dimethylammoniopropane sulfonate (CHAPS) with gentle stirring for 90 min. Insoluble material was removed by ultracentrifugation at 105,000 *g* for 60 min. Thus, about 900 nmoles of cytochrome P-450 were dialysed overnight against buffer A; 0.01 M Tris acetate (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol, 0.5% tergitol, 20% glycerol. This solution was applied to a column (3 × 30 cm) of DEAE-cellulose (DE52) previously equilibrated in buffer A. After a wash with buffer A (1 mL/mg protein), fractions containing isozyme P450IIIA were eluted with a linear gradient of K_2SO_4 (0–0.1 M, 500 mL) followed by two elutions using buffer A containing 0.3 and 0.4 M KCl. From the last eluate (0.4 M KCl), NADPH cytochrome P-450 reductase was purified according to the procedure of French and Coon [7]. The fractions eluted between 0.01 and 0.02 M K_2SO_4 were combined and dialysed overnight against buffer B: 0.01 M potassium phosphate (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol, 0.01% NaN_3, 0.5% tergitol, 20% glycerol. This solution was then applied to a CM-cellulose (CM52) column (2.2 × 20 cm) previously equilibrated in buffer B. The elution was then sequentially performed using buffer B containing 0.01 and 0.02 M potassium phosphate pH 7.4. Only the fraction eluting at 0.01 M phosphate contained large amount of P450IIIA as demonstrated by SDS–polyacrylamide gel electrophoresis analysis, and was used for the further purification. This fraction was dialysed overnight against buffer C (0.01 M potassium phosphate, pH 6.8, 1 mM EDTA, 0.1 mM dithiothreitol, 0.01% NaN_3, 0.5% tergitol, 20% glycerol) and applied to a column of hydroxylapatite (1.6 × 10 cm) previously equilibrated against the same buffer. Several fractions were eluted with buffer C containing 0.04, 0.08, 0.12, 0.16, 0.20, 0.25, 0.30 and 0.40 M phosphate. Isozyme P450IIIA was eluted in 0.16 M fraction. After dialysis against buffer B, this fraction was purified through a second CM-cellulose chromatography and P-450 was eluted in 0.004 M phosphate fraction. Elimination of tergitol and concentration of the fraction were simultaneously performed on a column of hydroxylapatite (1.6 × 10 cm). The column was previously equilibrated against buffer D: 0.01 M potassium phosphate (pH 6.8), 1 mM EDTA, 20% glycerol. After P-450 had been applied, the column was washed extensively with buffer D. This step allowed the elimination of tergitol whereas the purified hemoprotein was eluted as a sharp peak with buffer D containing 0.4 M phosphate and 0.1% cholate. This final fraction was dialysed overnight against buffer D containing 0.2 M phosphate to eliminate sodium cholate and stored at −75°.

Enzyme assay. Polyacrylamide gel electrophoresis were carried out at room temperature in the presence of SDS according to the method described elsewhere [8, 9]. The slab gel (14 × 11 × 0.15 cm) contained 9% acrylamide with 0.2% bis-acrylamide as the cross-linking reagent and 0.1% SDS. After incubation of microsomes, partly purified fractions or purified proteins with SDS and mercaptoethanol (5 min at 100°), the samples were applied and electrophoresis was performed 3 hr at 60 mA (200 V). The gel was fixed, stained with Coomassie Blue and destained with a mixture of water, acetic acid, methanol (80:10:10, v/v/v). Standard rabbit liver P450IIIA6 isoenzyme was included in each slab gel.

The absolute and difference spectra of P-450 in fractions and purified preparation were recorded on a Uvikon 860 Kontron spectrophotometrer equipped with a thermostated sample compartment. Sodium dithionite was used as reducing agent. Cytochrome P-450 concentrations were determined according to the procedure of Omura and Sato [10] using an extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm for the difference spectrum of Fe^{2+}–CO against Fe^{2+}.

The NH_2-terminal sequence of the purified P-450 was determined by Edman degradation on an Applied Biosystem model 470 A gas-phase sequencer coupled with a model 120 A phenylthiohydantoin analyser.

N-Demethylase activities of both microsomes and purified enzyme were carried out in 0.1 M potassium phosphate buffer (pH 7.4) at 37°. When microsomes from TAO-treated animals were used, P-450–TAO complex were dissociated by preincubation in presence of potassium ferricyanide (20 μM). In case of microsomal preparation, the final concentration of protein and substrate were respectively 1 mg/mL and 1 mM. The microsomal protein concentrations were previously estimated by using the procedure of Lowry *et al.* [11]. To discriminate the P450IIIA participation to the N-demethylation of the six veterinary drugs, the microsomal activities were compared in absence (incubation with potassium ferricyanide) or in presence of the reconstituted P-450–TAO complex (preincubation with TAO, 0.5 mM and NADPH, 0.5 mM) with further calculation of the percentage of inhibition due to the metabolic complex. Experiments on the reconstituted system were carried out as recommended [12]; cytochrome P-450 isozyme (0.1 μM) was incubated 5 min at room temperature with 30 μg/mL of sonicated lauroylphosphatidylcholine and a threefold molar excess of cytochrome P-450 reductase. This mixture was diluted to a final volume of 0.05 mL with buffer and substrate (1 mM) and the reaction

Table 1. *N*-Demethylase activities of liver microsomes from control, TAO treated sheep* and of reconstituted system

Substrate	Sheep liver microsomes			Reconstituted system (nmol product/ min/mol P-450)
	Control (nmol/min/mg)	TAO treated (nmol/min/mg)	Inhibition by TAO† (%)	
Aminopyrine	5.50 ± 1.24	8.73 ± 0.81‡		4.97
Benzphetamine	4.38 ± 0.58	3.62 ± 0.23		7.53
Erythromycin	1.75 ± 0.05	5.59 ± 1.46‡	65.6 ± 6.0	5.90
Ethylmorphine	4.27 ± 0.41	12.72 ± 2.71‡		4.27
Troleandomycin	1.07 ± 0.15	3.34 ± 1.07‡		7.30
Bromhexine	1.01 ± 0.11	2.15 ± 0.17‡	68.0 ± 1.3	4.04
Chlorpheniramine	3.47 ± 0.27	5.49 ± 0.69‡	60.4 ± 9.0	6.37
Chlorpromazine	1.57 ± 0.10	2.86 ± 0.43‡	61.9 ± 3.9	4.04
Ephedrine	4.40 ± 0.41	3.82 ± 0.56	17.2 ± 8.2	5.90
Ivermectine	0.75 ± 0.07	0.96 ± 0.08	36.4 ± 5.3	4.11
Spiramycin	1.50 ± 0.15	1.84 ± 0.11	24.4 ± 4.8	4.97

* Values are presented as means ± SD of three (control or TAO treated) individual liver fractions.

† Liver microsomes from TAO treated sheep were preincubated with potassium ferricyanide and furtherly with TAO and finally incubated in presence of each substrate for determining its N-demethylation.

‡ Values significantly different from corresponding control value ($P < 0.05$) as tested by Student's *t*-test.

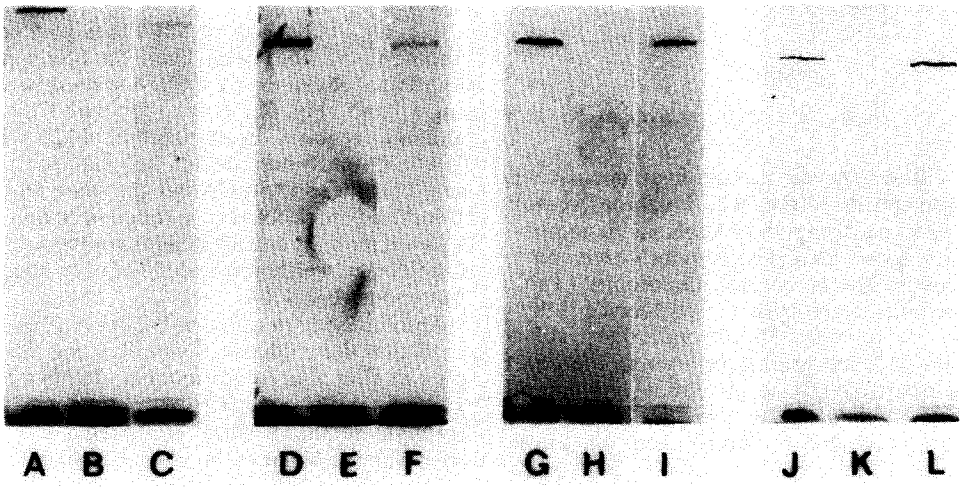

Fig. 1. Immunoblotting analysis of different run gels of liver microsomes from control (B, E, H, K) or TAO-treated sheeps (C, F, I, L). Microsomal proteins (5 μg) were assayed for cross reactivity towards anti-rabbit P450IA1 (B, C), IA2 (E, F), IIB1 (H, I) and IIIA6 (K, L) antibodies. Lanes A, D, G and J correspond to 0.5 pmoles of purified rabbit P450IA1, IA2, IIB1 and IIIA6 isozymes, respectively.

was initiated by the addition of NADPH to a final concentration of 0.5 mM. Incubations were carried out for 10 min. The activities determined from the linear part of kinetics were expressed as nmol product formed/min/nmole P-450. Demethylation of the substrates was determined by the method of Nash [13, 14].

Immunochemical studies. Antibodies against sheep liver cytochrome P450IIIA were raised in female rabbits by three multisites subcutaneous injection (1 mL) of 1 nmole of protein in 50% complete Freund adjuvant at one month intervals. Each week after the last injection, 20 mL of blood was drawn from a marginal ear vein and serum collected. IgG enriched fractions of sera were prepared by $(NH_4)_2SO_4$ precipitation. In order to assess the rise of P-450 antibodies in rabbit sera, classical Ouchterlony double diffusion analysis were carried out.

In order to determine the contribution of P450IIIA to *N*-demethylase activities, the IgG fraction prepared from rabbit antisera was compared to preimmune fractions for its ability to inhibit the monooxygenase activities catalysed by liver microsomes from TAO-treated sheep. Furthermore, liver microsomes prepared from 18 sheep including control animals and animals treated with oral TAO (100 mg/kg/day for 6 days) or phenobarbital (40 mg/kg/day for 6 days) were assayed for *N*-demethylase

Table 2. Purification procedure for the preparation of P450IIIA from TAO-treated sheep microsomes

Fraction	Total (nmol)	Cytochrome P-450 specific content (nmol/mg protein)	Yield (%)
(TAO) microsomes	877.0	0.64	100
DE cellulose Fraction 0.01 M K_2SO_4	295.0	0.90	33.6
CM cellulose (first) Fraction 0.01 M phosphate	51.8	1.69	5.9
Hydroxylapatite (first) Fraction 0.16 M phosphate	45.8	2.82	5.2
CM cellulose (second) Fraction 0.004 M phosphate	42.3	9.09	4.8
Hydroxylapatite (second) Fraction 0.4 M phosphate	26.0	13.19	2.9

activities (using 0.5 mg microsomal proteins, 20 min incubation, 1 mM substrate) and for specific content in P450IIIA by immunoblotting revealed with the above described IgG fraction prepared from rabbit antisera.

RESULTS

Induction of sheep liver monoxygenases by troleandomycin

The P-450 specific content of liver microsomes increased significantly from 0.37 ± 0.03 in controls to 0.63 ± 0.07 nmol/mg in TAO-treated animals. Furthermore some dealkylase activities were stimulated in comparison to control microsomes (Table 1); these included aminopyrine, erythromycin, ethylmorphine, troleandomycin, bromhexine, chlorpheniramine and chlorpromazine whereas there was no change in benzphetamine, ephedrine, ivermectine or spiramycin *N*-demethylase activities.

The comparative analysis of liver microsomes from control and TAO-treated sheep by SDS–polyacrylamide gel electrophoresis revealed a strong band occurring in the zone located above the band associated with P-450 induced by phenobarbital. On the basis of both its electrophoretic mobility and its unique cross immunoreactivity with antibodies against rabbit cytochrome P450IIIA6 as demonstrated by western blotting (Fig. 1), this band was tentatively assigned to a sheep P450IIIA form [15] induced by TAO.

Purification and characterization of hepatic P450IIIA from TAO-treated sheep

The sheep liver P450IIIA isozyme was purified to 13.2 nmol/mg of protein (Table 2). The DEAE cellulose chromatography was introduced to separate P-450 from more heavy microsomal proteins such as cytochrome P-450 reductase which was eluted at 0.4 M KCl. The first CM cellulose chromatography eliminated most of the protein contaminants which were less adsorbed to the column whereas the consecutive elution on a hydroxylapatite column and a second CM cellulose column resulted in an electrophoretically homogenous preparation of P450IIIA isoenzyme which was further purified by means of the last chromatography on hydroxylapatite.

Because of the lack of any other purified cytochrome P-450 from ovine liver, the electrophoretic mobility of sheep liver P450IIIA corresponded to a single band (Fig. 2), it was compared to that of authentic rabbit liver cytochrome IA2, IIB1 and IIIA6 previously described [5, 16]. Indeed, it is quite clear that sheep P450IIIA has the same mobility as the orthologous isoenzyme purified from rabbit liver. A plot of mobility against standard proteins indicated a molecular weight around 52 kD for sheep liver cytochrome P450IIIA.

Absolute spectra of ovine P450IIIA (Fig. 3) indicated that the oxidized cytochrome was essentially low spin with a soret maximum at 416 nm and α and β bands at 534 and 569 nm, respectively. In the ferrous state, the spectrum presented a maximum at 412 nm and at 445 nm. The ferrous carbonyl complex had maxima at 448 and 550 nm. The absorbance at 448 nm was similar to that of the ferric form at 416 nm.

Determination of the first 20 NH_2-terminal amino acid sequence represents a powerful means of comparing highly purified proteins even though such terminal sequences are generally less well conserved than the rest of protein. In this case, the comparison (Fig. 4) of sheep *versus* previously analysed human [17], rabbit [18] and rat [19] forms from the P450IIIA subfamily, led to the observation of a highly conserved region from amino acids 10 to 16 whereas methionine was generally the terminal residue. There were also strong homologies in position 3–4, 7–8, 10–16 and 19–20 between sheep and rabbit cytochromes. The major difference between these two sequences was insertion of proline and serine in position 5–6 in the ovine form.

Antibodies to sheep liver P450IIIA prepared from serum of immunized female rabbit gave a single band when reacting against the purified protein as

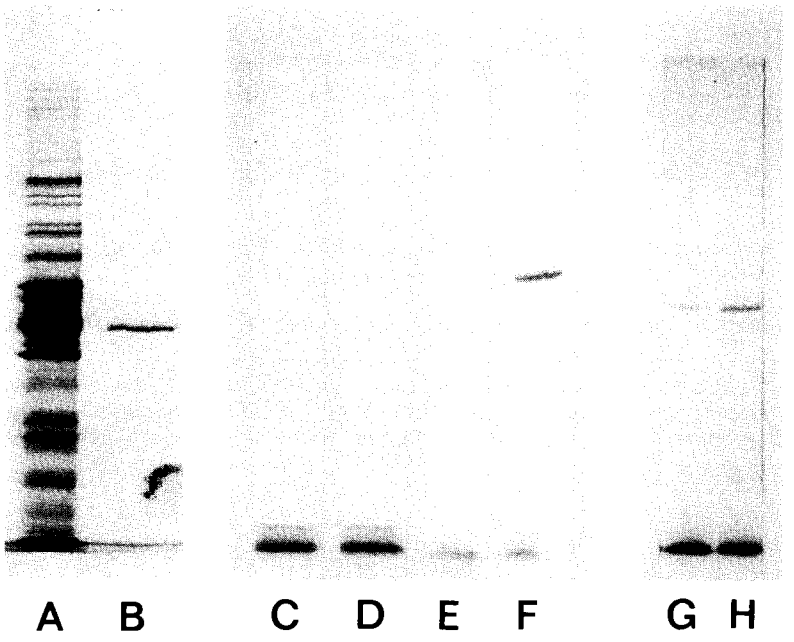

Fig. 2. SDS–polyacrylamide gel electrophoresis of microsomes from TAO-treated sheep (A) and 5 pmoles of purified P450IIIA protein from sheep (B). Western blotting analysis of 2 pmoles of purified ovine P450IIIA tested for cross reactivity towards anti-rabbit P450IA1 (C), P450IA2 (D), P450IIB1 (E) and P450IIIA (F). Immunoblotting analysis of the same 2 pmoles of purified ovine P450IIIA protein (G) and 5 μg of microsomal protein from TAO-treated sheep (H) revealed by anti-sheep P450IIIA obtained in female rabbit.

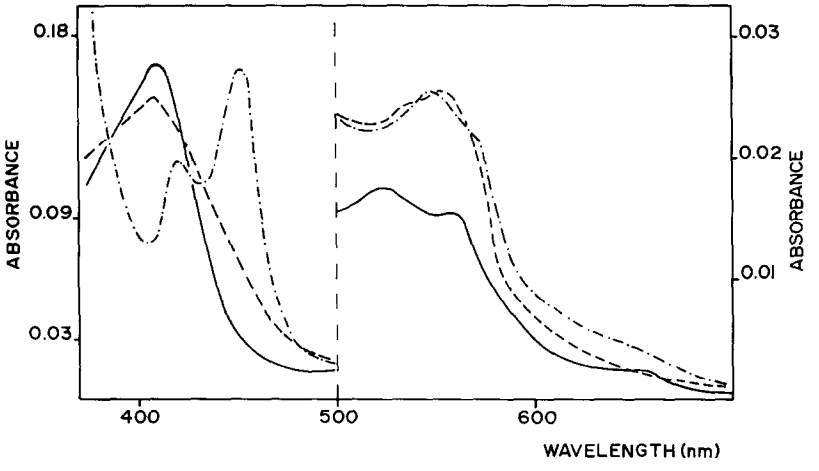

Fig. 3. Absolute spectra of ovine P450IIIA isozyme. Electrophoretically homogenous isozyme (0.79 nmol) was diluted in 0.1 M phosphate buffer pH 7.4, final volume 0.5 mL. After thermal equilibrium has been reached (t = +10°), the absolute spectra of the oxidized form was recorded (——). A small amount of sodium dithionite added to the solution allowed the redution for 30 min; the absolute reduced spectrum was then recorded (– – – –). Finally, carbon monoxide was bubbled into the cuvette for 1 min and the absolute spectrum of the ferrous carbonyl complex obtained (·–·–·–·).

observed by means of an Ouchterlony double diffusion experiment. They gave also a single band on Western blots of sheep liver microsomes (Fig. 2) and this band corresponded to the one detected by the antibodies of rabbit P450IIIA. These antibodies were used in Western blot to quantitate P450IIIA in 18 microsomal fractions from untreated sheep or from sheep treated with various inducers. This form accounted for 0.21 ± 0.04, 0.49 ± 0.11 and 0.50 ± 0.06 nmol/mg protein in untreated animals or

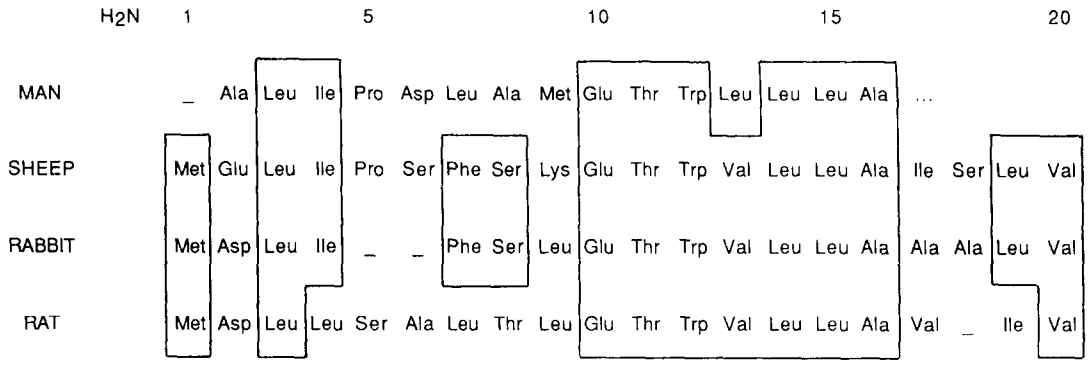

Fig. 4. Comparative N-terminal sequence of cytochrome P450IIIA isozyme in different animal species. Data from human, rabbit and rat forms are from Molowa *et al.* [17], Dalet *et al.* [18] and Gonzales *et al.* [19]. Homologies in the ovine cytochrome sequence with other animal and human P450IIIA are boxed.

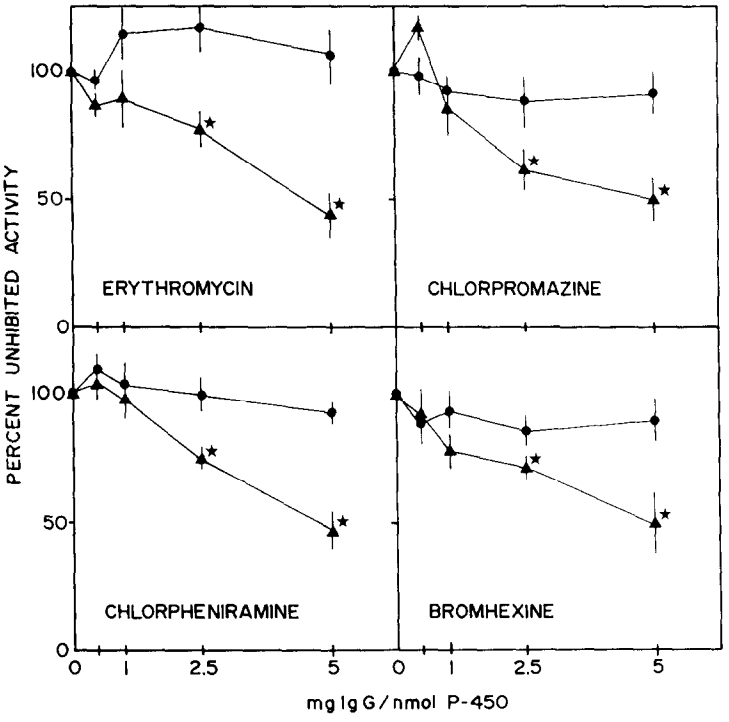

Fig. 5. Inhibition of erythromycin, chlorpromazine, chlorpheniramine and bromhexine *N*-demethylase activities by anti-sheep P450IIIA. The indicated amounts of the IgG fraction were added to standard incubations containing liver microsomal protein (0.5 mg) from TAO-treated sheep in the presence of 20 μM $Fe(CN)_6K_3$ (▲). The effect of a preimmune IgG fraction on the activity of the microsomal preparation is also shown (●). Activities in the absence of the IgG fractions are reported in Table 1. Asterisks indicate significant differences in *N*-demethylase activities as observed between incubations with preimmune or anti-P450IIIA IgG fractions, by using Student's *t*-test on absolute enzyme activities.

in animals receiving oral phenobarbital (40 mg/kg/day for 6 days) and TAO (100 mg/kg/day for 6 days), respectively.

Contribution of P450IIIA to N-*demethylase activities*

N-Demethylation of various substrates and veterinary drugs by the purified sheep P450IIIA in a reconstituted system is presented in Table 1. The turnover rates were quite similar from one substrate to another: 4–7 nmoles of formaldehyde produced per minute and per nmole of P-450 isozyme. It has been shown that P450IIIA1 in the rat and P450IIIA6 in the rabbit are selectively involved in the formation of an abortive complex with a metabolite of TAO. It was therefore of interest to determine to what extent such a complex inhibited various *N*-demethyl-

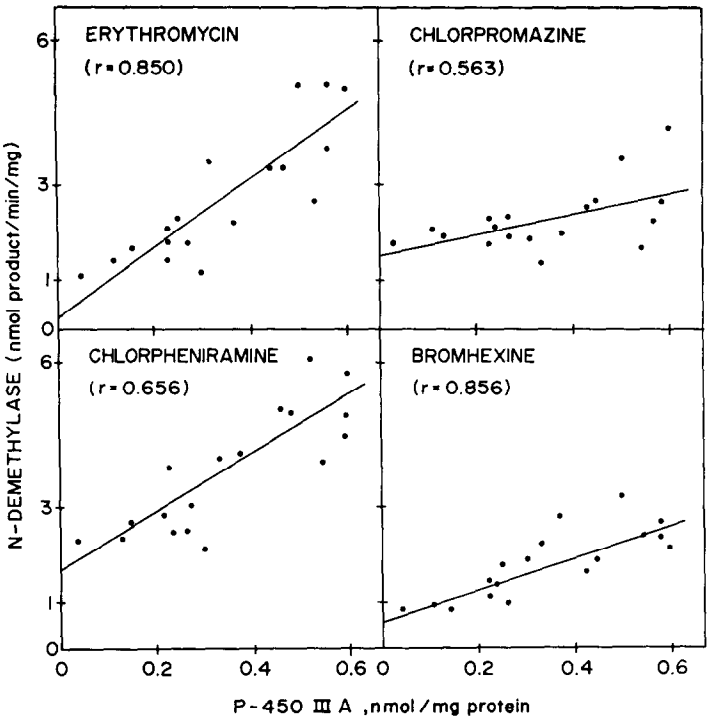

Fig. 6. Correlation of erythromycin, chlorpromazine, chlorpheniramine and bromhexine *N*-demethylase activities with immunochemically determined P450IIIA level in sheep liver microsomes. Microsomal samples prepared from 18 control and TAO or phenobarbital treated sheep were assayed for *N*-demethylase activities (using 0.5 mg microsomal protein, incubation time 20 min, substrate concentration 1 mM) and for P450IIIA by immunoblotting (5 μg of microsomal protein were electrophoresed in each well. Each gel also contained lanes with 1, 2, 4 and 10 pmol of purified sheep P450IIIA. The nitrocellulose sheets were treated with 1:100 dilution of rabbit anti-P450IIIA and revealed by peroxidase-IgG conjugate. The amounts of P450IIIA were estimated using the ratio of peaks obtained after densitometry. The lines through the points were drawn using linear regression analysis.

ase activities. For this purpose, the P-450–TAO complex was first generated in the microsomes by a preincubation in the presence of TAO and NADPH (control experiments indicated that the complex, absorbing at 456 nm, was formed in these conditions). Then the microsomes were incubated either with 20 μM $FeCN_6K_3$ or buffer alone. Finally, *N*-demethylase activities were determined in complexed or de-complexed microsomes. Results (Table 1) indicated that N-demethylation of bromhexine, chlorpheniramine, chlorpromazine and erythromycin were highly inhibited (60–68%) whereas moderate inhibition was observed with ivermectine, spiramycin and ephedrine (17–36%).

The IgG fraction prepared from rabbit antisera raised against ovine P450IIIA was examined for its ability to inhibit the N-demethylation of various drugs catalysed by microsomes from TAO treated sheep. When compared with rabbit preimmune fraction, no immunoinhibition was observed with substrates such as benzphetamine, ephedrine, ivermectin or spiramycin. In contrast, significant inhibitions (24–65%) occurred with the N-dealkylation of erythromycin, chlorpromazine, chlorpheniramine and bromhexine (Fig. 5).

Next, the level of P450IIIA determined by Western blot was plotted against various *N*-demethylase activities determined in 18 different ovine liver microsomal preparations. Correlation was highly significant (r = 0.563–0.856, N = 18, $P < 0.01$) with erythromycin, chlorpromazine, chlorpheniramine and bromhexine (Fig. 6) whereas no significant correlation was observed with benzphetamine, ephedrine, ivermectine and spiramycin.

DISCUSSION

In this report, we have identified and characterized a sheep liver cytochrome P-450 from the P450IIIA subfamily [15] involved in the N-demethylation of various substrates including erythromycin, chlorpromazine, chlorpheniramine and bromhexine. The isolation of this isozyme was possible because of both its inducibility by TAO in sheep and its immunoreactivity with antibodies raised against the previously isolated rabbit orthologous form [16]. Western blot analysis of liver microsomes from TAO-induced animals with different antibodies against rabbit P-450 demonstrated the possible induction by TAO of P450IA2 and IIB1 but above all of P450IIIA. This result was confirmed by both the selective Western blotting revelation with anti-rabbit P450IIIA6 and the selective inductibilities of animals erythromycin and TAO *N*-demethylases

whereas the N-demethylation of benzphetamine was not decreased in liver microsomes from TAO treated sheep.

As expected from orthologous forms, P450IIIA (sheep) and P450IIIA6 (rabbit) exhibited a high degree of similarity in terms of: (i) electrophoretic mobility and molecular weight; (ii) N-terminal sequence with 70% of homology for the first 20 amino acids; (iii) immunological properties; (iv) inducibility by TAO; and (v) implication in the demethylation of erythromycin. However, in contrast to the rabbit but as in the rat [19] and the human [20], the sheep P450IIIA isozyme was also inducible by phenobarbital. Evidence for the catalytic role of the sheep P450IIIA isozyme came from selective inhibition by TAO, immunoinhibition studies and the existence of correlations between its microsomal level and *N*-demethylase activities. The lack of significance of such studies when benzphetamine was used as a substrate, is consistent with the well established specificity of phenobarbital inducible P450IIB1 towards oxidation of this substrate [21]. In contrast, the significant inhibition by TAO and immunoinhibition obtained in the case of erythromycin supported the view that P-450 is the enzyme predominantly involved in the biotransformation of this macrolide antibiotic. The correlation between catalytic activity and P450IIIA level also supported this conclusion. Concerning the oxidation of alkylamines used in veterinary practice, if the reconstituted sheep enzyme metabolizes all substrates at similar rates, which could be explained by the particular experimental conditions of such determinations, all other results strongly suggested that this enzyme is involved in the N-dealkylation of chlorpromazine, chlorpheniramine and bromhexine, whereas it would be less responsible for the oxidation of either ephedrine, ivermectin or spiramycin. Concerning this last molecule, our conclusions might appear as rather paradoxical in view of the structural similarity between spiramycin and erythromycin or TAO. However, this is in agreement with previous observation on the inducing properties of macrolide antibiotics reported by Delaforge *et al.* [22] who showed that only the antibiotics containing desosamine and mycaminose aminosugars (erythromycin, TAO but not spiramycin) were able to give both induction of cytochrome P-450 anf formation of inhibitory P-450–metabolite complexes *in vivo* and *in vitro*. Finally, there is a risk of inductive effect in ruminant animals receiving a diet supplemented with macrolide antibiotics which are used as feed additives. Moreover, it is clear from our results that therapeutic effects of such drugs as chlorpromazine, chlorpheniramine and bromhexine could be lessened or greatly decreased in animals simultaneously treated with macrolide antibiotics.

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