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N-Hydroxylation of Benzamidine to Benzamidoxime by a Reconstituted Cytochrome P-450 Oxidase System from Rabbit Liver: Involvement of Cytochrome P-450 IIC3

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SUMMARY

Previous investigations have provided evidence for the participation of the cytochrome P-450 (P-450) enzyme system in the established *N*-hydroxylation of benzamidine to benzamidoxime by microsomal fractions from rabbit liver homogenates. In the present investigation, a representative mixture of P-450 isoenzymes was first isolated from the livers of untreated rabbits and then, together with purified NADPH-P-450 reductase, successfully used in a reconstituted enzyme system for the *N*-hydroxylation of benzamidine. In order to identify the participating isoenzyme, the P-450 mixture was separated by preparative high performance liquid chromatography on an anion exchange column. A P-450 fraction was obtained that was able to transform benzamidine with a specific activity >3-fold higher than that of the P-450 mixture. The electrophoretic and spectral properties, as well as the inhibition by monoclonal antibodies against P-450 IIC3, show that the isolated P-450 fraction must consist of one or more variants of the isoenzyme P-450 IIC3. By means of reconstitution experiments with highly purified variants of P-450 IIC3 from rabbit liver and with purified variants of P-450 IIC expressed by recombinant *Escherichia coli*, the participation of the two variants P-450 IIC3 (6βH) and P-450 IIC3 (6βL) in the *N*-hydroxylation of benzamidine was unequivocally confirmed.

Strongly basic amidine functional groups are components of numerous active compounds (1). The aromatic diamidines (pentamidine and diminazene) represent an important substance class and were developed principally because of their activity towards trypanosomes (2). Pentamidine has very recently become a subject of special interest in connection with the problem of human immunodeficiency virus infections. Today it is considered one of the agents of choice for the treatment of pneumonia caused by *Pneumocystis carinii*, one of the very frequently occurring secondary infections in patients with acquired immunodeficiency syndrome (3).

In previous *in vitro* biotransformation studies with microsomal fractions from rabbit liver homogenates (12,000 × *g* supernatant, microsomes), it was possible for the first time to demonstrate *N*-hydroxylation of very strongly basic amidine groups, using the model substance benzamidine (4) (Fig. 1). The metabolically formed benzamidoxime was initially detected qualitatively with the help of mass spectrometry after thin layer chromatographic separation and elution of the corresponding bands. Synthetically prepared benzamidoxime was used as a reference substance. Quantitative studies using HPLC analyses revealed that the microsomal *N*-oxidation of benzamidine (Fig. 1) followed Michaelis-Menten kinetics (5). The *N*-oxidation of benzamidine is of toxicologic relevance. The metabolite benzamidoxime proved to be mutagenic in the Ames test (in the presence of the 12,000 × *g* supernatant of a rabbit liver homogenate). The action of benzamidoxime brought about DNA single-strand breakage in rat hepatocytes and DNA amplification in SV-40-transformed hamster cells (6). *In vitro* experiments with the trypanocidal diamidines pentamidine and diminazene also demonstrated the occurrence of *N*-hydroxylation of amidine functional groups (7–10). Benzamidine is also *N*-hydroxylated *in vivo*, but this can only be detected after cleavage of conjugates.[1] On the other hand, a very strong retroreduction of amidoximes to amidines can also be observed *in vivo* (11, 12).

In principle, two different enzyme systems can participate in microsomal *N*-oxidative biotransformation processes, the P-450 system (EC 1.14.14.1) (13) and a FMO (EC 1.14.13.8) (14). The results of an incubation of benzamidine with purified FMO,

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[1] B. Clement *et al.* Biotransformations of benzamidine and benzamidoxine in vivo. Arch. Pharm. (Weinheim) (1993) in press.

ABBREVIATIONS: P-450, cytochrome P-450; HPLC, high performance liquid chromatography; FMO, flavine-containing monooxygenase; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SV-40, simian virus 40.

however, allowed the participation of FMO in the *N*-oxygenation of benzamidine to be excluded (4). Evidence in favor of participation of the P-450 system is the inhibition of the microsomal transformations by typical inhibitors of the P-450 system, such as SKF 525-A and carbon monoxide (4), and by antibodies against NADPH-P-450 reductase (9). Incubation experiments with phenobarbital, 3-methylcholanthrene, ethanol, and benzamidine itself did not provide any evidence for the participation of the P-450 isoenzymes thus inducible (5).

Reconstituted enzyme systems with purified P-450 and purified NADPH-P-450 reductase have previously been used successfully for the *N*-oxidation of, for example, 2-acetylaminofluorene (15, 16) and phentermine (17). However, nothing has yet been reported on the *N*-oxidation of amidines with reconstituted P-450 systems.

The present investigations are thus intended to determine whether the *N*-oxygenation of benzamidine that has previously been detected with microsomal fractions of rabbit liver homogenates can also be achieved with the reconstituted enzyme system from a P-450 isoenzyme mixture. By means of reconstitution experiments with purified P-450 isoenzymes, it should be possible ultimately to identify the isoenzymes participating in the reaction.

Materials and Methods

Organic syntheses. Benzamidoxime was prepared from benzonitrile and hydroxylamine hydrochloride according to the procedure described by Krüger (18). The purity of the compound was checked by the usual methods.

Preparation of rabbit liver microsomes. Untreated male and female rabbits of a mixed breed were used for the preparation of rabbit liver microsomes. The animals were obtained from the Behringwerke AG (Marburg, FRG) and weighed 3.0–4.5 kg. The rabbits were killed and the livers were removed and immediately placed in a ice-cooled 1.15% (w/v) solution of KCl (0.154 mM) for transport to the laboratory. All following separation steps were carried out at 0–4° in sucrose-containing phosphate buffer solution (20 mM potassium phosphate, 0.25 M sucrose, pH 7.4). The livers were cut into slices about 1–2-cm thick, washed several times with the buffer solution, minced using a commercial meat mincer, and finally homogenized in a flow-through homogenizer, according to the method of Ziegler and Pettit (19).

The separation of the microsomes was performed in analogy to the process described by Ziegler and Pettit (19), by fractional acid precipitation (preliminary precipitation at pH 6.3, main precipitation at pH 5.5) of the supernatant obtained from the liver homogenate after centrifugation at 12,000 × *g* for 30 min. The preliminary precipitation at pH 6.3 and separation of the initially precipitated components by centrifugation resulted in approximately 50% increased specific activities of P-450 and NADPH-P-450 reductase, in comparison with those obtained by precipitation only at pH 5.5.

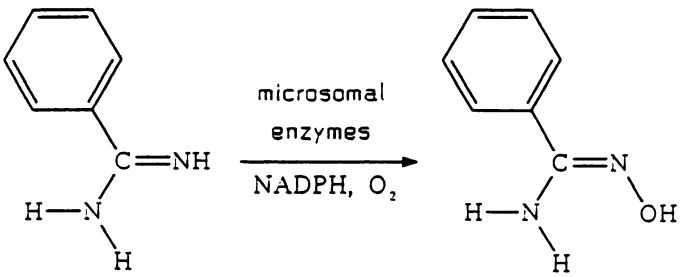

Fig. 1. *N*-Hydroxylation of benzamidine to benzamidoxime.

Preparation of a P-450 isoenzyme mixture and concomitant isolation of NADPH-P-450 reductase. A modification of the procedure described by Kling *et al.* (20) was used for the isolation of the P-450 isoenzyme mixture. All following purification steps were performed at 0–4°. Lubrol PX (Sigma, Deisenhofen, FRG) was used in place of Emulgen 913 for the solubilization of the microsomal proteins. The solubilized rabbit liver microsomes were placed on an octyl-Sepharose CL 4B (Pharmacia, Freiburg, FRG) column (i.d., 3.8 cm; length, 38 cm). Elution was performed by stepwise increases in the detergent concentration and decreases in the salt concentrations in elution buffers A, B, and C as described (see Fig. 2). Emulgen 913 in the elution buffers was replaced by Lubrol PX. The flow rate was 50 ml/hr and the volume of the fractions collected was 8.5 ml. The fractions were pooled according to their elution profile measured at 417 nm or according to the reductase activity (see Fig. 2). The combined reductase fractions (fractions 58–70, corresponding to peak 2 in the elution profile) were further processed by affinity chromatography on 2′,5′-ADP-Sepharose 4B (Pharmacia) and subsequently by gel filtration chromatography on acrylamide-agarose (AcA 44 Ultrogel; Pharmacia), according to the procedure described by Yasukochi and Masters (21). The combined P-450 fractions (fractions 146–153, corresponding to peak 4 of the elution profile) were applied to a hydroxylapatite (Hydroxylapatite fast flow; Calbiochem, Frankfurt, FRG) column (i.d., 2.5 cm; length, 10 cm) and freed from detergent and accompanying proteins by washing with 10 mM potassium phosphate buffer, pH 7.4, and 100 mM potassium phosphate buffer, pH 7.4. The bound P-450 was eluted in a sharp peak by 400 mM potassium phosphate buffer, pH 7.4. The buffers contained 20% (w/v) glycerol. The flow rate was 40 ml/hr.

Separation of P-450 isoenzymes by preparative HPLC on an anion exchange column. A conventional analytical HPLC system (655 A-11 HPLC gradient pump, L-5000 controller, 655 A-22 UV detector, and D-2000 integrator; Merck/Hitachi, Darmstadt, FRG) was used for preparative HPLC. The P-450 isoenzyme mixture was dialyzed overnight against 10 mM Tris-acetate buffer, pH 7.5, which contained 20% (w/v) glycerol, 0.5% (w/v) Lubrol PX, 1 mM EDTA, and 1 mM dithiothreitol (buffer D), and was then diluted to a protein concentration of approximately 0.25 mg/ml with the same buffer. Aliquots of 6 mg of protein were applied to a semipreparative Fractogel EMD DEAE-650(S) column (i.d., 10 mm; length, 150 mm; particle size, 25–40 μm; Merck, Darmstadt, FRG), which had been equilibrated with buffer D. The column was washed with 40 ml of buffer D and eluted with a linear gradient of 0 to 500 mM sodium acetate in buffer D for a period of 50 min, at a flow rate of 1 ml/min. The fractions obtained were pooled according to an elution profile measured at 280 nm (see Fig. 3), concentrated by ultrafiltration, and tested for their benzamidine *N*-hydroxylase activity in the reconstituted P-450 enzyme system.

Analytical procedures. The P-450 content was determined using the method of Omura and Sato (22). Cytochrome b_5 was determined according to the method described by Estabrook and Werringloer (23), by recording the reduced minus the oxidized spectrum (extinction coefficient $\epsilon = 185\ mM^{-1}\ cm^{-1}$). The activity of the NADPH-P-450 reductase was determined by the procedure of Williams and Kamin (24), in which the reduction of cytochrome *c* (type 4 horse heart; Sigma) is followed (extinction coefficient $\epsilon = 21\ mM^{-1}\ cm^{-1}$ for the reduced form of cytochrome *c*; 1 mU = 1 nmol of reduced cytochrome *c*/min). The protein contents were determined at the stage of the microsomes and the solubilized microsomes by the method of Gornall *et al.* (25) (reagent kit; Merck) and at all other stages by a bicinchoninic acid method (26) (reagent kit; Pierce, Rockford, IL). In both methods, bovine serum albumin was used as the standard. All photometric measurements were performed with a Kontron Uvikon 930 spectrophotometer. The SDS-PAGE analyses were carried out following the method of Laemmli (27), with a 3% stacking gel and an 8% separating gel (1.5-mm thickness). Staining was achieved with Coomassie brilliant blue R250 (Serva, Heidelberg, FRG). The following proteins were used as standards (low molecular weight calibration kit; Pharmacia): phos-

phorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,100).

Incubation conditions. The incubations were performed under aerobic conditions in 1.5-ml reaction vessels (Eppendorf, Hamburg, FRG), with shaking, in a water bath at 37°. A typical optimized incubation reaction mixture contained 0.05 nmol of P-450, 0.18 nmol (0.3 unit) of NADPH-P-450 reductase, 15 μg of L-α-dilauroyl phosphatidylcholine (final concentration, 40 μM, Sigma), 0.96 μmol of benzamidine (final concentration, 1.6 mM; EGA-Chemie, Steinheim, FRG), 1.98 μmol of $MgCl_2$ (final concentration, 3.3 mM), and 0.23 μmol of NADPH (final concentration, 0.38 mM), in 0.6 ml of a 50 mM phosphate buffer at pH 7.4. Reference incubations were performed by replacing the purified enzymes with an equivalent amount of the rabbit liver microsomal preparation that was used as the source for the enzyme purification. The effectors 5β-pregnan-3β,20α-diol and 16α-methylprogesterone (Sigma) were added in methanolic solution (volume, 10 μl) to the respective experiments (final concentration, 50 μM). After a 3-min preincubation period, the substrate benzamidine was added and the reaction was started by addition of the cofactor solution (NADPH and $MgCl_2$). The incubation time was 20 min. The formation of benzamidoxime during this time period was linear. The reaction was stopped by the addition of 0.4 g of NaCl and 0.5 ml of cooled diethyl ether.

HPLC. The previously developed HPLC method for the determination of benzamidoxime (5) had to be modified because of the smaller sample amounts used in the work with reconstituted P-450 systems. The sample preparation involved quantitative ether extraction. The ether extract was evaporated to dryness and then taken up in 100 μl of eluent. Aliquots of 10 μl were subjected to HPLC analysis (Waters 510 HPLC pump, Waters 486 UV detector, and Waters 746 integrator; Millipore GmbH, Eschborn, FRG) via an automatic sampler (AS-2000; Merck-Hitachi). Each sample was measured in triplicate. A commercial prepacked LiChrospher 60 RP-Select B column (125-mm length × 4-mm i.d.; particle size, 5 μm; Merck) with an RP-8 precolumn (4 mm × 4 mm; Merck) was used as the stationary phase. The separation was achieved at room temperature with 0.1 M ammonium acetate buffer, pH 6.5/methanol (90:10, v/v) as the mobile phase, at a flow rate of 1.0 ml/min. The UV detection was at 254 nm (the absorbance maximum of benzamidoxime). The retention times were 6.3 min for benzamidoxime and 4.0 min for benzamidine. For the determination of the recovery rate and the detection limit of the metabolite benzamidoxime, defined amounts of synthetic reference substance (0.05, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, or 2.0 nmol) were added to the usual incubation mixtures. These mixtures were then incubated and worked up under the same conditions as the experimental samples. The signals obtained (peak areas) were compared with those of the same amount of benzamidoxime dissolved in 0.1 M ammonium acetate buffer, pH 6.5/methanol (90:10, v/v). The calibration function was linear in the range from 5 pmol to 200 pmol of injected benzamidoxime, with a correlation coefficient of $r = 0.9997$ (48 determinations). The recovery rate after incubation and sample work-up amounted to 98.2 ± 2.6% (48 determinations). The detection limit was about 0.05 nmol of benzamidoxime/incubation experiment; this corresponds to 0.025 nmol of benzamidoxime formed/min/nmol of P-450.

Monoclonal antibodies against P-450 IIC3 and highly purified variants of P-450 IIC3. Monoclonal antibodies against P-450 IIC3, highly purified variants of P-450 IIC3 from rabbit liver, and the two variants P-450 IIC3 (6βH) and P-450 IIC3 (6βL) expressed by recombinant *Escherichia coli* were kindly provided by Dr. E. F. Johnson, Scripps Clinic and Research Foundation (La Jolla, CA). The procedures for the isolation of highly purified variants of P-450 IIC3 from the inbred strains (rabbits) IIIVO/J and III/J, as well as for the preparation of monoclonal antibodies, are described in the literature (28–31). The expression of P-450 IIC3 variants in *E. coli* will be reported elsewhere. A preliminary report has been made (32).

Results

The solubilization of rabbit liver microsomes with the non-ionic detergent Lubrol PX (final concentration, 0.2%, w/v) gave a yield of 91% for NADPH-P-450 reductase and of 81% for P-450. The solubilized rabbit liver microsomes could be separated into four fractions by hydrophobic interaction chromatography on octyl-Sepharose CL 4B (Fig. 2).

Whereas the first fraction (peak 1) contained only a small amount of reductase activity, cytochrome b_5, and P-450, the major part of the NADPH-cytochrome *c* reductase activity and small amounts of cytochrome b_5 were detected in the second fraction (peak 2). The subsequent bioaffinity chromatography of this prepurified reductase fraction on 2′,5′-ADP-Sepharose 4B, followed by gel filtration chromatography on AcA 44 Ultrogel, permitted the isolation of a highly purified reductase preparation with an NADPH-cytochrome *c* reductase activity of 31,000 mU/mg of protein. After SDS-PAGE, this fraction exhibited only one major single band, at M_r 78,000.

The third fraction (peak 3) consisted mainly of cytochrome b_5 (specific content, 10 nmol of cytochrome b_5/mg of protein). The fourth fraction (peak 4) had a high specific content of P-450 and did not contain any cytochrome b_5 and NADPH-cytochrome *c* reductase activity. After subsequent hydroxylapatite chromatography, an enriched P-450 preparation with a specific content of 6.0 nmol of P-450/mg of protein was obtained (see Table 2). SDS-PAGE analysis (see Fig. 4) showed that the P-450 preparation thus obtained contained a representative mixture of the P-450 isoenzymes of rabbit liver (protein bands in the range of M_r 48,000 to 58,000) and that it was free of cytochrome b_5 and NADPH-P-450 reductase. The P-450 isoenzyme mixture obtained, together with the purified reductase, could be successfully used in a reconstituted P-450

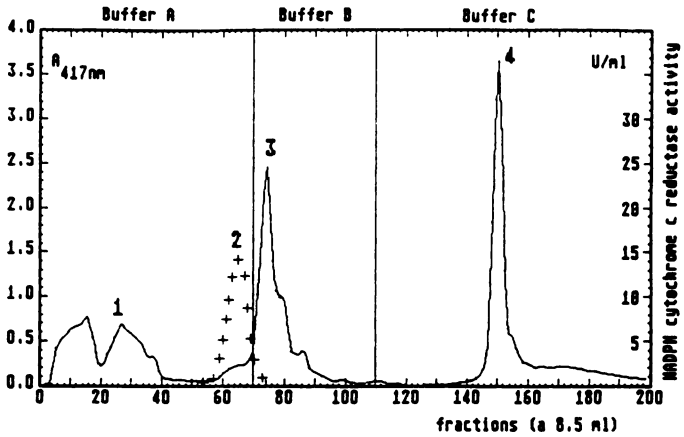

Fig. 2. Elution profile of solubilized rabbit liver microsomes after hydrophobic interaction chromatography on an octyl-Sepharose CL 4B column (i.d., 3.8 cm; length, 38 cm). Three thousand milligrams of solubilized microsomal protein were applied to the column. The elution was performed stepwise by changing the elution buffers (see Materials and Methods). The elution profile was prepared by measuring the absorbance at 417 nm (——) and the NADPH-cytochrome *c* reductase activity (+ + +), as described in Materials and Methods. The fractions related to peaks 1–4 in the elution profile were characterized as described in the text. Buffer A was 10 mM potassium phosphate, 1 mM Na_2EDTA, 500 mM NaCl, 20% (w/v) glycerol, 0.5% (w/v) sodium cholate, pH 7.4; buffer B was 10 mM potassium phosphate, 1 mM Na_2EDTA, 500 mM NaCl, 0.44% (w/v) sodium cholate, 0.2% (w/v) Lubrol PX, pH 7.4; and buffer C was 10 mM potassium phosphate, 1 mM Na_2EDTA, 20% (w/v) glycerol, 0.2% (w/v) sodium cholate, 2.0% (w/v) Lubrol PX, pH 7.4.

enzyme system for the *N*-hydroxylation of benzamidine (Table 1).

The determined conversion rate amounted to 0.63 ± 0.03 nmol of benzamidoxime formed/min/nmol of P-450. Reference incubations performed with the microsomal preparation resulted in a conversion rate of 0.58 ± 0.01 nmol of benzamidoxime/min/nmol of P-450 and 0.54 ± 0.01 nmol of benzamidoxime/min/mg of protein. Similar values were obtained in previous studies (5). The identity of the metabolite benzamidoxime was confirmed by comparison of its retention time with that of the synthetic reference compound, using the coinjection method and detection at two different wavelengths. For control purposes, the *N*-demethylation of benzphetamine, a reaction known from the literature to be dependent on P-450, was also followed using a reconstituted enzyme system made up of the same enzyme preparations. The observed conversion rate of 11.0 ± 0.8 nmol of metabolically formed formaldehyde/min/nmol of P-450 agreed well with the literature values (33). When either the P-450 preparation, the reductase preparation, or NADPH was omitted from the reconstituted enzyme system, no formation of benzamidoxime could be observed. The omission of dilauroyl phosphatidylcholine, however, did not lead to a significant reduction of the conversion rate. The addition of the nonionic detergent Lubrol PX gave rise to a marked reduction of the conversion rate only at a final concentration of 0.05% (w/v); no significant effects could be observed at a detergent concentration of 0.02% (w/v).

In order to isolate and identify the P-450 isoenzymes participating in the reaction, the P-450 isoenzyme mixture was separated into four fractions by preparative HPLC on a Fractogel EMD DEAE-650(S) anion exchange column (Fig. 3; Table 2). Examination of the benzamidine *N*-hydroxylase activity in the reconstituted P-450 system showed that the desired enzyme activity was present in fraction 2. Fraction 2 was able to transform benzamidine at a conversion rate of 2.24 ± 0.13 nmol/min/nmol of P-450 (see Fig. 6) and thus exhibited a >3-fold higher activity than the P-450 isoenzyme mixture. The other fractions were not active. It was not necessary to remove the detergent, because the P-450 fractions in the reconstituted system were so diluted that the concentration of the detergent was <0.02% (w/v).

TABLE 1

N-Hydroxylation of benzamidine by a mixture of P-450 isoenzymes prepared from rabbit liver microsomes

A complete incubation mixture consisted of 0.05 nmol of P-450, 0.3 unit (0.18 nmol) of NADPH-P-450 reductase, 15 μg of L-α-dilauroyl phosphatidylcholine (DLPC) (final concentration, 40 μM), 0.23 μmol of NADPH (final concentration, 0.38 mM), 1.98 μmol of $MgCl_2$ (final concentration, 3.3 mM), and 0.96 μmol of benzamidine (final concentration, 1.6 mM), in 0.6 ml of 50 mM potassium phosphate buffer, pH 7.4. Incubation, sample preparation, and HPLC were performed as described in Materials and Methods. Each incubation was measured in triplicate. At least two separate incubations were performed. Values are mean ± standard deviation. ND, not detectable; *n*, number of determinations.

Composition of incubation mixture	*n*	Activity	
		nmol of benzamidoxime/min/nmol of P-450	%
Complete system	18	0.63 ± 0.03	100.0*
−NADPH	6	ND	0.0
−P-450	6	ND	0.0
−Reductase	6	ND	0.0
−DLPC	6	0.62 ± 0.02	98.4 ± 3.2
+Lubrol PX, 0.02% (w/v)	6	0.69 ± 0.06	109.5 ± 9.5
+Lubrol PX, 0.05% (w/v)	6	0.43 ± 0.02	69.0 ± 3.2

* Based upon activity in the complete system.

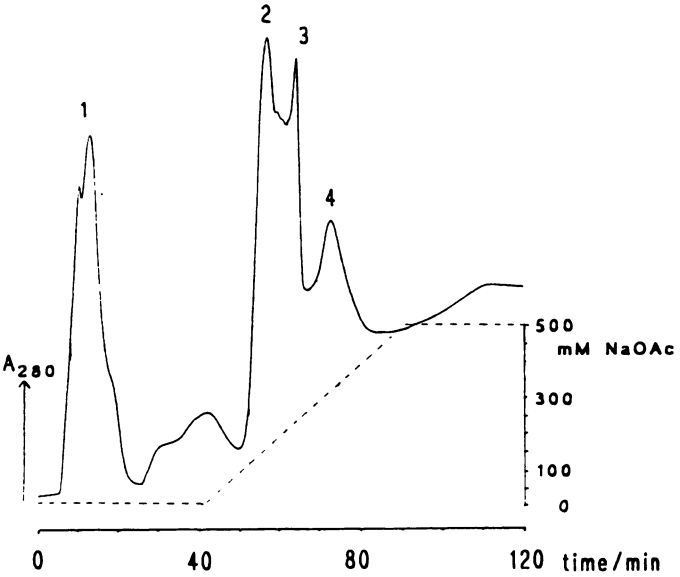

Fig. 3. Separation of P-450 isoenzymes by anion exchange HPLC. Aliquots of the P-450 mixture were applied to a semipreparative Fractogel EMD DEAE-650(S) HPLC column (i.d., 1.0 cm; length, 15.0 cm). The elution was performed by applying a linear gradient of sodium acetate (*NaOAc*). The elution profile was prepared by measuring the absorbance at 280 nm. Fractions 1–4 were tested for benzamidine *N*-hydroxylase activity as described in the text.

After SDS-PAGE (Fig. 4), fraction 2 showed a major band with an apparent molecular weight of 51,200 ± 500 and a weakly stainable impurity in the higher molecular weight region. The absorption spectrum was characteristic for a P-450 isoenzyme present in the low-spin form. The maximum in the CO difference spectrum was at exactly 450 nm. The specific P-450 content amounted to 7.0 nmol of P-450/mg of protein. The electrophoretic and spectral properties permit the assumption that the isolated P-450 fraction consists of one or more variants of the constitutive, noninducible, isoenzyme P-450 IIC3.

This isoenzyme form was first isolated from the livers of untreated rabbits and characterized in 1980 by Johnson (28) and Koop and Coon (34). Later investigations revealed that outbred New Zealand white rabbits contained two variants of P-450 IIC3 that could not be separated by the usual electrophoretic and chromatographic methods (29). However, the two variants differ in their structural and catalytic properties. Both subforms displayed very similar yet distinguishable peptide fingerprints.

Whereas one subform (6βH) is able to hydroxylate progesterone at the 6β- and 16α-positions with a high catalytic efficiency, the other subform (6βL) is associated with a less efficient progesterone 16α-hydroxylase activity. The 6β- and 16α-hydroxylase activities of the 6βH subform can be inhibited selectively by 16α-methylprogesterone (29, 30). The 16α-hydroxylase activity of the 6βL subform can be specifically stimulated by the allosteric effector 5β-pregnan-3β,20α-diol (30, 35). In certain inbred laboratory strains (IIIVO/J or B/J rabbits) only the latter of the two P-450 IIC3 subforms is present (30).

In 1984, Reubi *et al.* (31) prepared a monoclonal antibody against P-450 IIC3 that inhibited the catalytic activity of both P-450 IIC3 variants. After addition of this antibody preparation

TABLE 2
Purification of P-450 from rabbit liver microsomes

Purification step	Protein	P-450		Purification	Yield
		Total	Specific content		
	mg	*nmol*	*nmol/mg of protein*	*fold*	%
1. Microsomes	3276	3440	1.05	1.0	100.0
2. Solubilized supernatant	3067	2791	0.91	0.9	81.1
3. Octyl-Sepharose CL 4B	224	970	4.33	4.1	28.2
4. Hydroxylapatite	94	561	6.00	5.7	16.3
5. Anion exchange HPLC (fraction 2)	4	28	7.00	6.7	0.8

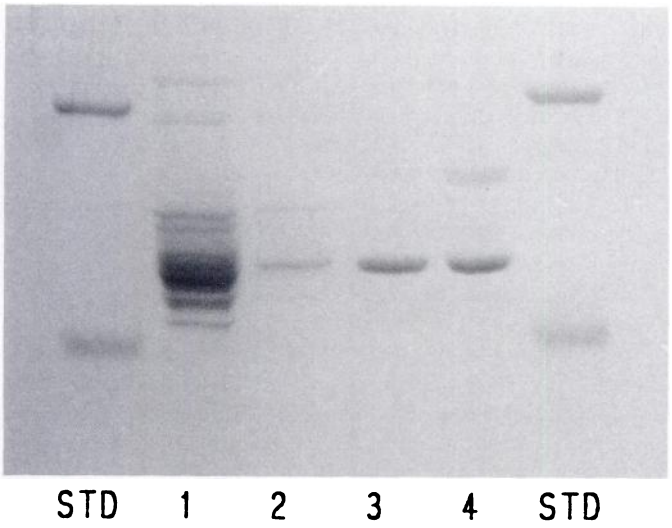

Fig. 4. SDS-PAGE of P-450 mixture and purified P-450 IIC3. The anode was at the bottom of the gel. Samples were electrophoresed on a 1.5-mm thick acrylamide gel (3% stacking gel, 8% resolving gel) and stained with Coomassie blue. *Lane 1*, mixture of rabbit liver microsomal P-450 isoenzymes as obtained after hydrophobic interaction chromatography and hydroxylapatite chromatography (10 μg); *lane 2*, P-450 fraction 2 as obtained after anion exchange HPLC (2 μg). Highly purified variants of P-450 IIC3 isolated from rabbit inbred strain III/J (*lane 3*, 2 μg) and from rabbit inbred strain IIIVO/J (*lane 4*, 2 μg) are given for comparison. The outer lanes show protein standards (*STD*) with molecular weights of 67,000 and 43,000.

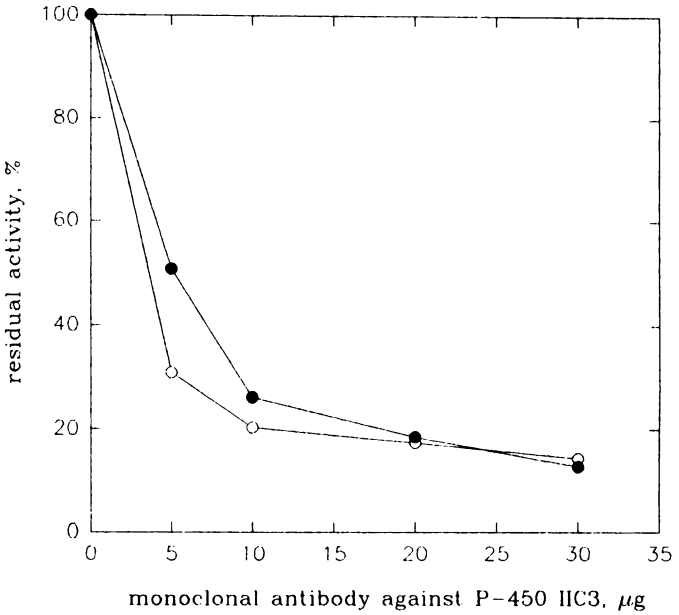

Fig. 5. Inhibition by a monoclonal antibody against P-450 IIC3 of the *N*-hydroxylation of benzamidine catalyzed by reconstituted preparations of P-450. The indicated amounts of the monoclonal antibody were incubated at 37° with 0.05 nmol of P-450 reconstituted with 0.3 unit of NADPH-P-450 reductase and L-α-dilauroyl phosphatidylcholine. After a 3-min preincubation period benzamidine and NADPH were added as described in Materials and Methods. The reaction was stopped after 20 min and the benzamidoxime formed was analyzed as described. Data are expressed as the percentage of benzamidine *N*-hydroxylase activity catalyzed by the mixture of P-450 isoenzymes (○) and by P-450 fraction 2 as obtained after anion exchange HPLC (●), compared with the original activity in an incubation containing no antibody. Each point represents the mean value of three determinations. The relative standard deviation was <5% in each case. The benzamidine *N*-hydroxylase activity without antibody was 0.63 ± 0.03 nmol of benzamidoxime formed/min/nmol of P-450 for the P-450 mixture and 2.24 ± 0.13 nmol/min/nmol of P-450 for P-450 fraction 2.

to our reconstituted P-450 systems, we observed an impressive inhibition of the *N*-hydroxylation of benzamidine (Fig. 5). The inhibition curves showed that as little as 20 μg of the antibody were sufficient to inhibit the benzamidine *N*-hydroxylase activity of the P-450 isoenzyme mixture and of the P-450 fraction 2 isolated therefrom by >80%. The addition of the same amount of antibody to reference incubations with the rabbit liver microsomal preparation resulted in a 93.8 ± 1.1% inhibition of the rabbit liver microsomal benzamidine *N*-hydroxylase activity.

The results of the reconstitution experiments performed with highly purified variants of P-450 IIC3 for comparison purposes are shown in Fig. 6. Comparable conversion rates for the *N*-hydroxylation of benzamidine were obtained with the variants isolated by Johnson (28–31) and co-workers from the inbred rabbit strains III/J and IIIVO/J, with the variants IIC3 (6βL) and IIC3 (6βH) expressed by recombinant *E. coli*, and with the IIC3 preparation (fraction 2) isolated in this work.

Under the influence of 16α-methylprogesterone (final concentration, 50 μM), the conversion by fraction 2 was inhibited by 28 ± 2%. The addition of 5β-pregnan-3β,20α-diol (final concentration, 50 μM), on the other hand, resulted in a stimulation of 112 ± 4%. Effects on the same order of magnitude were observed with the IIC3 preparation isolated from the inbred strain III/J; the latter is a mixture of the two variants IIC3 (6βH) and IIC3 (6βL) (30). In the cases of the variant IIC3 (6βL) isolated from the inbred strain IIIVO/J, as well as the variant IIC3 (6βL) expressed by recombinant *E. coli*, only a very weak stimulation by 5β-pregnan-3β,20α-diol and no significant inhibition by 16α-methylprogesterone were observed. In contrast, the effects with the variant IIC3 (6βH) expressed by recombinant *E. coli* were much more pronounced, i.e., a 342 ± 91% stimulation by 5β-pregnan-3β,20α-diol and a 73 ± 14% inhibition by 16α-methylprogesterone.

For comparison purposes, we have applied the highly purified P-450 IIC3 variants from the inbred strains III/J and IIIVO/J,

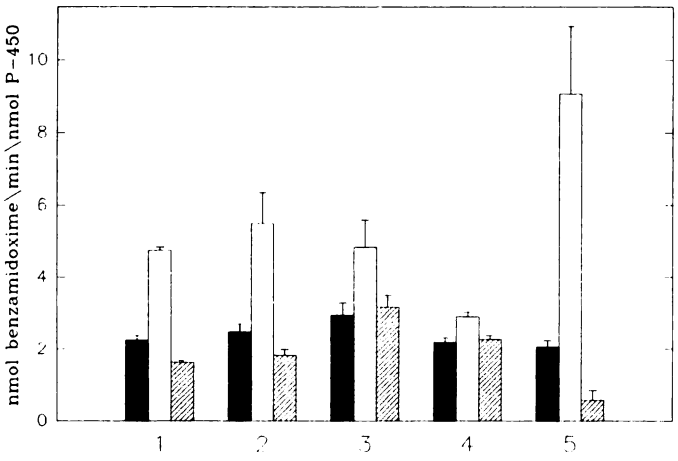

Fig. 6. *N*-Hydroxylation of benzamidine by purified variants of P-450 IIC3. The incubation mixtures contained 0.3 unit of purified NADPH-P-450 reductase and 0.05 nmol of purified P-450 IIC3 from different origin. The exact composition of a complete incubation mixture is described in the legend to Table 1 and in Materials and Methods. The effectors 5β-pregnan-3β, 20α-diol and 16α-methylprogesterone were added to the incubation mixture in methanol (10 μl). The final concentration of the effectors was 50 μM. *1*, P-450 fraction 2 as obtained after anion exchange HPLC; *2*, P-450 IIC3 prepared from rabbit inbred strain III/J; *3*, P-450 IIC3 prepared from rabbit inbred strain IIIVO/J; *4*, P-450 IIC3 (6βL) expressed by recombinant *E. coli*; *5*, P-450 IIC3 (6βH) expressed by recombinant *E. coli*. ■, Benzamidine-*N*-hydroxylase activity of the complete system expressed as the mean value of 12 determinations from four separate incubations (n = 12, mean ± standard deviation, x = 4); □, activity after addition of 5β-pregnan-3β,20α-diol (n = 6, mean ± standard deviation, x = 2); ▨, activity after addition of 16α-methylprogesterone (n = 6, mean ± standard deviation, x = 2) (n, number of determinations; x, number of separate incubations).

as well as the IIC3 preparation (fraction 2) isolated in the present work, to SDS-PAGE (Fig. 4).

Discussion

Because the previous investigations (5) had provided no information about the P-450 isoenzymes participating in the *N*-hydroxylation of benzamidine and because pretreatment with typical P-450 inducers did not result in an increase in the conversion rates (5), we initially attempted to obtain a P-450 preparation that would contain all the constitutionally present P-450 isoenzymes. The purification procedures described enable a rapid enrichment of the naturally occurring P-450 isoenzymes and the concomitant isolation of the NADPH-P-450 reductase from rabbit liver.

The *N*-oxidation of benzamidine with microsomal fractions from rabbit liver observed in the previous investigations (4) could also be demonstrated with a P-450 enzyme system reconstituted from the purified reductase and the enriched P-450 isoenzyme mixture. The dependence of the reaction on the P-450 system was confirmed by the fact that the conversion of benzamidine to benzamidoxime took place only in the presence of the P-450 preparation and the reductase preparation. In the case of participation of the FMO or of another oxidizing enzyme that could have been present as an impurity in the P-450 preparation, similar conversion rates would have been obtained with the sole addition of the P-450 preparation and with the complete reconstituted enzyme system.

The observation that the catalytic activity of the reconstituted enzyme system was not dependent on the presence of the phospholipid dilauroyl phosphatidylcholine can be explained by the possible presence of residual traces of detergent in the enzyme preparations used. Other authors have reported that the role of phospholipids in reconstituted P-450 enzyme systems can be taken over by low concentrations of nonionic detergents (33).

By means of the HPLC separation on the semipreparative Fractogel EMD DEAE-650(S) ion exchange column, a rapid separation of the P-450 isoenzyme mixture and the enrichment of the specific benzamidine *N*-hydroxylase activity by a factor of 3.6 in fraction 2 of the eluate were achieved. Because fraction 2 in the SDS gels shows only one additional, weakly stainable, band apart from the major band, the relatively low specific P-450 content of 7.00 nmol/mg of protein is presumably due to a loss of heme by contact with the metal-surfaces of the conventional analytical HPLC apparatus, rather than to a possible contamination by accompanying proteins.

The assignment of the benzamidine *N*-hydroxylase activity to the known constitutive P-450 isoenzyme form IIC3 on the basis of electrophoretic and spectral properties was confirmed by inhibition studies with monoclonal antibodies against P-450 IIC3. The extent of the inhibition of the benzamidine *N*-hydroxylase activity of fraction 2 by the monoclonal antibodies against P-450 IIC3 correlated with the results of Reubi *et al.* (31) for the inhibition of the P-450 IIC3-dependent 6β- and 16α-hydroxylation of progesterone. Because the benzamidine *N*-hydroxylase activity of the P-450 isoenzyme mixture was inhibited by the antibodies to a similarly large degree, it is improbable that other isoenzyme forms apart from P-450 IIC3 participate in the *N*-hydroxylation of benzamidine. The almost complete inhibition (93.8 ± 1.1%) of the microsomal benzamidine *N*-hydroxylase activity by the monoclonal antibody against P-450 IIC3 provides a link to the metabolism of benzamidine in rabbit liver microsomes. The specific content of P-450 IIC3 in microsomes obtained from untreated rabbits ranges from 0.15 to 0.25 nmol/mg of protein, as determined by a competitive binding assay using a monoclonal antibody to P-450 IIC3 (36). The specific content (0.25 nmol of P-450 IIC3/mg of protein) times the specific benzamidine *N*-hydroxylase activity of P-450 IIC3 (2.24 ± 0.13 nmol of benzamidoxime formed/min/nmol of P-450 IIC3) could well explain the observed total microsomal activity of 0.54 ± 0.01 nmol of benzamidoxime/min/mg of protein. Taken together, these findings suggest that P-450 IIC3 is exclusively responsible for the *N*-hydroxylation of benzamidine in rabbit liver microsomes.

The reconstitution experiments performed for comparative purposes with highly purified variants of P-450 IIC3 unequivocally confirmed the participation of the isoenzyme form IIC3 in the *N*-hydroxylation of benzamidine. They also revealed that the two variants P-450 IIC3 (6βH) and P-450 IIC3 (6βL) are equally able to catalyze the reaction. A similar catalytic behavior of the two subforms in the metabolism of xenobiotic agents had previously been reported by Dieter and Johnson (29) for the 4-hydroxylation of biphenyl and the 7-hydroxylation of acetylaminofluorene. The two subforms exhibited different catalytic behavior towards the substrate benzamidine only under the influence of the effectors 5β-pregnan-3β,20α-diol and 16α-methylprogesterone. The P-450 IIC3 preparation isolated in the present work, which exhibited moderate stimulation of the benzamidine *N*-hydroxylase activity by 5β-pregnan-3β,20α-diol and poor inhibition by 16α-methylprogesterone, showed behav-

ior similar to that of the highly purified P-450 IIC3 preparation isolated from the inbred strain III/J, which contains both P-450 IIC3 variants (30). This indicates that the IIC3 preparation isolated in the present work also consists of a mixture of the two subforms.

Benzamidine has a pK_a value of 11.6 (37). It is thus even more strongly basic than the aliphatic amines. This is, above all, the consequence of the mesomerically stabilized cation (9), which is also the predominant form at pH 7.4. Therefore, amidines are very soluble in water and have highly hydrophilic properties. The present investigations have shown for the first time that the P-450 forms IIC3 (6βH) and IIC3 (6βL), which participate in the 6β- and 16α-hydroxylation of progesterone, are also able to *N*-hydroxylate hydrophilic amidine groups. It was also shown that low concentrations of 5β-pregnan-3β,20α-diol, a naturally occuring catabolic product of progesterone, can stimulate the *in vitro* *N*-hydroxylation of benzamidine catalyzed by P-450 IIC3. Lasker *et al.* (38) have demonstrated the potential role of positive effectors in the regulation of *in vivo* metabolism by showing that flavone can stimulate the whole-body metabolism of zoxazolamine by the P-450 system. It might be speculated that the established microsomal *N*-hydroxylation of the trypanocidal diamidines pentamidine and diminazene (7–10) is probably also catalyzed by P-450 IIC3. Thus, it might be possible that the *N*-oxidative metabolic pathway of these drugs is activated during pregnancy.

Preliminary *in vitro* studies in our laboratories have shown that benzamidine can also be *N*-hydroxylated by human liver microsomes. However, the *in vivo* relevance of this *N*-oxidative pathway remains to be established, because there is the possibility of a simultaneously occurring retro-reduction of benzamidoxime to benzamidine (11, 12). The identification of P-450 IIC3 as the rabbit liver microsomal benzamidine *N*-hydroxylase facilitates the search for the human equivalent. At present at least three different human P-450 isoenzymes belonging to the P-450 IIC subfamily have been characterized as proteins, with even more members as cDNAs (39). Because it is well established that even a single amino acid difference can cause a dramatic change in the substrate specificity of P-450 isoenzymes (40), it cannot be predicted that members of the human P-450 IIC subfamily must be responsible for the *N*-hydroxylation of benzamidine in human liver microsomes. Nevertheless, the members of this subfamily should be the first candidates to be checked for amidine *N*-hydroxylase activity.

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