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MECHANISM OF THE MICROSOMAL *N*-HYDROXYLATION OF *PARA*-SUBSTITUTED BENZAMIDINES

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Abstract—With the aid of HPLC analyses and simple Michaelis–Menten kinetics, the maximum rates of the microsomal *N*-oxygenation of various *para*-substituted benzamidines **1** to benzamidoximes **2** were determined. The presence of electron-donating substituents increased the rates whereas the presence of electron-accepting substituents decreased them. A significant correlation between the logarithm of the maximum rates with the Hammett σ_p constants was found for a reaction constant of $\rho = -0.88$. These results support the postulated radical mechanism for the *N*-oxygenation by the cytochrome P-450 enzyme system.

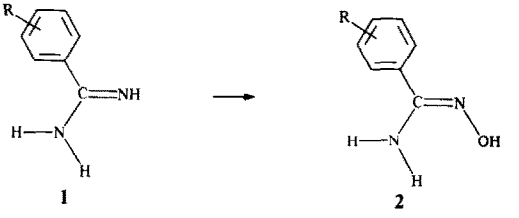
Practically all drugs and environmentally relevant chemicals contain nitrogen in the form of widely different functional groups [1]. In the human body, the enzymatic oxidation of these nitrogen atoms represents an important step in the metabolism [2]. Although the strongly basic amidine function is a component of numerous active principles [3], evidence for the *N*-oxygenation of some *N,N*-unsubstituted benzamidines **1** to the corresponding benzamidoximes **2** (Fig. 1) was obtained only recently [4]. This transformation exhibits all the characteristics of a reaction catalysed by the cytochrome P-450 enzyme system [5] and is the first example of the microsomal *N*-oxygenation of a very strongly basic function (benzamidine possesses a pK_a value of 11.6 [6]).

In view of the major significance of cytochrome P-450 for the biotransformations of most foreign substances (xenobiotica) [7–9], we became interested in the mechanism of enzymatic *N*-hydroxylations for the example of the corresponding reactions of benzamidines. In the course of initial qualitative experiments on some *N,N*-unsubstituted benzamidines, it was found that, on the one hand, no *N*-oxygenated metabolite could be detected in the case of *p*-nitrobenzamidine **1g** whereas, on the other hand, this microsomal *N*-oxygenation could be observed not only for the parent benzamidine **1a** but also for *p*-methylbenzamidine **1b** and *p*-methoxybenzamidine **1c** [4]. Thus, it was reasonable to assume that the presence of electron-donating substituents (donors) on the aromatic ring increased the reaction rates of the *N*-oxygenations of **1** to **2** while the presence of electron-accepting substituents (acceptors) reduced them. This assumption has been validated by the present investigations in which further benzamidines **1d–f** having electron-accepting groups were studied and the postulated *N*-oxygenations were confirmed by means of TLC/MS† analyses. Finally, the maximum rates of the *N*-oxygenations of the variously substituted benzamidines **1a–f** were determined with the help of HPLC† analysis methods; furthermore, structure–activity relationships were sought.

MATERIALS AND METHODS

Reagents and biochemicals. NADPH (tetrasodium salt) was purchased from Boehringer-Mannheim GmbH (Mannheim, F.R.G.). Benzamidine hydrochloride **1a** and *p*-aminobenzamidine hydrochloride were products from EGA-Chemie (Steinheim, F.R.G.). All other chemicals and solvents (GR) were obtained from Merck AG (Darmstadt, F.R.G.).

Synthesis. The amidines **1b, c, g** and the amidoximes **2a, b, c, g** were synthesised as reported previously [4]. *p*-Bromo- and *p*-chlorobenzamidine **1d** and **1e** were prepared by the method of Pinner and



a: R = H b: R = p-CH_3 c: R = p-OCH_3
d: R = p-Br e: R = p-Cl f: R = p-CN
g: R = p-NO_2

Fig. 1. *N*-Oxygenation of *para*-substituted benzamidines **1** to form the corresponding benzamidoximes **2**.

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† Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; MS, mass spectrometry.

isolated as the hydrochlorides [10, 11]. *p*-Cyanobenzamidine **1f** was obtained by diazotisation of *p*-aminobenzamidine hydrochloride with subsequent exchange of the diazonium group using CuCN according to the procedure of Sandmeyer [12]. *p*-Bromo, *p*-chloro-, and *p*-cyanobenzamidoximes **2d, e,** and **f** were prepared from hydroxylamine and the corresponding nitrile [4, 10, 13]. All compounds were characterised using standard methods.

Preparation of liver homogenates. Liver homogenates of untreated rabbits and 12,000 *g* supernatant fractions were prepared and analysed as described previously [14].

Incubations. Incubations were carried out in unstoppered 25 ml Erlenmeyer flasks at 37° in a shaking water-bath. Incubation mixtures contained the following components: 3.0 ml of phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), 1.0 ml of cofactor solution (containing 2 mg of NADPH and 1.9 mg of $MgCl_2$), 1.0 ml of the 12,000 *g* supernatant fraction of rabbit liver homogenate (corresponding to about 20 mg of protein), and 1.0 ml of respective benzamidine hydrochloride solution (5, 7.5, 10, 15, 20, and 40 μmol/ml for **1a, b, c, d,** and **e**; 20, 40, 50, 75, 80, 90, 100, 120, and 150 μmol/ml for **1f**; 100 and 200 μmol/ml for **1g**) in a total volume of 6.0 ml. The incubation samples were preincubated for 2 min at 37° and the reaction was started by addition of the 12,000 *g* supernatant fraction. An incubation time of 30 min was used routinely with the exception of **1f** and **1g** where 40 min incubation times were employed. The incubations were terminated by the addition of 5.0 ml of freshly-distilled diethyl ether and chilling the mixture on ice.

For all detected *N*-oxygenations, investigations on the dependency on the enzyme source (amount of protein) were carried out. It was found that, in the presence of a sufficient amount of cofactor, the formation of the respective amidoxime was proportional to the amount of protein and that, when 10–30 mg of protein were used, a cofactor amount of 2.0 mg of NADPH was sufficient for all *N*-oxygenations. Studies on the dependency of the reaction on the amount of cofactor also demonstrated a proportionality between the rate of formation of the various amidoximes and the amount of NADPH employed. Since preliminary experiments had shown that the incubation of microsomes in the presence of either NADPH or an NADPH-generating system gave similar results, NADPH was used in all of the experiments reported here. In all metabolic studies, the presence of Mg^{2+} ions, in addition to oxygen, microsomal enzymes, and NADPH, was necessary for an optimal enzyme activity.

HPLC analsysis. Terminated incubations were extracted and analysed by HPLC as described previously for benzamidoxime (for experimental details, see [5]). In contrast to the reported procedure [5], the extraction process was repeated four times. This was to ensure that all amidoximes had approximately the same recovery ratios. Standard curves (peak height) were constructed by introducing known amounts (in the range between 10 and 800 nmol) of the particular benzamidoxime into the usual incubation mixture at 0° and then treating the resultant mixture in the same manner as an experimental sample. The standard curves were linear over these ranges with correlation factors of 0.995 or higher (N = 32). The recovery ratios of the amidoximes from such incubation mixtures enriched with amidoximes were 96.5% or greater. The levels of benzamidoximes in unknown incubation mixtures were determined directly from the standard curves whereby the standard samples were run in parallel with the experimental samples. The retention times were 7.5 min for **2a**, 8.3 min for **2b**, 8.1 min for **2c**, 8.0 min for **2d**, 8.1 min for **2e**, 6.7 min for **2f**, and 6.9 min for **2g**. The detection limits for all of the amidoximes were 1.0 nmol or less per sample.

Control incubations in which substrate, cofactor, and the 12,000 *g* supernatant fraction were individually excluded from the incubation mixture were carried out concurrently in order to check for interfering substances as well as for cofactor- and enzyme-dependencies of the products formed.

The initial rate versus substrate concentration data were analysed by means for Lineweaver–Burk plots. Linear regression lines yielded the enzymatic kinetic parameter V_{max}.

Since the experiments on the *N*-oxygenations of the various benzamidines constitute a comparative investigation, they were performed with the same cell fraction (12,000 *g* supernatant) of one rabbit liver homogenate in order to ensure the same activity of the enzyme source. All experiments were repeated with two further 12,000 *g* supernatant fractions from two different rabbits in order to take the biological variability into account.

TLC–MS analysis. The qualitative TLC–MS detection of the benzamidoximes **2a–c** formed from the corresponding benzamidines has already been described in Ref. 4 (for experimental details, see Ref. 4). The detection of **2d–f** was performed analogously with the help of synthesized reference substances. For the detection of **2f**, in contrast to the other amidoximes, 80 μmol of **1f** per incubation experiment, an incubation time of 60 min, and 32 runs were necessary in order to obtain an amount sufficient for the TLC–MS analysis. For the case of *p*-nitrobenzamidine **1g**, 100 and 200 μmol of substrate, an incubation time of 60 min, and 32 runs were selected. Chloroform–methanol (8.5:1.5, v/v) served as the solvent mixture for the TLC separation (R_f values: **2d** = 0.54; **2e** = 0.48; **2f** = 0.62). Direct inlet mass spectra were recorded on a Finnegan 4000 mass spectrometer operating at an ionising potential of 70 eV. The mass spectra obtained contained molecular ion peaks as well as ion peaks for the typical fragments [4] of *N,N*-unsubstituted benzamidoximes.

RESULTS

TLC–MS analysis

By means of the TLC–MS analytical procedure developed previously [4] for the metabolites **2a–c**, the benzamidoximes **2d, e,** and **f** were identified for the first time as metabolites of the respective benzamidines. The MS data and the chromatographic behaviour are each in accord with those of synthetic reference substances.

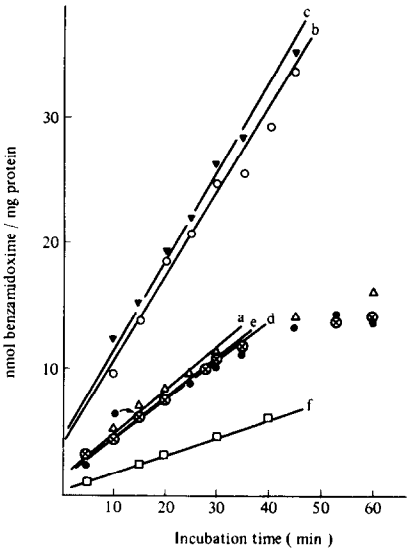

Fig. 2. Formation of the benzamidoxime **2** by 12,000 *g* supernatant fractions of rabbit liver homogenates as a function of time. Each point is the mean of three incubations. See Materials and Methods for details of the contents of the reaction mixtures, incubations, sampling, and analyses. **a** (△): benzamidoxime; **b** (○): *p*-methylbenzamidoxime; **c** (▼): *p*-methoxybenzamidoxime; **d** (⊗): *p*-bromobenzamidoxime; **e** (●): *p*-chlorobenzamidoxime; **f** (□): *p*-cyanobenzamidoxime.

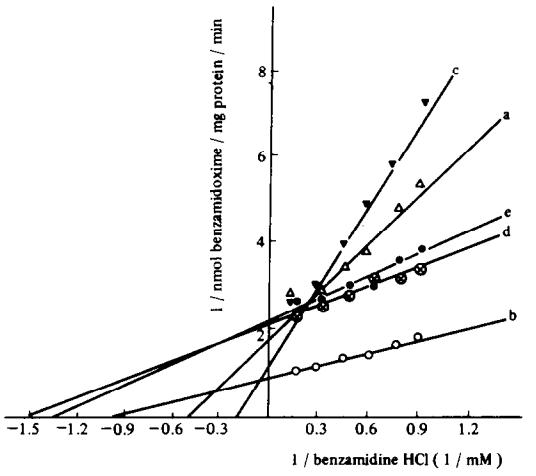

Fig. 3. Lineweaver–Burk plots of the *N*-oxygenations of the benzamidines **1a–e**, as measured by the formation of the amidoximes **2a–e** in the reaction mixtures. Each point is the mean of three determinations. The reaction mixtures contained the components listed in Materials and Methods. For an explanation of the symbols, see Fig. 2.

In the case of the reaction of *p*-nitrobenzamidine **1g**, no *p*-nitrobenzamidoxime **2g** could be detected even after use of larger amounts of substance, longer incubation times, and the combination of a large number of runs; this had also been observed previously in the course of an initial investigation [4].

HPLC analysis

The quantitative HPLC analytical method developed previously for benzamidoxime [5] could also be used successfully for the ring-substituted benzamidoximes. The analyses confirmed the microsomal formation of the benzamidoximes **2a–f** from the corresponding benzamidines. Again, *p*-nitrobenzamidoxime **2g** could not be detected. Hence, the enzymatic transformations of the benzamidines **1a–f** were included in the quantitative structure–activity relationships. No further metabolites or decomposition products of the amidines **1** or the amidoximes **2** could be detected by either TLC or HPLC analyses in any of the studied reactions of the benzamidines **1a–f** with the 12,000 *g* supernatant fraction of rabbit liver homogenates; this has also been confirmed by other investigations [15].

Kinetics of the N*-oxygenations*

The formation of the respective benzamidoxime was linear over a period of at least 30 min (Fig. 2).

The *N*-oxygenations of the benzamidines **1a–f** by the 12,000 *g* supernatant fraction of rabbit liver homogenates in the presence of NADPH obey Michaelis–Menten kinetics. Lineweaver–Burk plots for one 12,000 *g* supernatant fraction are given in Figs 3 and 4. Comparable results were obtained using the cell fractions from two other rabbits. The apparent K_m values and the apparent V_{max} values calculated from the Lineweaver–Burk plots are given in Table 1 (average values ± SD of the three different experiments, three different animals).

Table 1. K_m, $V_{max}X$, and log $V_{max}X$ data for the microsomal *N*-oxygenations of *para*-substituted benzamidines **1** and the corresponding σ_{para} values

Amidine	X (= substituent R)	K_m (mM)	$V_{max}X$ (nmol/min/mg protein)	log $V_{max}X$	σ_{para} [22]
1a	H	1.95 ± 0.30	0.54 ± 0.17	−0.27	0
1b	CH_3	1.04 ± 0.11	1.03 ± 0.14	+0.01	−0.17
1c	OCH_3	4.45 ± 0.47	0.93 ± 0.17	−0.03	−0.27
1d	Br	0.67 ± 0.09	0.42 ± 0.08	−0.38	+0.23
1e	Cl	0.73 ± 0.09	0.41 ± 0.11	−0.39	+0.23
1f	CN	6.67 ± 0.81	0.15 ± 0.03	−0.82	+0.66

Values are presented as the means of determinations from three animals ± SD; apparent K_m values and apparent $V_{max}X$ values were calculated from the Lineweaver–Burk plots.

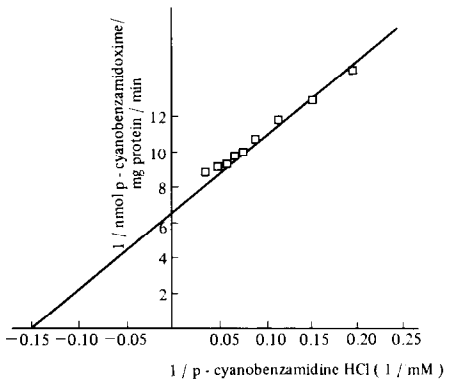

Fig. 4. Lineweaver–Burk plot of the *N*-oxygenation of *p*-cyanobenzamidine **1f**, as measured by the formation of *p*-cyanobenzamidoxime **2f** in the reaction mixture. Each point is the mean of three determinations. The reaction mixtures contained the components listed in Material and Methods.

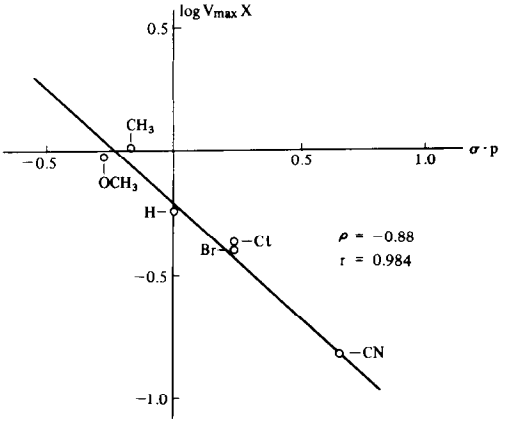

Fig. 5. Correlations of log $V_{max}X$ with σ_{para} for the *N*-oxygenations of *para*-substituted benzamidines by the 12,000 *g* supernatant fractions of rabbit liver homogenates.

Correlation of the log V_{max} *value with the Hammett substituent parameter* σ_{para}

A significant correlation was found between the logarithms of the maximum rates (log $V_{max}X$) with the Hammett σ_p constants [16] (N = 6; correlation coefficient $r = 0.984$) which confirms the validity of the Hammett or the free enthalpy relationship, respectively [16], for the *N*-oxygenation of *para*-substituted benzamidines **1** (Table 1, Fig. 5).

DISCUSSION

Structure–activity relationships were sought for an investigation of the mechanism of the microsomal *N*-oxygenation of *N,N*-unsubstituted benzamidines **1** (Fig. 1) by apparent cytochrome P-450 [5].

Only *para*-substituted benzamidines were selected as substrates for the reaction in order to avoid possible steric influences of the substituents. In addition, the spatial requirements of the substrates should be of a similar size in order to keep any differences on approach to the enzyme as small as possible. Finally, it was ensured that the selected benzamidines **1a–g** and the formed benzamidoximes **2a–g** did not undergo any additional or further enzymatic or chemical transformations which could have influenced these quantitative investigations. Since work is still in progress to identify and purify the isoenzyme of cytochrome P-450 responsible for the *N*-oxygenation of the benzamidines **1**, it was also necessary to exclude competing reactions of other enzymes of the 12,000 *g* supernatant fraction of rabbit liver homogenates employed. This was achieved for benzamidine **1a** and benzamidoxime **2a**, in particular, by a separate investigation [15]. However, also with the HPLC and TLC/MS analysis methods discussed here, no chemical or enzymatic reactions were found for any of the compounds apart from the *N*-oxygenation observed for all of the benzamidines except *p*-nitrobenzamidine **1g**. Even so, a possible further hydroxylation of the CH_3 group of *p*-methylbenzamidoxime **2b** and an also feasible *O*-dealkylation of *p*-methoxybenzamidoxime **2c** were excluded in the course of a separate investigation*.

Thus, the correlaion found (Fig. 5) for the microsomal *N*-oxygenations of the benzamidines **1** with the Hammett σ_{para} constants was not influenced by other reactions. An even better correlation (N = 5; $r = 0.987$) was obtained when the relatively low maximum rate of the *N*-oxygenation of *p*-methoxybenzamidine **1c** was not taken into consideration. In this case, the result could have been falsified by an interaction between the oxygen atom of the methoxy group and the iron atom of the catalytic centre of cytochrome P-450 [17].

The correlation between the log $V_{max}X$ values and the σ_p^+ constants of Brown and Okomoto [18] was considerably less satisfactory (N = 6; $r = 0.916$). Further significant correlations with other physicochemical parameters such as, for example, the Hansch π-constants [19, 20] could not be established. Since an *N*-oxygenation was observed only for *N,N*-unsubstituted benzamidines and *N*-phenylbenzamidine but not for *N*-substituted benzamidines with alpha-hydrogen atoms, we suggested a

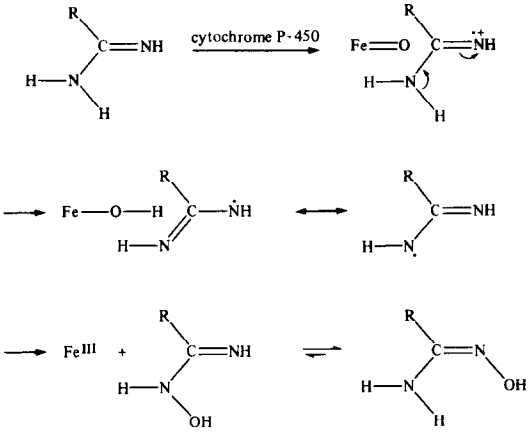

Fig. 6. Proposed mechanism for the *N*-oxygenation of benzamidines [21].

* Work in preparation.

radical mechanism (Fig. 6) for the reaction by the catalytic centre of cytochrome P-450 [21]. On the basis of this mechanism (Fig. 6), an *N*-dealkylation was postulated for *N*-alkylbenzamidines with alpha-hydrogen atoms and this has been unequivocally confirmed in the mean time [14]. Thus, it seemed reasonable to assume that the presence of electron-donating substituents (donors) on the aromatic ring must increase the reaction rates of the *N*-oxygenations of **1** whereas the presence of electron-accepting substituents (acceptors) must reduce them. A high electron density on the amidine system should therefore be advantageous for the progress of the reaction.

From the slope of the line in Fig. 5, a reaction constant of $\rho = -0.88$ was obtained. The negative sign signifies [22] that, as postulated, the presence of electron-donating groups facilitates the attack of the electrophilic enzyme (cytochrome P-450) on the amidine system whereas the presence of acceptors hinders it. The experimentally-determined ρ value thus serves to support the proposed mechanism (Fig. 6) for the *N*-oxygenation of amidines since reactions with negative [23] or low [22] values of ρ often proceed by way of radical intermediates.

Of course, substituents also affect the pK_a values of the amidine functions [24–26] whereby the presence of electron donors should increase the basicity. In the present case, this would mean that, under physiological conditions (pH = 7.4), for example in the case of *p*-methylbenzamidine **1b** in comparison to benzamidine **1a**, larger amounts of the protonated form are present. However, for the metabolisation of amidines by electrophilic enzymes (cytochrome P-450), this effect would have the opposite action since it must be assumed that the free bases rather than the protonated forms of the amidine undergo this reaction [21]. Hence it is improbable that the differing reaction rates are the result only of the changed basicities.

Such structure–activity relationships are essential for obtaining indirect evidence of radical intermediates; this has also been demonstrated by a recently-reported investigation on the *N*-dealkylation of *para*-substituted *N,N*-dimethylanilines which gave comparable values [27]. Using conventional techniques, radical intermediates can only be detected with certainty in exceptional cases [28]. For example, the reported evidence that the oxygen atom transferred to the methyl group in the *N*-demethylation reaction can only originate from oxygen activated by the enzyme [29, 30] shows that the possibly formed radicals are able to react very rapidly with the oxygen bonded to iron before they are able to leave the "enzyme–substrate cage."

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