Isolation and Characterization of the Protein Components of the Liver Microsomal O₂-insensitive NADH-Benzamidoxime Reductase*

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Drugs containing strong basic nitrogen functional groups can be N-orangeyated to genotoxic products. The reduction of such products is of considerable toxico logical significance, most in vitro studies have focused on oxygen-sensitive reductase systems. However, an oxygen-insensitive microsomal hydroxylamine reductase consisting of NADH, cytochrome b₅, its reductase, and a third unidentified protein component has been known for some time (Kadlubar, F. F., and Ziegler, D. M. (1974) Arch. Biochem. Biophys. 162, 83–92). This report describes the isolation and identification of all of the components required for the reconstitution of an oxygen-insensitive liver microsomal system capable of catalyzing the efficient reduction of primary N-hydroxylated structures such as amidines, guanidines, amidinohydrazones, and similar functional groups. In addition to cytochrome b₅ and its reductase, the reconstituted system requires phosphatidylcholine and a P₄₅₀ isoenzyme that has been purified to homogeneity from pig liver. The participation of cytochrome b₅ and NADH cytochrome b₅ reductase in cytochrome P₄₅₀-dependent biotransformations has previously only been described for oxidative processes. The data presented suggest that this system may be an important catalyst in the reduction of genotoxic N-hydroxylated nitrogen components in liver. Their facile reduction by cellular NADH may be the reason why N-hydroxylated products can be missed by studies in vitro. Furthermore, the enzyme system is involved in the reduction of amidoximes and similar functional groups, which can be used as prodrug functionalities for amidines and related groups.

The metabolism of nitrogen-containing functional groups has become a topic of considerable interest since the early discovery that N-hydroxylated intermediates are often responsible for the toxic and/or carcinogenic properties of aromatic amines, hydrazines, and amidines (1). On the other hand, the more facile N-oxgenation of secondary and tertiary alkylamines to hydroxylamines and N-oxides was considered as a route for detoxication (2, 3), and it was generally assumed that the strongly basic nitrogen compounds were metabolically stable. However, we have demonstrated that even the protonated hydrophilic amidines (4–6), as well as diamidines such as pentamidine and dimenazine and also guanidines and amidinohydrazone, are capable of undergoing metabolic N-oxgenation by liver microsomal cytochrome P₄₅₀ monooxygenases (7, 8). The N-oxgenation of these functional groups produces more reactive metabolites, and the genotoxic properties of benzamidoxime are well known (9). During investigations of the metabolic fate of strongly basic N-hydroxylated xenobiotics, we observed that they were readily reduced both in vivo and in vitro by a microsomal system present in all mammalian species (rats, rabbits, pigs, and humans) tested to date (10–13).

Preliminary experiments indicated that this system (10) had many of the characteristics of the microsomal O₂-insensitive hydroxylamine reductase described by Kadlubar et al. (14, 15), which required NADH-cytochrome b₅ reductase, cytochrome b₅, and a third unidentified protein component. In this report, we describe the isolation, purification, and characterization of this component from pig liver microsomes and show that the system catalyzing the O₂-insensitive reduction of benzamidoxime requires NADH-cytochrome b₅ reductase, cytochrome b₅, a cytochrome P₄₅₀, and phospholipid (Fig. 1).

EXPERIMENTAL PROCEDURES

Purification

Pig Liver Microsomes—Pig liver microsomes were prepared by fractional acid precipitation according to the procedure of Ziegler and Pettit (16) with slight modifications (7).

Cytochrome b₅, NADH-Cytochrome b₅ Reductase and NADPH-Cytochrome b₅ Reductase—Cytochrome b₅, NADH-cytochrome b₅ reductase, and NADPH-cytochrome P₄₅₀ reductase were separated by modifications of the procedure described by Kling et al. (17). This procedure (Boehringer Mannheim) was used in place of Emulen 913 to solubilize the microsomal proteins and in the elution buffers. All purification steps were performed at 4 °C. The solubilized pig liver microsomes were applied to an octyl-Sepharose CL 4B (Pharmacia, Freiburg, FRG) column (inner diameter, 3.8 cm; length, 38 cm), and enzymes were eluted by a stepwise increase in the detergent concentration and a decrease in the salt concentration in elution buffers (buffer A: 10 mM potassium phosphate, pH 7.4, 1 mM EDTA (Serva, Heidelberg, FRG), 1 mM dithiothreitol, 20% (w/v) glycerol, 0.5% (w/v) sodium cholate, and 0.5 mM NaCl; buffer B: 10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20% (w/v) glycerol, 0.44% (w/v) sodium cholate, 0.2% (w/v) Thesit, and 0.5 mM NaCl; buffer C: 10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20% (w/v) glycerol, 0.2% (w/v) sodium cholate, and 2% (w/v) Thesit). The flow rate was 50 ml/h; the volume of the fractions collected was 8.5 ml; and the eluate was monitored at 280 nm (Fig. 2). The fractions containing NADPH-cytochrome P₄₅₀ reductase activity (Fig. 2, peak 2) and those containing NADH-ferricyanide reductase activity (Fig. 2, peak 4) were collected. In addition, the fractions with the highest absorbance at 417 nm (cytochrome b₅) were combined (Fig. 2, peak 3).

NADH-Cytochrome b₅ Reductase—NADH-cytochrome b₅ reductase (Fig. 2, peak 4) was purified to homogeneity by affinity chromatography on 5'-AMP-Sepharose 4B (Pharmacia) similar to the procedure described for the purification of NADPH-P₄₅₀ reductase (18). The fractions containing the highest NADH-ferricyanide reductase activity were combined and concentrated, followed by gel filtration (NAP 10, Pharmacia). The specific activity of the purified reductase was 27 units/mg, and only one band was detectable on SDS-PAGE* (data not shown).

Cytochrome b₅—Cytochrome b₅ present in the highest concentration

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NADPH-cytochrome P450 reductase activity (c) was further purified by affinity chromatography by anion exchange HPLC. The elution profile was recorded by measuring the absorbance at 280 nm. Fractions TM1 and TM2 were tested for benzamidoxime reductase activity and NADH ferricyanide reductase activity as described under “Experimental Procedures.”

Fraction TM1—Fraction TM1 (Fig. 3, peak 1), which contained the third component required for benzamidoxime reductase activity in the reconstituted system and the UDP-glucuronosyltransferase (both M, 50,000), was applied to a UDP-hexanolamine-Sepharose (Sigma) column (inner diameter, 1.2 cm; length, 4.5 cm) equilibrated with 10 mM Tris acetate buffer, pH 7.4, and washed with 50 ml of buffer F, consisting of 50 mM KCl and 40 μM phosphatidylcholine in 10 mM Tris acetate buffer, pH 7.4. Activity was recovered quantitatively in the effluent, which showed only one band (50 kDa) on SDS-PAGE (like TM1 in Fig. 4). This fraction is named the third protein or the third component.

Detergents were removed from the purified enzymes by shaking the concentrated fractions for 4 h with Calbiosorb (Calbiochem, La Jolla, CA) at 4 °C.

Analytical Procedures

Materials—Benzamidoxime was synthesized by a published method (19). Benzamidine was obtained from Aldrich-Chemie (Steinheim, FRG), dextromethorphan was from Sigma, and dextrophan-D-tartrate was from ICN Biochemicals Inc. NADPH and NADH were purchased from Merck. Benzamidine was from Sigma, benzamidine, 99%, dextromethorphan was from Sigma, dextrophan-D-tartrate was from ICN Biochemicals Inc. NADPH and NADH were purchased from Merck.

Spectrophotometric measurements were performed with a Kontron Uvicord 930 spectrophotometer.

For HPLC analysis, a conventional system was used: L-5000 control.
for the reconstituted enzyme system consisted of 50 pmol of cytochrome P450 reductase activity was measured by following the reduction of reduced ferricyanide/min) as described by Mihara and Sato (24). Modifications (18) (1 milliunit 5 concentration was determined by recording the reduced minus the oxidized spectrum (absorbance 185 nm). SDS-PAGE analyses were carried out by the method of Laemmli (21), with a 3% stacking gel. For cytochrome b preparations a 12% separation gel was used, while all other preparations were separated on an 8% gel (1.5-mm thickness). Staining was carried out by the method described by Smith, 657 A-11 HPLC sampler, and D-2500 integrator (Merck/Hitachi).

Protein Concentrations—All protein concentrations were measured using the method described by Smith et al. (20) with bicinchoninic acid (BCA reagent kit; Pierce).

SDS-PAGE—The SDS-PAGE analyses were conducted by the method of Laemmli (21), with a 3% stacking gel. For cytochrome b preparations a 12% separation gel was used, while all other preparations were separated on an 8% gel (1.5-mm thickness). Staining was performed with Coomassie Brilliant Blue R250 (Serva, Heidelberg, FRG). Standards and samples were pretreated with β-mercaptoethanol for 5 min at 90 °C. The following proteins were used as standards (high molecular weight calibration kit; Pharmacia): myosin (Mr 205,000), β-galactosidase (Mr 116,000), phosphorylase b (Mr 94,000), albumin (Mr 67,000), ovalbumin (Mr 45,000), and carbonic anhydrase (Mr 29,000).

Cytochrome b Concentration—Cytochrome b concentration was determined by recording the reduced minus the oxidized spectrum (absorbance 185 nm) as described by Estabrook and Werringloer (22).

Cytochrome P450 Concentrations—Cytochrome P450 concentrations were determined by measuring the carbon monoxide difference spectra after reduction with dithionite as described by Omura and Sato (23). In the case of TM1 protein, cytochrome P450 was also quantified by CO differential absorption at 91 (type 4 horse heart; Sigma) as described in Fig. 2 and (25) with minor modifications (18) (1 milliunit = 1 nmol of reduced cytochrome c/min).

Assay for the Reduction of Benzamidoxime—The incubation mixture for the reconstituted enzyme system consisted of 50 pmol of cytochrome P450 reductase, 5 µg of fraction TM1 (Fig. 3), 40 µl DLPC, and 3.3 mM MgCl2 in a final volume of 300 µl. After 5 min preincubation period at 37 °C, the reaction was initiated by the addition of NADH (final concentration, 1 mM) to a total volume of 300 µl. After 20 min, the reaction was terminated by adding 300 µl of methanol on ice. Precipitated proteins were sedimented by centrifugation at 10,000 × g for 5 min, and the supernatant was analyzed by HPLC (26). Aliquot of 10 µl were injected into a LiChrospher RP-Select B column (125 × 5 mm, 5 µm; Merck) with an RP-Select B precolumn (4 × 4 mm; Merck). The separation was carried out at room temperature with 3 mM 1-octanesulfonic acid, pH 2.5 (adjusted with 85% phosphoric acid)/acetonitrile (88:12, v/v) as the mobile phase, at a flow rate of 0.7 ml/min. The effluent was monitored at 229 nm. The retention time for benzamidine was 16.5 ± 0.4 min and 14.3 ± 0.2 min for benzamidoxime when injected as controls (Fig. 5). The rate of product formation was linear for about 60 min, and the limit of detection of benzamidine was 10 pmol/injection. Standard curves with 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, and 50.0 µM benzamidine were constructed and found to be linear over this range with correlation coefficients >0.9993. The recovery of benzamidine from incubation mixtures was 98.5 ± 1.3% (n = 36) of that obtained using samples that contained the same amount of benzamidine dissolved in phosphate buffer.

Microsomal Reductase Activities—The reductase activities of pig and human liver microsomes were measured by following the reduction of benzamidoxime, as described above for the reconstituted system, in 300 µl of 100 mM phosphate, pH 6.3, containing 500 µM benzamidoxime, 1 mM NADH, and 0.05–0.1 mg of human liver microsomes or 0.2 mg of pig liver microsomes. Ten different samples of human liver microsomes obtained from Human Biologics, Inc. (Phoenix, AZ) were tested. Various enzymatic activities related to specific human P450 subfamilies had been determined by Human Biologics, Inc.

O-Demethylation of Dextromethorphan—The O-demethylation of dextromethorphan was carried out essentially as described (27) in 100 mM phosphate, pH 7.4, containing 0.5 units of NADPH-cytochrome P450 reductase, 5 µg of third protein component or 5 µg of fraction TM1 (Fig. 3), 40 µl DLPC, and 3.3 mM MgCl2 in a final volume of 300 µl.

FIG. 5. Representative HPLC chromatograms of benzamidoxime metabolism by pig liver microsomes. See “Experimental Procedures” for details of reaction mixture content, incubation, sampling, and analysis. A, omission of NADH; B, complete system. Retention times were 14.3 min for benzamidoxime and 16.5 min for benzamidine.

$\text{b}_p$, 0.5 unit of NADH cytochrome $b_5$ reductase, 5 µg of fraction TM1 (Fig. 3, peak 1) or 5 µg of third protein, 7.5 µg of DLPC (final concentration, 40 µM), and 150 nmol of benzamidoxime (final concentration, 0.5 mM) in a 100 mM potassium phosphate buffer, pH 6.3. After a 5-min preincubation period at 37 °C, the reaction was initiated by the addition of NADH (final concentration, 1 mM) to a total volume of 300 µl. After 20 min, the reaction was terminated by adding 300 µl of methanol on ice. Precipitated proteins were sedimented by centrifugation at 10,000 × g for 5 min, and the supernatant was analyzed by HPLC (26). Aliquot of 10 µl were injected into a LiChrospher RP-Select B column (125 × 5 mm, 5 µm; Merck) with an RP-Select B precolumn (4 × 4 mm; Merck). The separation was carried out at room temperature with 3 mM 1-octanesulfonic acid, pH 2.5 (adjusted with 85% phosphoric acid)/acetonitrile (88:12, v/v) as the mobile phase, at a flow rate of 0.7 ml/min. The effluent was monitored at 229 nm. The retention time for benzamidine was 16.5 ± 0.4 min and 14.3 ± 0.2 min for benzamidoxime when injected as controls (Fig. 5). The rate of product formation was linear for about 60 min, and the limit of detection of benzamidine was 10 pmol/injection. Standard curves with 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, and 50.0 µM benzamidine were constructed and found to be linear over this range with correlation coefficients >0.9993. The recovery of benzamidine from incubation mixtures was 98.5 ± 1.3% (n = 36) of that obtained using samples that contained the same amount of benzamidine dissolved in phosphate buffer.

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A complete incubation mixture for optimized conditions consisted of 50 pmol of cytochrome $b_5$, 0.5 unit of NADH cytochrome $b_5$ reductase, 5 µg of third protein, 0.5 mM benzamidoxime, 1 mM NADH, 7.5 µg of DLPC (final concentration, 40 µM), in 0.5 ml of 100 mM phosphate buffer, pH 5.5. Incubation, sample preparation, and HPLC were performed as described under "Experimental Procedures." Each incubation was measured in triplicate. At least two separate incubations were performed. Values are mean ± S.D.

### TABLE I

| Composition of incubation mixture | Activity | Percentage | n* |
|----------------------------------|----------|------------|----|
| Complete system 12                | 98.8 ± 7.8 | 100        | 6 |
| Without NADH 9                   | ND       | 0.0        | 6 |
| Without NADH/with NADPH (1 mM)   | 37.2 ± 4.9 | 37.7 ± 5.0 | 6 |
| Without cytochrome $b_5$ 9       | 2.1 ± 0.7 | 2.1 ± 0.7  | 6 |
| Without NADH cytochrome $b_5$ 9  | ND       | 0.0        | 6 |
| Without third protein 9          | 10.6 ± 0.9 | 10.7 ± 0.9 | 6 |
| Without DLPC 9                   | 70.3 ± 1.6 | 71.1 ± 1.6 | 6 |
| With superoxide dismutase (500 units/ml) 9 | 148.2 ± 2.3 | 150 ± 2.5 | 6 |

* Number of determinations.
Activity values are in nmol of benzamidine/min/mg of third protein ± S.D.
Based upon activity in the complete system.
ND, not detectable.

### RESULTS

**Reconstitution Studies**—The data summarized in Table I demonstrate that reduction of benzamidoxime by NADH requires three proteins purified to apparent homogeneity from pig liver microsomes. The fastest rates were consistently observed with mixtures containing 0.5 units of NADH-cytochrome $b_5$ reductase, 50 pmol of cytochrome $b_5$, and 5 µg of the third protein isolated from peak 4 (Fig. 2) along with DLPC in phosphate buffer. DLPC was the least essential ingredient, and omission of this phospholipid decreased activity by almost 30%. On the other hand, omitting either cytochrome $b_5$ or its reductase virtually abolished activity, and omission of the third protein component decreased activity by almost 90% compared with rates obtained with the complete system (Table I). Replacing NADH with NADPH at the same initial concentration (1 mM) reduced activity by almost 60%. The consumption of NADH was linear during the incubation with the complete system. The molar ratio of benzamidine production and depletion of NADH was 1. In the absence of the third protein component, the decrease of absorbance was the same as the blank (without substrate).

Benzamidoxime reductase activity of the reconstituted system was insensitive to catalase and cyanide. A complete inhibition of the benzamidoxime reductase activity was observed upon the addition of 0.5 mM N-methylhydroxylamine (data not shown). Superoxide dismutase (500 units/ml) increased the rate of reduction with some preparations as much as 50%.

We also investigated the addition of NADPH-cytochrome P450 reductase. The presence of NADPH-cytochrome P450 reductase in the reconstituted system decreased the benzamidoxime reductase activity by 35% (Table II). In addition to this, added NADPH (1 mM) resulted in a further 25% inhibition compared with the complete system. The inhibitory effect of NADPH-P450 reductase was reversed by the addition of superoxide dismutase. The replacement of NADH-cytochrome $b_5$ reductase by NADPH-P450 reductase in presence of superoxide dismutase diminished activity by about 50%; in the absence of superoxide dismutase, however, there was no detectable activity (Table II).

The addition of dextromethorphan (final concentration, 0.5 mM), NADPH-P450 reductase (0.5 unit), and NADPH (final concentration, 1 mM) to the reconstituted benzamidoxime reductase system diminished the appearance of the benzamidine product by about 30% in presence of superoxide dismutase (500 units/ml) (data not shown).

In addition to benzamidoxime, the reconstituted system also catalyzed the reduction of guanoxabenz and N-hydroxydebrisoquine (data not shown). Although the reduction of these N-hydroxylated substrates by NADH required the same components, the optimal pH for reduction of N-hydroxydebrisoquine (near pH 7) was higher than that for the reduction of guanoxabenz (pH 6) or benzamidoxime (pH 5.5).

**Composition of Third Protein Component**—Spectra of this protein purified to apparent homogeneity from peak TM1 (Fig. 3) were similar to those of other cytochrome P450 isoenzymes and, like the latter, gave typical reduced minus oxidized difference spectra in the presence of CO (data not shown). The amino acid sequences of three peptides isolated from a proteolytic digest of the purified protein are shown in Table III. The sequence of peptide 2 is identical to that of a sequence in P450 #57.8), CYP1A (≤57.8%), CYP1A (≤57.8%) and CYP2J2 (≤56.3%), while the sequence of peptide 2 shows a slight similarity to the subfamily CYP3A.

The data summarized in Table IV demonstrate that the third protein component can function as a monooxygenase in the system reconstituted with the NADPH-cytochrome P450 reductase. The results show that the protein purified to homogeneity from fraction TM1 also has the catalytic activity expected of this class of P450-dependent monooxygenases.

**Human Liver Microsomes**—Activity measurements with human liver microsomes show that these preparations also catalyze oxygen-insensitive reduction of benzamidoxime by NADH. The activity of 10 samples of human microsomes varied between 0.2 and 0.7 nmol of benzamidine/min/mg of protein. The stoichiometric ratio of NADPH-cytochrome $b_5$ to NADPH-cytochrome P450 reductase to third protein component in the reconstituted system from pig liver was 10:2:1. However, there was still an increase in amidoxime reductase activity by the addition of cytochrome $b_5$ to this reconstituted system, while the stoichiometric ratio of NADH-cytochrome $b_5$ reductase to third protein component was optimal. Since the relative composition of the three protein components of benzamidoxime reductase varies

### TABLE II

| Composition of incubation mixture | n* | Activity | Percentage |
|----------------------------------|----|----------|------------|
| Complete system 12               | 12 | 147.9 ± 5.3 | 100 ± 3.6 |
| Without NADPH 8                 | 8  | 95.4 ± 8.7  | 64.5 ± 5.9 |
| Without SOD 8                   | 4  | 200.7 ± 3.7 | 149.2 ± 2.5 |
| Without NAPDH 8                 | 8  | 61.5 ± 14.5 | 41.6 ± 9.8 |
| Without SOD 8                   | 4  | 29.1 ± 1.8  | 20.1 ± 1.2 |

* Based upon activity in the complete system.

ND, not detectable.
and Ziegler (15) also indicated that this hydroxylamine reduc-
tive enzyme. A comparison with all known cytochrome P450 iso-
zymes shows that its amino acid sequence is very close to isoenzymes of the subfamily 2D from other species. Similarities with other isoenzymes are less striking. The assumption that the isolated enzyme is a P450 isoenzyme was confirmed by the CO difference spectra. The identity of the homogenous third component as a cytochrome P450 was also confirmed by its ability to catalyze the demethylation of dextromethorphan upon reconstitution with NADPH-P450 reductase and phospholipid (Table IV). This reaction is a characteristic marker activity of the P450 2D subfamily (31). The addition of dextromethorphan and the components, which demethylate dextromethorphan, to the benzamidoxime reduction system diminished the benzamidoxime reductase activity. This indicates that benzamidoxime and dextromethorphan are both sub-
strates for the purified enzyme. Furthermore, the mass of the purified third component, 50 kDa, is consistent with the size of other P450 isoforms isolated from other species. Cytochrome P450 2D isoenzymes from pig have not been described previ-
ously. Attempts to reconstitute the benzamidoxime reductase from human liver and to identify the responsible human P450 isoenzyme are in progress. Human hepatic microsomes cata-
lyzed the reduction of benzamidoxime, and the conversion rates observed were of an order of magnitude comparable with those determined for microsomes from porcine liver. We can there-
fore expect that the results of these investigations will be comparable with those from experiments with isolated human enzymes.

The participation of cytochrome P450 and NADPH-cyto-

The complete incubation mixture consisted of 5 µg of third protein (TM1 or UDP1), 0.3 units of NADPH cytochrome P450 reductase, 7.5 µg of DLPC (final concentration, 40 mM), 3.3 mM MgCl2, 200 mM NADPH, and 50 µM dextromethorphan in 0.3 ml of 100 mM phosphate buffer, pH 7.4, as described under “Experimental Procedures.” Each incubation

in all human samples and each of these proteins can be rate-
limiting (data not shown), no clear correlation between benz-
amidoxime reductase activity and the content of the various P450 isoenzymes was observed.

Microsomal preparations from rabbit and rat liver (10), as well as those from pig and human livers catalyze the NADH-depen
dent reduction of benzamidoxime to benzamidine. The reduction of other N-oxygenated basic nitrogen-containing functional groups by these liver preparations has also been described for N-hydroxylated guanidine, N-hydroxycarbodio-
quine (12), and amidinohydrazide guanoxabenz (30). Previous studies (4, 7) have also shown that liver microsomal prepara-
tions can also catalyze NADPH-dependent N-hydroxylation of these compounds, but the rates of N-hydroxylation were about 2 orders of magnitude lower than rates of reduction, which suggests that the latter reaction may prevent accumulation of toxic N-hydroxylated products. The in vivo relevance of such processes has been confirmed previously with studies on live animals (13).

Preliminary studies on the biochemical properties of the benzamidoxime reductase suggested that it shared character-
istics reported by Kadlubar and Ziegler (15) for pig liver micro-
somal hydroxylamine reductase. Both were completely oxygen-
sensitive, preferentially use NADH as reductant, and are

Discussion

The present report clearly demonstrates that the oxygen-
sensitive benzamidoxime reductase purified and reconsti-
tuted from pig liver microsomes requires cytochrome b5, its
reductase, and a third electrophoretically pure protein (Fig. 4 and Table I). This protein also has characteristics of a P450

Table III

Amino acid sequences of peptides of third protein component compared to sequences of CYP 2D and CYP 2D6

| Peptide 1 | Met Leu Lys Leu Leu Asp Leu Val Leu Glu Gly Leu Lys Glu Glu |
| Peptide 2 | Ile Ile Asp Arg Arg Arg Arg Thr Glu Asp Met |
| Peptide 3 | Met Ile His Pro Asp Val Gin Arg Arg Val Gin Gin Glu Ile Asp |
| Peptide 4 | Phe Arg Arg Arg Arg Arg Arg Arg Arg |
| Peptide 5 | Ser Thr Thr Thr Thr Thr Thr Thr Thr |

a Ref. 28. See “Experimental Procedures.”
b –, same amino acid as in third protein component.
c Ref. 29. See “Experimental Procedures.”

Table IV

Demethylation of dextromethorphan

| Composition of incubation mixture | Activitya |
|----------------------------------|-----------|
| Complete system                  | 10 ± 0.5  |
| TM1                              | 8         |
| Third protein                    | 8         |
| Without NADPH                    | 5         |
| Without P450 reductase           | 8         |
| Without third protein            | 8         |

a Number of determinations.
b Activity values are in nmol of dextrophan/min/mg of third protein.
c ND, not detectable.
system inhibited the reductase activity, as was also described for the microsomal reaction (12). It has been reported by Cribb et al. (35) that sulfamethoxazole hydroxylamine is reduced aerobically by human liver microsomes. The authors employed the same microsomal liver preparations (10 different samples of human liver microsomes provided by Human Biologics, Inc.). The reaction rates obtained in this study on the reduction of benzamidoxime are very similar to those described for the reduction of sulfamethoxazole hydroxylamine (correlation coefficient, 0.9835), strongly suggesting that the same enzyme system that reduces amidoximes is also capable of reducing hydroxylamines.

It has been shown that the presence of the three protein components, cytochrome b5, its reductase, and cytochrome P450, as well as DLPC and NADH is required for the N-reduction of benzamidoxime (Table I). The observation that the absence of DLPC in reconstituted systems does not lead to a pronounced lowering of the conversion rates can be explained by the possibility that traces of detergent are still present in the enzyme fractions. The role of phospholipids in reconstituted P450 enzyme systems can be partially fulfilled by a low concentration of nonionic surfactants (36).

The presence of superoxide anions evidently gives rise to inhibiting reactions, as demonstrated by the stimulation of benzamidoxime reduction by superoxide dismutase (Table I). This observation also demonstrates that a nonenzymatic, iron-catalyzed reduction of benzamidoxime by superoxide does not contribute to product formation.

The electron transport of the benzamidoxime reduction could be partially effected by the NADPH-cytochrome P450 reductase, although only in the presence of superoxide dismutase. Furthermore, the conversion rate is only 20% of that of the complete system. The reduction in activity upon the addition of this flavoprotein to the complete reconstituted system, which is further accentuated by the addition of NADPH, can be explained by the formation of superoxide (Table II).

The reconstitution of the three protein components is essential for the electron transfer, as was also shown by measuring the consumption of NADH.

The evidence presented suggests that the reductase activity of the reconstituted system is solely enzymatic. The proteins cytochrome b5, NADH-cytochrome b5 reductase, and cytochrome P450, isolated to electrophoretic purity, in a reconstituted system under aerobic conditions are highly efficient as a catalyst for the reduction of N-hydroxylated compounds, such as N-oxygenated amidines, guanidines, and amidinoxyhydrzones. We expect that this reduction also occurs with weakly basic N-hydroxylated substances that often exhibit a genotoxic potential, as is the case for the above mentioned compounds with strongly basic functional groups. These biotransformations can be considered as a physiological detoxification reaction.

The facile reduction of amidoximes and similar functional groups in vivo by the microsomal enzyme system described here and possibly by other reductive systems is responsible for the pharmacological activity of N-hydroxylated compounds such as the amidoximes of pentamidine (37). Thus an amidoxime group can serve as a prodrug functionality for an amido group. The same concept can be applied to guanidines and amidinoxyhydrzones and their N-oxygenated derivatives (12, 30). Therefore, strong basic nitrogen-containing functional groups, which are protonated under physiological conditions and are not absorbed as cations, can be made orally available by introducing a hydroxy group, which lowers the pH values significantly. The amidoximes and similar functionalities are absorbed as the free bases and reduced in the liver to the active principles by the enzyme system described here. Meanwhile, this prodrug approach has not only been applied to pentamidine but also to orally active fibrinogen receptor antagonists (38).

The studies summarized in this report completely characterize for the first time all of the components of the microsomal enzyme system catalyzing the reduction of a wide variety of N-oxygenated metabolites. The observation that human liver microsomal benzamidoxime and sulfamethoxazole hydroxylamine reductase (35) are similar to the purified pig liver system suggests that this multicomponent reductase may have a vital role protecting humans and other mammals against accumulation of potentially genotoxic N-hydroxylated metabolites.

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