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Oxygen-insensitive enzymatic reduction of oximes to imines

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ABSTRACT

The reduction of oximes to imines under anaerobic and aerobic conditions was studied using (*E*)- and (*Z*)-2,4,6-trimethylacetophenone oxime, benzaldoxime and (*E*)-2,4,6-trimethylbenzaldoxime. Pig and human liver microsomes, pig liver mitochondria and cytosol to a minor extent catalyzed the conversion of both isomeric ketoximes to the corresponding stable imine, the (*E*)-isomer being the better substrate. All reactions were oxygen-insensitive and required active protein and NADH or NADPH; however, NADH was preferred as cofactor. The reconstituted liver microsomal system of a pig liver CYP2D enzyme (NADH-benzamidoxime reductase), which is known to reduce *N*-hydroxylated derivatives of strongly basic functional groups, such as amidoximes, is also capable of reducing oximes. As expected, the corresponding imine was detected in relevant amounts when incubating 2,4,6-trimethylacetophenone oxime using the reconstituted enzyme system, but reduction rates were significantly lower compared to rates obtained when incubating benzamidoxime. Steric hindrance due to the methyl groups in *ortho* position to the oxime functionality could be excluded as being responsible for the lower conversion rates according to results obtained in incubations of 2,4,6-trimethylbenzamidoxime. When incubating benzaldoxime, only benzoic acid could be detected as metabolite, since the aldehyde is easily oxidized during incubation procedures, whereas incubations of (*E*)-2,4,6-trimethylbenzaldoxime also showed the formation of the corresponding aldehyde. These results allow us to postulate that the metabolism of aldoximes like 2,4,6-trimethylbenzaldoxime most likely proceeds through enzymatic reduction of the oxime to yield the intermediate imine, which is subsequently hydrolyzed to the aldehyde and then oxidized to the corresponding benzoic acid.

1. Introduction

Previous studies have shown that *N*-hydroxylated derivatives of strongly basic functional groups were reduced both in vivo and in vitro by a microsomal enzyme system present in all mammalian species (rats, rabbits, pigs and humans) tested to date [1,2]. This oxygen-insensitive liver microsomal enzyme system, named benzamidoxime reductase, was shown to be responsible for the efficient reduction of primary *N*-hydroxylated structures, such as amidoximes and *N*-hydroxyguanidines [1–4]. It requires NADH cytochrome b_5-reductase, cytochrome b_5 and a third protein component, which, isolated and purified from pig liver microsomes, has repeatedly been identified as an isoenzyme of the cytochrome P450 2D subfamily [5,6]. The enzyme has not been obtained in a recombinant form to date and the purified protein has been employed for the various studies [2]. The human equivalent has not been identified to date and human CYP2D6 is definitely not the responsible catalyst [5]. It could also be shown that cytochrome b_5 and cytochrome b_5-reductase alone are capable of reducing

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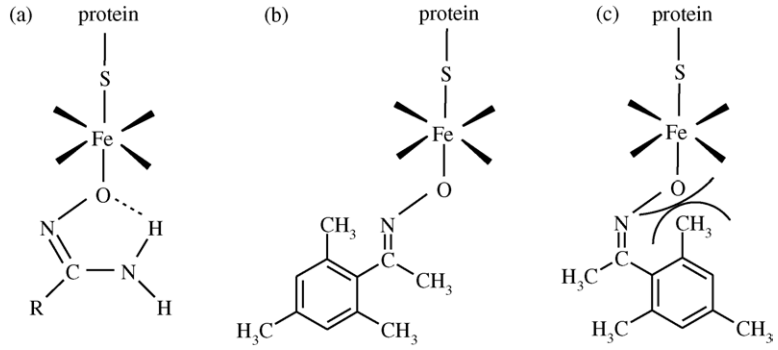

Fig. 1 – Possible structures for the complexes formed between (a) amidoximes or *N*-hydroxyguanidines (2) or (b) (*E*)- or (c) (*Z*)-ketoximes and P450 enzymes.

the *N*-hydroxylated structures [2,5]. Concerning reactions of *N*-hydroxylated structures with P450 enzymes it has been discussed that an additional amino group geminal to the oxime function which is found in amidoximes or *N*-hydroxyguanidines stabilizes the enzyme substrate complex via a five-membered transition state as shown in Fig. 1a [2]. Regarding this mechanistic proposal, it was also of interest whether this reconstituted liver microsomal system is capable of reducing oximes in spite of the fact that the five-membered transition state cannot be formed because of the missing geminal amino group. Oximes, such as 2-pralidoxime, are known as antidotal agents since they are able to reactivate organophosphate–cholinesterase conjugates, giving rise to the functional enzyme [7]. Recent studies show further fields of application for oximes, for example, the use of an oxime as antiepileptic drug [8]. The possible use of hydroxy- or methoxy-substituted benzaldoximes and benzaldehyde-*O*-alkyloximes as tyrosinase inhibitors is discussed as well [9]. Furthermore, oximes are common metabolites for a variety of primary aliphatic amines [10–12]. Only limited information is available regarding oxime biotransformation. Tatsumi and Ishigai [13] reported that liver aldehyde oxidase in the presence of its electron donor exhibits a significant oxime-metabolizing activity under anaerobic conditions. They proposed that the enzyme catalyzes the reduction of oximes to the corresponding ketimines, which in turn, depending on their chemical stability, are hydrolyzed non-enzymatically to the corresponding oxo compounds and ammonia. Alkyl- and arylaldoximes undergo a P450 Fe(II)-catalyzed dehydration to their corresponding nitriles when incubated in the absence of oxygen [14]. Stiff et al. [15] and Nakasa et al. [16–18] showed that the metabolism of zonisamide, an anticonvulsant agent containing a 1,2-benzisoxazole heterocycle, most likely proceeds through reductive cleavage of the N–O bond to yield the intermediate imine. The imine itself could not be detected since it is easily hydrolyzed to the corresponding ketone. Thus, no direct proof was available concerning the metabolic reduction of oximes to imines as they are usually hydrolyzed too rapidly to be detected. The involvement of stable imines should solve this problem. So, it was of interest whether the model compound 2,4,6-trimethylacetophenone oxime is reduced to the corresponding stable imine. The *N*-hydroxylation of this imine has already been described [19,20]. In addition, the metabolic reduction of two aldoximes, benzaldoxime and (*E*)-2,4,6-trimethylbenzaldoxime was examined in the presence of different pig liver homogenates, human liver microsomes and the reconstituted liver microsomal system of a pig liver CYP2D enzyme.

This report gives the first direct proof for the formation of an imine as primary reduction product under aerobic conditions. Thus, oximes can be reduced to imines representing a new metabolic pathway, which has to be considered when incorporating oxime functional groups into new drug candidates. The present study also focuses on the stereospecifity of the enzymatic reduction.

2. Materials and methods

2.1. Physical measurements

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 300 NMR spectrometer (Rheinstetten, Germany) at 300 K. Electron impact mass spectra were recorded on a Hewlett Packard 5989 A MS engine (Waldbronn, Germany). Electrospray ionization (ESI) mass spectra were recorded on a Bruker Esquire ~LC mass spectrometer (Bremen, Germany).

2.2. Chemicals

2,4,6-Trimethylacetophenone and NADPH were purchased from Merck (Darmstadt, Germany) and 2,4,6-trimethylbenzaldehyde, 2,4,6-trimethylbenzoic acid and 2,4,6-trimethylbenzonitrile from Lancaster (Frankfurt, Germany). NADH and (*E*)-benzaldoxime were obtained from Fluka (Buchs, Switzerland). Benzamidine, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (DLPC) and hydrobenzamide were acquired from Sigma–Aldrich (Steinheim, Germany). (*Z*)-Benzaldoxime was purchased from TRC (Toronto, Canada). All other chemicals and solvents were of analytical grade, acetonitrile and methanol were of HPLC grade.

2.3. Synthesis of (*E*)-2,4,6-trimethylbenzaldoxime

2,4,6-Trimethylbenzaldoxime was prepared according to procedure of Liu et al. [21] and identified as the (*E*)-isomer with m.p. 121–123 °C.

2.4. Synthesis of (E)- and (Z)-2,4,6-trimethylacetophenone oxime

A mixture of (Z)- and (E)-2,4,6-trimethylacetophenone oxime was obtained as described previously [22,23]. Pure (E)- and (Z)-2,4,6-trimethylacetophenone oxime was obtained by chromatography on silica using a mixture of cyclohexane/ethylacetate (3/1) as solvent: (Z)-isomer, R_f 0.32 and (E)-isomer, R_f 0.42. The structures of the two isomers were unequivocally assigned by a two-dimensional NOESY experiment in DMSO as the solvent.

2.5. Synthesis of 2,4,6-trimethylacetophenone imine

2,4,6-Trimethylacetophenone imine was prepared by reaction of methyl magnesium iodide with 2,4,6-trimethylbenzonitrile as described by Pickard and Tolbert [24].

2.6. Synthesis of benzamidoxime

The synthesis of benzamidoxime is described elsewhere [25,26].

2.7. Synthesis of 2,4,6-trimethylbenzamidoxime

Although reported in the literature [27,28], neither full experimental protocols nor spectroscopic characterization is given for this compound: into a solution of 1.80 g (11.2 mmol) of trimethylbenzonitrile-*N*-oxide [21] in 60 mL of methanol is blown gaseous ammonia for 1/2 h, while keeping the temperature of the solution below 0 °C. Then the excess of ammonia was allowed to evaporate at r. t., and, after evaporation of the remaining solvent, the residue was recrystallized (from cyclohexane/ethyl acetate) to afford 1.20 g (6.7 mmol, 58%) of 2,4,6-trimethylbenzamidoxime, m.p. 174–175 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.20 (s, 6 H, *o*-Me), 2.22 (s, 3 H, *p*-Me), 5.62 (br. s, 2 H, NH_2), 6.94 (s, 2 H, aryl-H), 9.05 (s, 1 H, OH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 19.0 (*o*-Me), 20.6 (*p*-Me), 127.4 (C_{aryl}-3), 131.6 (C_{aryl}-1), 136.5 (C_{aryl}-2), 137.0 (C_{aryl}-4), 151.0 ($C(NH_2)OH$). MS (EI, 70 eV) *m/z* (I, %B) 178 (M^+, 100), 163 (25), 161 (50), 160 (21), 146 (74), 145 (36), 144 (98), 130 (21), 91 (26), 77 (20), 73 (23).

2.8. Synthesis of 2,4,6-trimethylbenzamidine

The previously unknown amidine was obtained by catalytic reduction of 2,4,6-trimethylbenzamidoxime analogous to the procedure described by Judkins et al. [29] for similar benzamidines. 1.0 g (5.6 mmol) of 2,4,6-trimethylbenzamidoxime and 1 mL of acetic anhydride were dissolved in 50 mL of acetic acid, and 0.50 g of Pd/C (10%) were added. This mixture was shaken for 24 h under an atmosphere of H_2 at 4 bar. The suspension was then filtered and the filtrate was concentrated under vacuum using a rotary evaporator. Then ca. 20 mL of heptane were added and the solution was concentrated to dryness. The remainder was dissolved in a small amount of hot ethyl acetate and any insoluble material was filtered off. After addition of acetone the solution was allowed to cool to afford a white powder of 2,4,6-trimethylbenzamidinium acetate with m.p. of 185–187 °C in two crops and a yield of 0.42 g (1.9 mmol, 34%). Calculated for $C_{12}H_{18}N_2O_2$ (M_r = 222.29) C, 64.84; H, 8.16; N, 12.60; found C, 64.22; H, 8.29; N, 12.63%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.69 (s, 3 H, $MeCO_2^-$), 2.26 (s, 3 H, *p*-Me), 2.28 (s, 6 H, *o*-Me), 6.99 (s, 2 H, aryl-H), 10.2 (very broad s, ca. 4 H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 18.5 (*o*-Me), 20.6 (*p*-Me), 24.9 ($MeCO_2^-$), 128.0 (C_{aryl}-3), 129.5 (C_{aryl}-1), 134.3 (C_{aryl}-2), 139.3 (C_{aryl}-4), 167.5 ($C(NH_2)_2$), 176.6 ($MeCO_2^-$). MS (EI, 70 eV) *m/z* (I, %B) 162 (M^+, 100), 161 (22), 146 (23), 145 (68), 144 (33), 131 (23), 130 (82), 60 (31), 45 (46), 43 (65).

2.9. Human liver microsomes

Human liver microsomes were obtained from BD Gentest (San Jose, CA, USA) containing 20 mg/mL of protein in 250 mM sucrose. The P450 concentration was specified with 420 pmol P450 per mg of protein.

2.10. Preparation of pig liver microsomes, mitochondria and cytosol

Microsomes and cytosol were obtained by ultracentrifugation as published before [30]. Mitochondria were prepared according to the procedure of Beattie [31] and Kline et al. [32] with slight modifications [33].

2.11. Preparation of cytochrome b_5, NADH cytochrome b_5-reductase and CYP2D enzyme

Cytochrome b_5, NADH cytochrome b_5-reductase and CYP2D enzyme were fractionated by the procedure of Clement et al. [5,34]. Thesit (Fluka) was used to solubilize the microsomal proteins in the elution buffers. All purification steps were performed at 4 °C.

2.12. Cytochrome b_5

Cytochrome b_5 was purified on a DEAE-cellulose column as described previously [5]. The final cytochrome b_5 stocks contained 26.4 nmol (system I) and 38.9 nmol (system II) of cytochrome b_5 per mg of protein, respectively.

2.13. CYP2D enzyme

Benzamidoxime reductase was chromatographed on an anion exchange column by preparative HPLC with a conventional HPLC system (L-6210 Intelligent Pump, 655 A-22 UV detector, D-2500 integrator; Merck/Hitachi) as described before [34] using a semi-preparative Fractogel EMD TMAE 650 (S) column (160 mm × 50 mm; particle size, 25–40 μm; Merck). The first fraction contained the CYP2D enzyme, the second fraction contained the NADH cytochrome b_5-reductase.

2.14. NADH cytochrome b_5-reductase

NADH cytochrome b_5-reductase was purified to homogeneity by affinity chromatography on 5′-AMP-Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the procedure of Yasukochi and Masters [35]. The fractions containing the highest NADH-ferricyanide-reductase activity were combined and concentrated, followed by gel filtration

(NAP™ 25, Amersham Pharmacia Biotech AB). The specific activity of the purified reductase was 99 units/mg of protein.

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

The microsomal and the enzyme fractions were stored at −80 °C in aliquots.

2.15. Analytical procedures

(1) Protein concentration: Protein concentrations were measured using the method described by Smith et al. [36] with bicinchoninic acid (BCA reagent kit, Pierce Chemical Co., Rockford, IL, USA). All photometric measurements were performed with an Uvicon 930 (Kontron Instruments, Neufahrn, Germany) spectrophotometer.
(2) Cytochrome P450 concentrations: The P450 concentration was analyzed using the method of Omura and Sato [37].
(3) Cytochrome b_5 concentrations: The cytochrome b_5 concentration was determined by recording the reduced minus the oxidized spectrum (absorbance at 185 nM^{-1} cm^{-1}) as described by Estabrook and Werringloer [38].
(4) NADH cytochrome b_5-reductase: NADH cytochrome-reductase was assessed by its NADH-ferricyanide-reductase activity (1 unit = 1 μmol of reduced ferricyanide/min) as described by Mihara and Sato [39].
(5) CYP2D enzyme: CYP2D enzyme activity was measured by reduction of benzamidoxime to benzamidine and monitored by HPLC [5].

2.16. Incubation procedures

A typical incubation procedure with pig liver microsomes, cytosol or mitochondria is as follows: incubation mixtures consisted of 220 μg active protein and substrate either 3 mM (purified (*E*)- and (*Z*)-2,4,6-trimethylacetophenone oxime, aldoximes and amidoximes) or 4 mM (mixture of (*E*)- and (*Z*)-isomers, 54:46), respectively, in 100 mM phosphate buffer pH 6.3 or 7.4. The substances were solubilized in DMSO, if necessary (maximum final concentration of DMSO: 3.3%). After a pre-incubation time of 2 min at 37 °C, NADH or NADPH (final concentration: 1 mM) was added to a total volume of 150 μL and incubations were performed for 30 min at 37 °C. Incubations were terminated by the addition of an equal volume of cold methanol and centrifuged for 5 min at 9000 rpm. Ten microliter aliquots of the resulting supernatant were analyzed by reversed-phase HPLC.

Two differently composed enzymes systems were used for the batches, containing different stocks of purified cytochrome b_5. A typical incubation using the reconstituted enzyme system (benzamidoxime reductase) procedure is as follows: to a buffered suspension (phosphate buffer, 100 mM, pH 6.3) of 75 pmol (system I) and 100 pmol (system II) of cytochrome b_5, respectively, NADH cytochrome b_5-reductase (0.55 U) and 5 μg of the CYP2D enzyme, DLPC (40 μM) was added. After a pre-incubation time of 3 min at 37 °C, the substrate (final concentration: 3 mM, maximum final concentration of DMSO as solvent: 3.3%) was added and the mixture was pre-incubated again for another 2 min. The reaction was started adding NADH (1 mM), and incubations were performed for 30 min. Reactions were terminated by the addition of an equal volume of cold methanol. The samples were centrifuged for 5 min at 9000 rpm, and the supernatants were used for HPLC analysis.

Aerobic incubations were exposed to laboratory air while anaerobic incubations were performed in argon-degassed buffers, were gassed with argon and the reaction tubes were closed during incubation. The effectiveness of oxygen removal by this procedure was controlled via test incubations. As described [14], benzaldoxime undergoes a P450 Fe(II)-catalyzed dehydration to its corresponding nitrile when incubated anaerobically.

2.17. HPLC analysis

The HPLC system used consisted of a 600 HPLC pump and a controller 600 E from Waters (Milford, CT, USA), equipped with an autosampler (Waters 700 satellite WISP) and a variable wavelength spectrophotometric detector (Waters 486 TAD). The areas under the peak were integrated with the EZChrom Chromatography Data System (EZChrom Elite SS42ox Interface, Scientific Software Inc., Pleasanton, CA, USA).

(1) Benzoic acid, benzaldehyde/hydrobenzamide (*E*)- and (*Z*)-benzaldoxime (retention times of 9.8, 23.7, 26.2 and 31.2 min, respectively) were separated using a LiChrocart® 125 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 92/8 (v/v) mixture of water containing 100 mM potassium dihydrogenphosphate pH 4.5 and acetonitrile as eluent. The flow rate was 1 mL/min, and detection was performed at 226 nm.
(2) 2,4,6-Trimethylbenzoic acid (*E*)-2,4,6-trimethylbenzaldoxime and 2,4,6-trimethylbenzaldehyde (retention times of 9.3, 16.5 and 26.4 min, respectively) were separated using a LiChrocart® 250 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 60/40 (v/v) mixture of water containing 100 mM potassium dihydrogenphosphate and acetonitrile as eluent. The flow rate was 1 mL/min, and detection was performed at 210 nm.
(3) 2,4,6-Trimethylacetophenone imine (*Z*)- and (*E*)-2,4,6-trimethylacetophenone oxime (retention times of 8.3, 15.9 and 17.3 min, respectively) were separated using a LiChrocart® 125 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 72/28 (v/v) mixture of water containing 10 mM octane sulfonic acid (pH 3.0) and acetonitrile as eluent. The flow rate was 1 mL/min, and detection was performed at 210 nm.
(4) Benzamidoxime and benzamidine (retention times of 6.5 and 21.2 min, respectively) were separated using a LiChrocart® 250 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 83/17 (v/v) mixture of water containing 10 mM octane sulfonic acid and acetonitrile as eluent.

The flow rate was 1 mL/min, and detection was performed at 229 nm.

(5) 2,4,6-Trimethylbenzamidoxime and 2,4,6-trimethylbenzamidine (retention times of 20.8 and 23.7 min, respectively) were separated using a LiChrocart® 250 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 76/24 (v/v) mixture of water containing 10 mM octane sulfonic acid (pH 3.0) and acetonitrile as eluent. The flow rate was 1 mL/min, and detection was performed at 229 nm.

(6) (*E*)- and (*Z*)-Benzaldoxime and benzonitrile (retention times of 28.0, 30.9 and 39.7 min, respectively) were separated using a LiChrocart® 250 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 85/15 (v/v) mixture of water containing 100 mM potassium dihydrogenphosphate and acetonitrile as eluent. The flow rate was 1.3 mL/min, and detection was performed at 226 nm.

2.18. Product quantitation

In order to evaluate the recovery of 2,4,6-trimethylacetophenonimine, 2,4,6-trimethylbenzaldehyde and 2,4,6-trimethylbenzoic acid, respectively, from protein samples and to quantify the amount of metabolite formed, known amounts of metabolite in the range of 0.25–150 and 0.2–15 μM were spiked into usual incubation mixtures. Instead of active microsomes inactivated boiled microsomes were used and the cofactor was omitted. The mixture was incubated and the work-up was performed as described in Section 2. In order to evaluate the recovery, the results were compared to a calibration curve prepared using the imine, aldehyde and acid, respectively. Recovery of 2,4,6-trimethylacetophenonimine and 2,4,6-trimethylbenzoic acid from protein samples was <99%, recovery of 2,4,6-trimethylbenzaldehyde was 80%.

2.19. HPLC-MS analysis

The HPLC-MS system consisted of an Agilent 1100 binary pump (Waldbronn) with a split (1:5.5) to the ESI mass spectrometer operating in the positive ion mode, with a scan range of *m/z* 50–600 with nominal resolution. Mass spectrometric conditions were optimized for *m/z* 180.

(1) 2,4,6-Trimethylacetophenone imine, affording a signal at *m/z* 162 $[M+H]^+$, and (*E*)-2,4,6-trimethylacetophenone oxime (retention times of 11.6 and 19.7 min, respectively) were separated using a LiChrocart® 125 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a gradient elution system: solvent A: ammonium acetate 25 mM, pH 4.4 and acetonitrile 20%, solvent B: acetonitrile. The elution profile started at 100% solvent A (0–10 min), then linearly changing to 50% A (10–15 min), continuing with 50% for 10 min before returning to starting conditions (25–30 min) and equilibration (30–35 min). The flow rate was set at 1 mL/min.

(2) 2,4,6-Trimethylbenzamidine and 2,4,6-trimethylbenzamidoxime (retention times of 9.9 and 14.6 min, respectively) were separated using a LiChrocart® 250 mm × 4 mm LiChrospher® 60 RP-select B column (5 μm) (Merck) with a RP-select B pre-column (4 mm × 4 mm). Separations were carried out at room temperature using a 85/15 (v/v) mixture of water containing 25 mM ammonium acetate (pH 4.4) and acetonitrile as eluent. The flow rate was 1 mL/min. 2,4,6-Trimethylbenzamidine afforded a signal at *m/z* 163 $[M+H]^+$ and a fragment with low abundance of approximately 5% at *m/z* 146 $[M-NH_3+H]^+$.

2.20. UV–vis studies of the interaction of CYP2D with different oxime compounds

Studies were carried out at 25 °C in a Varian Cary 50 spectrophotometer (Varian, Germany) in 1 cm path-length disposable cuvettes (100 μL total volume) containing 1.07–1.50 μM cytochrome P450.

Absolute spectra were recorded against phosphate buffer, difference spectra against protein dilution in the reference cuvette.

Assayed compounds were first dissolved in DMSO and then in 100 mM potassium dihydrogenphosphate buffer pH 7.4, added stepwise to the sample cuvette up to a final concentration of 50 mM and equivalent volumes of DMSO and buffer were added to the reference cuvette.

3. Results

3.1. Reduction of (E)- and (Z)-benzaldoxime

(*E*)- and (*Z*)-benzaldoxime were stable to degradation and hydrolysis in aqueous solutions for at least 24 h, even when heated to 37 °C. Benzaldehyde is unstable and undergoes oxidation to benzoic acid. Experiments on the stability of benzaldehyde comparing the amounts of acid formed by autoxidation and the amounts formed by enzymatic reaction showed that the small amounts of aldehyde, which are formed during incubations of benzaldoxime with microsomes are readily oxidized to benzoic acid.

Aerobic incubations of 3 mM (*E*)- and (*Z*)-benzaldoxime containing NADH in the presence of liver microsomes (220 μg) or cytosol (220 μg) were analyzed by reversed-phase HPLC. In addition to the starting aldoxime, only benzoic acid was observed as a metabolite (Fig. 2). 0.18 ± 0.01 and 0.12 ± 0.01 nmol of benzoic acid for the (*E*)-isomer and 0.19 ± 0.01 and 0.13 ± 0.01 nmol of acid for the (*Z*)-isomer were formed per minute per mg of protein from aerobic incubations using pig liver microsomes and pig liver cytosol, respectively, in the presence of NADH. Omitting the cofactor led to the formation of benzoic acid as well, but to a minor extent, as 0.13 ± 0.01 nmol of acid was formed per minute per mg protein from each isomer in the presence of pig liver microsomes. The retention time for the metabolite agreed with that of synthetic material. Neither benzaldehyde nor hydrobenzamide, a product formed via condensation reaction of benzaldimine with benzaldehyde, could be detected as metabolite. Control incubations without protein showed a

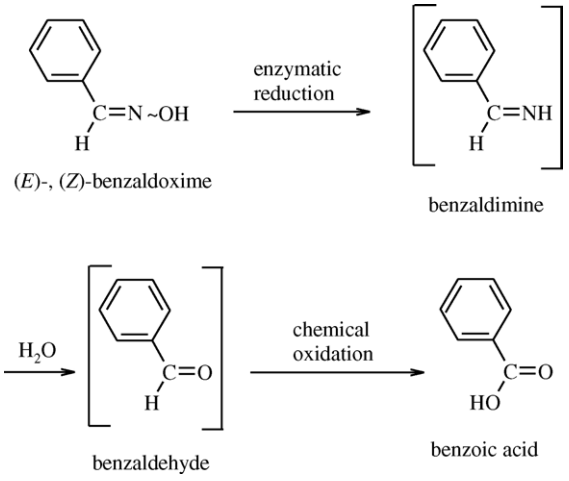

Fig. 2 – In vitro metabolism of benzaldoxime: only benzoic acid was identified as stable metabolite.

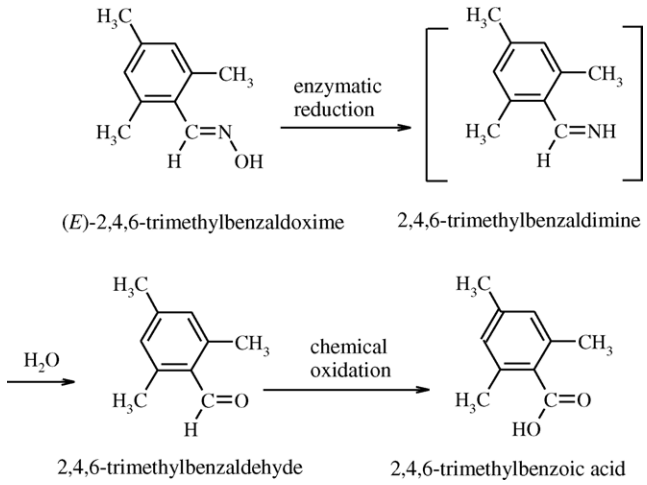

Fig. 3 – In vitro metabolism of (*E*)-2,4,6-trimethylbenzaldoxime: 2,4,6-trimethylbenzaldehyde and -benzoic acid were identified as stable metabolites.

smaller peak for the acid, which resulted from impurities of the substrate benzaldoxime with benzoic acid.

3.2. *Reduction of (E)-2,4,6-trimethylbenzaldoxime*

Aerobic and anaerobic incubations of (*E*)-2,4,6-trimethylbenzaldoxime in the presence of pig liver microsomes (220 μg), cytosol (220 μg) or the liver microsomal enzyme system were performed, and 2,4,6-trimethylbenzaldehyde and 2,4,6-trimethylbenzoic acid were analyzed by reversed-phase HPLC. The retention times for the metabolites formed, agreed with those of synthetic materials and addition of the reference substances to the incubation mixtures led to an increase in the areas of the metabolite peaks.

Studies concerning the stability of the aldehyde have shown, as expected, that it is partially oxidized to its corresponding acid, when incubated at 37 °C for 30 min (Fig. 3).

The formation of 2,4,6-trimethylbenzaldehyde from incubations of (*E*)-2,4,6-trimethylbenzaldoxime (3 mM) in the presence of pig liver microsomes (220 μg) was an oxygen-insensitive and cofactor-dependent (NADH or NADPH; final concentration: 1 mM) reaction. NADH was preferred as cofactor as 0.37 ± 0.09 nmol of the aldehyde were formed per minute per mg protein from anaerobic incubations with liver microsomes (220 μg) in the presence of NADH, whereas 0.19 ± 0.02 nmol per minute per mg protein could be detected under identical conditions but with NADPH as cofactor. 2,4,6-Trimethylbenzaldehyde was oxidized to the benzoic acid, but to a minor extent only.

The ability of the liver purified benzamidoxime reductase to catalyze the conversion of (*E*)-2,4,6-trimethylbenzaldoxime has been demonstrated (Table 1). Two different batches (systems I and II) of this enzyme system containing different

Table 1 – In vitro conversion of (*E*)-2,4,6-trimethylbenzaldoxime by pig liver microsomes, pig liver cytosol and benzamidoxime reductase

Preparation	Composition	Aldehyde[a]	N[b]	Acid[a]	N[b]
Pig liver microsomes	Complete system	0.37 ± 0.06	12	0.01 ± 0.01	8
	– NADH	0.13 ± 0.17	12	0.01 ± 0.01	8
	– NADH + NADPH	0.17 ± 0.11	8	0.05 ± 0.02	8
	– pH 7.4	0.26 ± 0.02	4	0.01 ± 0.02	4
	Complete system – O_2	0.37 ± 0.09	4	0.06 ± 0.04	4
	– NADH	ND[c]	4	ND[c]	4
	– NADH + NADPH	0.19 ± 0.02	4	0.17 ± 0.19	4
	– pH 7.4	0.27 ± 0.04	4	0.01 ± 0.02	4
Pig liver cytosol	Complete system	0.07 ± 0.01	4	0.01 ± 0.01	8
Benzamidoxime reductase	Complete system (I)	0.31 ± 0.12	4	0.12 ± 0.04	4
	– CYP2D	0.20 ± 0.14	4	ND[c]	4
	Complete system (II)	0.52 ± 0.06	4	0.07 ± 0.04	4

See Section 2.
[a] Measured in nmol/min/mg protein.
[b] Number of determinations.
[c] Not detected.

amounts of cytochrome b_5 were used, as mentioned in Materials and Methods.

3.3. *Reduction of (E)- and (Z)-2,4,6-trimethylacetophenone oxime*

(E)- and (Z)-2,4,6-trimethylacetophenone oxime and the corresponding imine were stable to isomerization, degradation and hydrolysis in buffered solutions for at least 24 h, even when heated to 37 °C. Aerobic and anaerobic incubations of 4 mM 2,4,6-trimethylacetophenone oxime, which existed as a 54:46 mixture of (E)- and (Z)-isomers, in the presence of pig liver microsomes (1.93 ± 0.26 nmol/min/mg protein), mitochondria (3.53 ± 0.23 nmol/min/mg protein) and also cytosol (0.16 ± 0.01 nmol/min/mg protein) led to the formation of the corresponding imine (Fig. 4). Formation of the imine was an oxygen-insensitive enzymatic reaction as incubations without protein did not show a peak for the metabolite at all. The presence of a reducing agent, NADH or NADPH, was also required. The use of NADPH instead of NADH gave a much lower activity (for pig liver microsomes: 37%). For incubation times of 10–45 min, the enzymatic reduction of 2,4,6-trimethylacetophenone oxime proceeded linearly (data not shown). The retention time for the metabolite (8.3 min) agreed with that of the synthetic material. This was confirmed by the observation of the correct masses using LC–MS coupling.

Human liver microsomes were also capable of catalyzing this reduction as 0.025 ± 0.001 nmol of imine per minute per mg protein and, accordingly, 0.126 ± 0.013 nmol of imine per minute per nmol P450 were formed from aerobic incubations.

In order to determine the role of the stereochemistry of ketoximes in that reaction, pure (E)- and (Z)-isomers were prepared and their fate was studied in the presence of liver microsomes. Much higher conversion rates could be observed for the reduction of the (E)-isomer; 2.14 ± 0.29 nmol of imine were formed per minute per mg protein from aerobic incubations of (E)-2,4,6-trimethylacetophenone oxime (3 mM) with liver microsomes (220 μg) in the presence of NADH, whereas only 1.34 ± 0.01 nmol per minute per mg protein of imine could be detected under identical conditions but with the (Z)-isomer as substrate.

The reduction of the pure (E)-isomer (3 mM) to the corresponding imine in vitro by the highly purified reconstituted liver microsomal enzyme system (benzamidoxime reductase) has been demonstrated (Table 2). Omitting either one of the components of the reconstituted system or NADH resulted in a complete loss of activity.

X = Me, NH_2

Fig. 4 – In vitro metabolism of either (E)- and (Z)-2,4,6-trimethylacetophenone oxime (X = Me) or 2,4,6-trimethylbenzamidoxime (X = NH_2).

Table 2 – In vitro reduction of (E)-2,4,6-trimethylacetophenone oxime by benzamidoxime reductase

Composition	2,4,6-Trimethylacetophenone imine (nmol/min/mg protein)
Complete system (I)	0.37 ± 0.05
– b_5-Reductase	0.02 ± 0.05
– Cytochrome b_5	ND[a]
– CYP2D	ND[a]
– NADH	0.03 ± 0.06
Complete system (II)	0.77 ± 0.04
– b_5-Reductase	ND[a]
– Cytochrome b_5	ND[a]
– CYP2D	ND[a]

See Section 2. Data are means ± standard deviation from $N = 4$ different determinations.
[a] Not detected.

3.4. *Reduction of benzamidoxime*

In order to compare the results obtained from the reduction of the oximes with those when incubating benzamidoxime, aerobic incubations of benzamidoxime (3 mM) were performed using the same enzyme batches and concentrations of the purified reconstituted liver microsomal system, which has been used for the oximes. The results of the reduction of benzamidoxime catalyzed by pig liver microsomes and the benzamidoxime reductase are listed in Table 3.

3.5. *Reduction of 2,4,6-trimethylbenzamidoxime*

9.41 ± 0.14 and 0.54 ± 0.03 nmol of 2,4,6-trimethylbenzamidine (Fig. 4) were formed per min per mg protein from aerobic incubations of 3 mM 2,4,6-trimethylbenzamidoxime containing NADH (final concentration: 1 mM) with liver microsomes (220 μg) and cytosol (220 μg), respectively (Table 3). The retention time for the metabolite agreed with that of the synthetic material. This was confirmed by LC–MS analysis as well. Control incubations without protein, substrate or cofactor did not show a peak for the amidine.

The reduction of 2,4,6-trimethylbenzamidoxime (3 mM) in vitro by the two differently composed purified reconstituted liver microsomal systems of the CYP2D enzyme was demonstrated. Omitting either one of the components of the enzyme system resulted in a significant reduction in activity (Table 3). In order to determine the amount of DMSO tolerated by the enzymes without loss in activity, incubations containing 3.3% and less than 1% DMSO were performed and the amount of amidine formed was determined. No significant differences in the amount of amidine formed could be found indicating that up to 3.3% of DMSO per incubation batch is tolerable when using the reconstituted enzyme system.

3.6. *Test incubations for anaerobic conditions*

In order to determine the effectiveness of oxygen removal, the procedure was controlled via test incubations. As described [14], benzaldoxime undergoes a P450 Fe(II)-catalyzed dehydration to its corresponding nitrile when incubated anaerobically. Contrary to the studies of Boucher et al., who applied

Table 3 – In vitro reduction of benzamidoxime and 2,4,6-trimethylbenzamidoxime by pig liver microsomes, pig liver cytosol and the purified liver microsomal enzyme system

Pig liver preparation	Composition	Benzamidoxime		2,4,6-Trimethylbenzamidoxime	
		Benzamidine[a]	N[b]	2,4,6-Trimethylbenzamidine[a]	N[b]
Microsomes	Complete system	7.13 ± 0.08	4	9.41 ± 0.14	4
	– NADH	–[d]		ND[c]	4
Cytosol	Complete system	–[d]		0.54 ± 0.03	4
Enzyme system	Complete system (I)	9.24 ± 0.73	4	12.3 ± 1.4	4
	– b_5-Reductase	0.57 ± 0.17	4	0.53 ± 0.56	4
	– Cytochrome b_5	0.05 ± 0.03	4	ND[c]	4
	– CYP2D	0.42 ± 0.13	4	3.39 ± 0.72	4
	Complete system (II)	19.2 ± 2.0	4	20.55 ± 0.82	4
	– b_5-Reductase	1.66 ± 0.25	4	–[d]	
	– Cytochrome b_5	ND[c]	4	–[d]	
	– CYP2D	1.19 ± 0.09	4	3.50 ± 0.66	4

See Section 2.
[a] Measured in nmol metabolite/min/mg protein.
[b] Number of determinations.
[c] Not detected.
[d] Not measured.

phenobarbital-induced rat liver microsomes, our reaction was tested using pig liver microsomes. Conversion rates using pig liver microsomes were well below those employing induced rat liver microsomes. However, the effectiveness of oxygen removal was demonstrated as 0.48 ± 0.03 nmol nitrile were formed per minute per nmol P450 from anaerobic incubations of (*Z*)-benzaldoxime (1 mM) with pig liver microsomes (1 μM P450) in 100 mM potassium dihydrogenphosphate buffer (pH 7.4) in the presence of NADPH (1 mM), whereas 0.11 ± 0.03 nmol of nitrile per minute per nmol P450 could be detected under identical composition but in the presence of oxygen.

3.7. *Study by UV–vis spectroscopy of the interaction between CYP2D and different amidoximes and aldoximes*

The UV–vis absolute spectrum of CYP2D exhibited a broad Soret peak around 413 nm (spectrum not shown), indicating that its iron(III) existed in an equilibrium between the low spin and the high spin state as already described for cytochromes P450 of other species by Kumaki et al. [40].

The stepwise addition of the substrates benzaldoxime and benzamidoxime to CYP2D led to the difference spectra characterized by peaks at 418–422 nm and troughs at 390–393 nm (Table 4). These difference spectra may be considered as reverse type I (peak at 420 nm, trough at 385–390 nm) or type II spectra (peak at 425–430, trough at 390–410 nm), which are both due to coordination of the added compound as ligand to the iron ion in the ferric state, displacing the equilibrium between high and low spin state in favour of the low spin state [41]. Reverse type I spectra describe the ligation via an oxygen atom and type II via the nitrogen. A red shift in the absolute absorption spectrum could not be observed, most likely due to the fact that the interaction is not very strong as can be seen in the faint absorption differences between peaks and valleys in the difference spectra.

2,4,6-Trimethylbenzamidoxime was also able to form reverse type I or II binding spectra with CYP2D, the results are shown in Table 4, whereas in case of the compounds (*E*)- and (*Z*)-2,4,6-trimethylacetophenone oxime as well as 2,4,6-trimethylbenzaldoxime no spectra could be obtained because of poor water solubility.

4. Discussion

The objective of this study was to investigate the reduction of oximes by various pig liver homogenates and human liver microsomes. In the present paper, the in vitro reduction of 2,4,6-trimethylacetophenone oxime to its corresponding imine has been demonstrated, giving the first direct proof for the formation of an imine as primary reduction product.

Table 4 – Characteristics of the UV–vis difference spectra obtained upon addition of benzaldoxime and two amidoxime compounds to CYP2D at pH 7.4.

Compound	λ_{max} (nm)	λ_{min} (nm)	ΔA_{max}[a]	Spectrum type[b]
Benzaldoxime	422	390	0.031	rI/II
Benzamidoxime	418	393	0.009	rI/II
2,4,6-Trimethylbenzamidoxime	425	395	0.015	rI/II

[a] ΔA_{max}: maximum absorbance observed between λ_{max} and λ_{min} at saturating ligand concentrations.
[b] Spectrum type: reverse type I spectra are characterized by a peak (λ_{max}) at 420 nm and a trough (λ_{min}) around 385–390 nm, type II difference spectra are characterized by a peak (λ_{max}) around 425–430 nm and a trough (λ_{min}) around 390–410 nm.

For aromatic aldoximes the corresponding metabolite, that are the aldimines, are hydrolyzed to the aldehyde, the enzymatic formation of which can be quantified in the case of (*E*)-2,4,6-trimethylbenzaldoxime. Steric hindrance is responsible for the higher stability of the metabolite 2,4,6-trimethylbenzaldehyde, compared to the parent compound benzaldehyde.

Thus, oximes can be reduced to imines representing a new metabolic pathway, which has to be considered when incorporating oxime functionalities into new drug candidates.

Human and pig liver microsomes, pig liver mitochondria and cytosol, were capable of catalyzing the reduction of 2,4,6-trimethylacetophenone oxime. This enzyme-dependent reaction was oxygen-insensitive, it required NADH or NADPH. However, NADH was preferred as cofactor. The highest reduction activity was obtained when incubating the ketoxime at pH values between 5.5 and 6.5, but also at physiological pH value 7.4 measurable amounts of the corresponding imine could be detected. Because of the fact that optimized conditions (pH, cofactor) for this reduction are close to those for the pig liver microsomal enzyme system of the CYP2D enzyme, called benzamidoxime reductase, and for mechanistic reasons, it was of interest whether this enzyme system is capable of catalyzing this reaction. Previous reports have shown that this oxygen-insensitive enzyme system is responsible for the efficient reduction of primary *N*-hydroxylated structures, such as amidoximes and *N*-hydroxyguanidines [2]. It has been discussed, that an additional amino group geminal to the oxime function, which is part of an amidoxime or *N*-hydroxyguanidine, is able to stabilize the enzyme substrate complex via a five-membered transition state (Fig. 1a) [2]. The complex is formed via the oxygen atom after deprotonation or weakening of the (N)–OH bond (Fig. 5) [42], a complexation that has first been described for fluorenone oxime by Wang et al. [43]. This type of interaction is also known for tetrahydrobiopterine-free-NOS [44]. NO synthases like P450 are heme thiolated proteins. One can also consider that the oxime-hydrogen is accepted by the double-bonded nitrogen in an equilibrium thus facilitating the decomposition of the complex to the amidine and the resting state of the P450 enzyme.

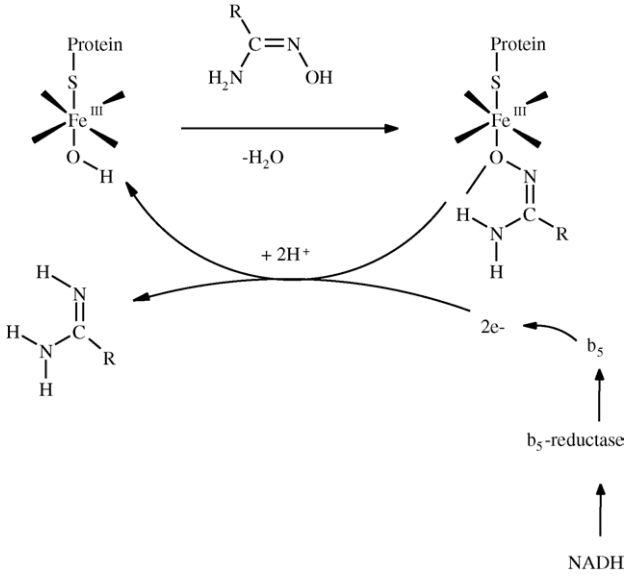

Fig. 5 – Proposed mechanism for the N-reduction of arylamidoximes (2).

Consistent with this mechanistic proposal, substrate properties of oximes concerning this enzymatic reaction catalyzed by benzamidoxime reductase were inferior to those of amidoximes. 2,4,6-Trimethylacetophenone imine was detected in relevant amounts when incubating the corresponding oxime using the reconstituted enzyme system, but reduction rates were significantly lower compared to rates received when incubating benzamidoxime under identical conditions. In particular for (*E*)-2,4,6-trimethylbenzaldoxime it is clear that the reduction is also performed by cytochrome b_5-reductase and cytochrome b_5 alone, because in the presence of the P450 enzyme the reaction rate is not significantly higher (Table 1). This is in contrast to the amidoximes (Table 3) where reductions can also be observed in the presence of b_5 and its reductase alone but rates are extremely higher when the P450 enzyme has been added. This is in agreement with previous studies [5]. Obviously, *N*-hydroxylated derivatives behave differently: *N*-hydroxylated structures bearing an additional NH_2-group like amidoximes, *N*-hydroxyguanidines and *N*-hydroxyamidinohydrazones are reduced by cytochrome b_5 and cytochrome b_5-reductase which can be extremely improved by P450 enzymes, whereas oximes (Fig. 1b and c) and also simple hydroxylamines, not able to form the complex shown in Fig. 1a, can be reduced by cytochrome b_5 and its reductase alone. The reduction of *N*-hydroxylated structures alone by cytochrome b_5 and its reductase could be shown by us before [2,5] and has been confirmed for aromatic hydroxylamines and amidoximes in recent studies [45,46].

Steric hindrance due to the methyl groups in *ortho* position to the oxime functionality could be excluded as being responsible for the lower conversion rates according to results obtained in incubations of 2,4,6-trimethylbenzamidoxime. Quite the contrary, incubation results shown in Table 4 demonstrate that methyl groups in *ortho* or *para* position benefit the catalytic activity of the liver microsomal enzyme system.

Studies focussing on the stereochemistry of this enzymatic reaction display the differences of the catalytic activity dependent on the geometrical constitution of the substrate. Higher reduction rates were obtained for (*E*)-2,4,6-trimethylacetophenone oxime, bearing the bulky aryl substituent in position *trans* to the hydroxy group (Fig. 1b), compared to the (*Z*)-isomer indicating that a trimethylated substituent in position *cis* to the oxime functionality might impair the approach to the active site of the enzyme because of steric hindrance (Fig. 1c).

In conclusion, the problem of identifying imines as metabolites of oximes has been solved by studying the metabolism of the oxime leading to the stable imine 2,4,6-trimethylacetophenone imine. The identification of this imine as product of a new reductive metabolic pathway allows the assumption that also other oximes are transformed in a similar way although only stable chemical degradation products of the primarily formed imines can be detected. The reduction of oximes to imines is insensitive to oxygen. Thus, the in vivo relevance is out of question. Oxygen-insensitive oxime reductases are present mainly in micro-

somes and mitochondria. One enzyme system responsible for this conversion consists of cytochrome b_5, cytochrome b_5-reductase and a third component, a P450 enzyme [5]. The proposed mechanism [2] for this unusual reductive process is confirmed by the presented studies as amidoximes are better substrates than oximes because of the additional amino group of amidoximes stabilizing the proposed enzyme substrate complex via a five-membered transition state (Fig. 1a). Steric hindrance as an influence was excluded by studying the metabolism of 2,4,6-trimethylbenzamidoxime simultaneously.

The results of the spectroscopic analysis clearly show the formation of low spin iron(III) complexes between CYP2D and oxime compounds, whereas it could not be proved if the ligation is due to binding via the oxygen or the nitrogen atom. Irrespective of the presence of the additional amino group of amidoximes the difference spectrum obtained for benzamidoxime was very similar to that of benzaldoxime, with an even higher absorption difference in case of benzaldoxime.

Since the greater the amount of high spin P450 iron (at the beginning of the experiment, without adding any substrates) the greater is also the peak-to-trough spectral height produced by type II compounds added [40], one can understand the rather faint absorption differences in case of CYP2D, where no predominant high spin state existed. This fact made it impossible to determine spectral dissociation constants and did not lead to reproducible results.

The involvement of a P450 enzyme in the reduction of amidoximes and hydroxylamines has been questioned since none of the known recombinant human isoenzymes tested so far were capable of performing the reaction, nor did all inhibition studies lead to any conclusive results [2,6,47–52]. Problems in the identification of responsible human isoenzymes are not unusual, the most popular example being the human Vitamin D_3 hydroxylase. Whereas porcine CYP 2D25 has been found to be responsible for the hydroxylation reaction, the corresponding human isoenzyme still remains a matter of debate [53–57]. Recently, interest has grown in the question about the participation and nature of a third component reducing a new class of amidoxime prodrugs [6,45,46,52,58]. Currently, at least three different enzyme systems reducing *N*-hydroxylated derivatives are discussed. One is composed of only two compounds [45,46,51], one is located in adipose tissue [58] and the three-component system presented by Andersson et al. and our group [5,6,30,47,48,52]. Kurian et al. and Saulter et al. found in case of hydroxylamines and also amidoximes, especially a newly developed furamidine prodrug DB 289, that there is no third component required and the reduction works perfectly with cytochrome b_5 and b_5-reductase only [45,46]. The two-component system can achieve high reduction rates when reconstituted in a 10:1 molar ratio [45,46,51], a proportion that has been claimed physiological [45,59] determined by a Job plot [60], but has never been confirmed so far. This will be a subject of further investigation. If only cytochrome b_5 and b_5-reductase are responsible for the reduction a mechanism is very hard to propose. How can cytochrome b_5 bind amidoximes and similar compounds as this protein is always viewed as ''helper enzyme'', which has not been shown to have a major role in xenobiotic metabolism and does not even bind simple compounds like carbon monoxide [61,62]?

Others also believe that a third component is necessary [52,58,63]. It is possible that in fat tissue the reduction is again performed by cytochrome b_5/b_5-reductase and a third component, possibly a desaturase [58]. There is no P450 activity in fat tissue. However, in microsomes and in mitochondria usually no desaturase activity can be detected. Andersson et al. found the reduction of benzamidoxime in mitochondria clearly depending on the presence of a third component which seems no P450 enzyme because of the failure of various inhibition studies, but which has not been identified so far [52]. However, they were not able to identify the third component again demonstrating the difficulties of purifying this component. We also purified the system from mitochondria [47] and again identified cytochrome b_5/b_5-reductase and a third component, which we also could not identify so far. However, it is obvious, that in mitochondria P450 is not involved [47] in agreement with the findings of Andersson et al.

Desaturases are known to reduce several fatty acyl-CoAs and to oxidize phenolic compounds by monooxygenase-type reactions [64] as well as P450 enzymes, well known of being capable of accepting a whole range of compounds. So, all this is in agreement that in different organs and organelles different third components, all capable of accepting electrons from cytochrome b_5/b_5-reductase, are involved.

Concerning the failure of P450 inhibition studies, this can be explained by the proposed mechanism. During the catalytic cycle the iron(II) state of the third component is never achieved. The ligand binding was detected in the iron(III) state. Carbon monoxide and other P450 inhibitors only have great affinities to the iron(II) of the P450 enzyme [41]. This might also explain the insensitivity to molecular oxygen of the reduction.

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