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Stereoselectivity in the *N'*-Oxidation of Nicotine Isomers by Flavin-Containing Monooxygenase

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SUMMARY

N'-Oxidation of nicotine isomers by porcine liver flavin-containing monooxygenase shows a clear stereoselectivity in the formation of the diastereomeric *N'*-oxides. (*S*)-(−)-Nicotine exhibited no stereoselectivity in the formation of *cis*-1'*R*,2'*S*- and *trans*-1'*S*,2'*S*-products, whereas with (*R*)-(+)-nicotine, only the *trans*-1'*R*,2'*R*-*N'*-oxide was formed. The concentration of each isomer required for half maximal activity differs significantly, and access of (*S*)-(−)-nicotine to the active site appears to be more restricted than for (*R*)-(+)-nicotine as judged from the observed K_m values (K_m = 181 and 70 μM, respectively, for the (*S*)-(−)- and (*R*)-(+)-isomers). These results indicate that a region adjacent to the active site may sterically prohibit binding of (*R*)-(+)-nicotine when the *N'*-methyl and pyridyl groups are in a *cis*-orientation. *N*-Methylnicotinium ion (both *R*- and *S*-isomers) is not a substrate for either porcine flavin monooxygenase, guinea pig liver microsomes, or ram seminal vesicular microsomes.

It is now well established that the *N*-oxidation of most tertiary amines requires a direct two-electron oxidation of the heteroatom via an ionic mechanism, which is carried out almost exclusively in mammals by the FMO (1). This reaction appears to be a major route for the detoxification of a number of medicinal agents.

The *N'*-oxidation of nicotine by crude hepatic preparations has received specific attention because of the interesting stereoselectivity observed in the formation of the diastereomeric products (2, 3). Although studies using hepatic microsomes and specific activators and inhibitors of flavoprotein monooxygenase support the view that nicotine *N'*-oxidation is mediated via this enzyme (4), no studies have yet been carried out with a pure enzyme preparation. Thus, the stereochemical aspects of nicotine *N'*-oxidation by the FMO remain to be established.

This communication describes the *in vitro* biotransformation of (*S*)-(−)- and (*R*)-(+)-nicotine, (**1a** and **2a**, respectively, in Scheme 1), and the related compounds (*S*)-(−)- and (*R*)-(+)-*N*-methylnicotinium diacetate (**1b** and **2b**, respectively), by purified porcine FMO.

Materials and Methods

Materials. (*R*)-(+)-[*N'-methyl*-^{3}H]nicotine (specific activity, 76.5 Ci/mmol) and (*S*)-(−)-[*N'-methyl*-^{3}H]nicotine (specific activity, 68.6 Ci/mmole) were obtained from New England Nuclear, Boston, MA; (*R*)-(+)-[*N-methyl*-^{14}C]*N*-methylnicotinium diacetate (specific activity, 10 mCi/mmole) was prepared as previously described (5). The optical purity of these radionuclides was demonstrated to be better than 99% (6). All synthetic chromatographic standards (7, 8) and guinea pig microsomes were prepared as previously described (9). The FMO was isolated from porcine liver microsomes by the procedure described by Ziegler and Poulsen (10). The preparation of ram seminal vesicular microsomal cyclooxygenase followed the procedure described by Takeguchi and Sih (11).

***In vitro* experiments.** Guinea pig liver microsome incubations contained the appropriate substrate (10 μl, 0.015 μmol, 1 μCi), cofactor solution (150 μl; NADPH, 1 mmol; G6PD, 0.1 unit; G6P, 5 mmol; and $MgCl_2$, 12.5 mmol), and microsomes (25 mg of protein/ml in 140 μl of 0.1 M phosphate buffer, pH 7.6). Controls were prepared containing deactivated enzyme (2 min at 100°). All incubation mixtures were preincubated for 5 min at 37° in a circulating water bath before addition of substrate. Incubations were then continued for 10 min and quenched by addition of 1.0 *N* HCl (50 μl). Samples were stored at −20° before analysis.

Incubation mixtures of purified porcine liver flavin-containing monooxygenase contained the following: either (*S*)-(−)- or (*R*)-(+)-[*N'-methyl*-^{3}H]nicotine (50 μl, 0.01 μmol, 1 μCi), cofactor solution (50 μl; NADPH, 1 mmol; G6PD, 0.1 unit; G6P, 5 mmol; and $MgCl_2$, 12.5 mmol), and monooxygenase (2.49 mg of protein/ml in 100 μl of 0.1 M phosphate buffer, pH 7.4). Reactions were also carried out with (*R*)-(+)-[*N-methyl*-^{14}C]*N*-methylnicotinium diacetate (33 μl, 0.01 μmol, 1.0 μCi) as substrate instead of the nicotine isomers. Control incubations with deactivated enzyme (heating at 100° for 2–3 min) were also performed. All mixtures were preincubated for 5 min at 37° before substrate addition. Incubations were then carried out for 10 min before

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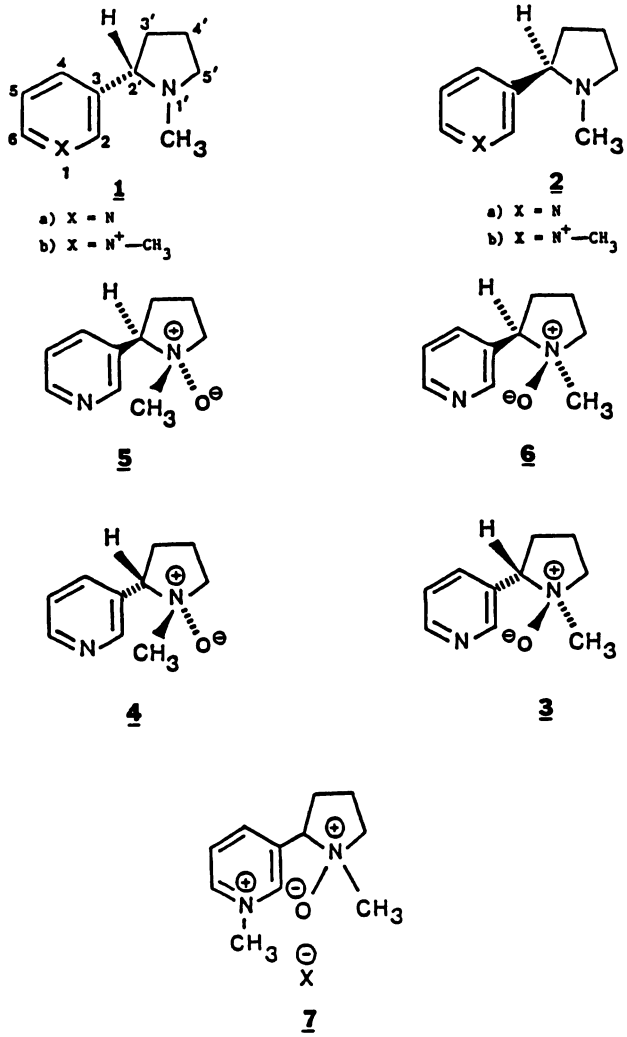
ABBREVIATIONS: FMO, flavin-containing monooxygenase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase.



Scheme 1.

quenching with 1 N HCl (50 μl). After quenching, samples were stored at −20° before analysis.

Kinetic constants for the oxidation of nicotine enantiomers catalyzed by the porcine liver monooxygenase were calculated from double reciprocal plots of activity (initial velocity) versus substrate concentration. Activity was measured by following substrate-dependent oxygen uptake at 37°, pH 7.4, in media containing 100 mM potassium phosphate, 2.3 mM G6P, 1 unit of *Leuconostoc mesenteroides* G6PD (Sigma Type XXIII), 0.15 mM $NADP^+$, and 0.5 nM flavin (FAD)-containing monooxygenase. After 3–4 min temperature equilibration, nicotine (in no more than 40 μl) was added and oxygen uptake was recorded for an additional 3–4 min. For each enantiomer, velocities were measured at substrate concentrations 10 times above and below the K_m.

Experiments with ram seminal vesiculer microsomes used incubation mixtures containing either (*S*)-(−)- or (*R*)-(+)-[*N′-methyl*-^{3}H]nicotine (50 μl, 0.01 μmol, 1 μCi), arachidonate (50 μl, 0.4 mM), microsomes (100 μl, 13 mg of protein/ml), hemoglobin (5 μl, 0.004 mM), and 0.1 M phosphate buffer, pH 7.8 (to 205 μl). In another series of experiments, either (*S*)-(−)- or (*R*)-(+)-[*N-methyl*-^{14}C]*N*-methylnicotinium diacetate (50 μl, 0.015 mmol, 0.2 μCi) was used as substrate instead of the nicotine isomers. Incubations were also performed in which either arachidonate or microsomes were omitted or in which deactivated enzyme (heating at 100° for 2–3 min) was utilized. All incubation mixtures were preincubated for 5 min at 37° before addition of arachidonate and were then allowed to proceed for 10 min before quenching with 1 N HCl (50 μl). After quenching, all samples were stored at −20° before analysis.

Analytical methodology. Analysis of the diastereomeric nicotine *N′*-oxides was carried out using a modification of the high performance liquid chromatography procedure described by Thompson *et al.* (12). The chromatographic parameters were as follows: column, Partisil PAC (25 cm × 0.4 cm; Whatman, Clifton, New Jersey); mobile phase, isocratic methanol/water (95:5); flow rate, 1.0 ml/min. Retention times for the *cis*- and *trans*-isomers were 7.4 and 9.2 min, respectively.

Determination of *N*-methyl-*N′*-oxonicotinium ion formation was carried out using the following high performance liquid chromatography procedure: column, Partisil SCX (25 cm × 0.4 cm; Whatman); mobile phase, 0.3 M sodium acetate/methanol (70:30) containing 1% triethylamine and adjusted to pH 4.5 with glacial acetic acid, and then pH 5.5 with ammonium hydroxide; flow rate, 2 ml/min. Retention times for *N*-methyl-nicotinium ion and *N*-methyl-*N′*-oxonicotinium ion were 4.8 and 6.0 min, respectively. The *cis*- and *trans*-isomers of *N*-methyl-*N′*-oxonicotinium ion (**7**) co-eluted on this chromatographic system.

The identities of radiolabeled oxidation products from the above incubations were established by co-elution of radioactivity in quenched samples with authentic standards (UV absorption at 254 nm).

Results and Discussion

The kinetic constants (Table 1) were based on data collected from three different preparations of the purified monooxygenase, and the difference in K_m for the nicotine isomers was real and reproducible (less than 2% variation with all three preparations). V_{max} values were identical for both nicotine isomers in all experiments. Values were within 2% for experiments using the same enzyme preparation.

As can be seen by the data listed in Table 1, the purified porcine monooxygenase catalyzes the oxidation of both enantiomers of nicotine but concentrations required for half-maximal activity differ significantly. Access of (*S*)-(−)-nicotine to the active site appears somewhat more restricted than that of (*R*)-(+)-nicotine, as indicated by the lower K_m of the latter. However, at infinite concentrations both enantiomers are oxidized at the same velocity. Unlike acyclic aliphatic tertiary amines, nicotine is not self-activating and the oxidation of both enantiomers is stimulated to the same extent by the positive effector, *n*-octylamine (Table 1).

Interesting differences in the stereochemistry of formation of the diastereomeric *N′*-oxides from nicotine were observed (Table 2). With the (*S*)-(−)-isomer (**1a**), no stereoselectivity was exhibited in the formation of *cis*-1′*R*, 2′*S* and *trans*-1′*S*, 2′*S* products (**3** and **4**, respectively). However, with (*R*)-(+)-nicotine (**2a**) product stereospecificity was observed, in that only the *trans*-1′*R*, 2′*RN′*-oxide (**6**) was formed. This observation is quite different from the results obtained with guinea pig microsomes (see Table 2), which, consistent with previous reports (2, 3), indicate a preference for both nicotine enantiomers to form predominantly the *N′*-oxide with the 1′*R* config-

TABLE 1

Kinetic constants for *N′*-oxygenation of nicotine enantiomers catalyzed by purified porcine liver flavin-containing monooxygenase

Substrate	K_m[a,b]	V_{max}[b]	
		− *n*-Octylamine	+ *n*-Octylamine
	μM	nmol/min · nmol of FAD	
(*S*)-(−)-Nicotine	181	22	37
(*R*)-(+)-Nicotine	70	22	41

[a] Values were calculated from double reciprocal plots of initial rates versus substrate concentration at pH 7.5 and 37°.

[b] Three experiments; values did not vary by more than 2%.

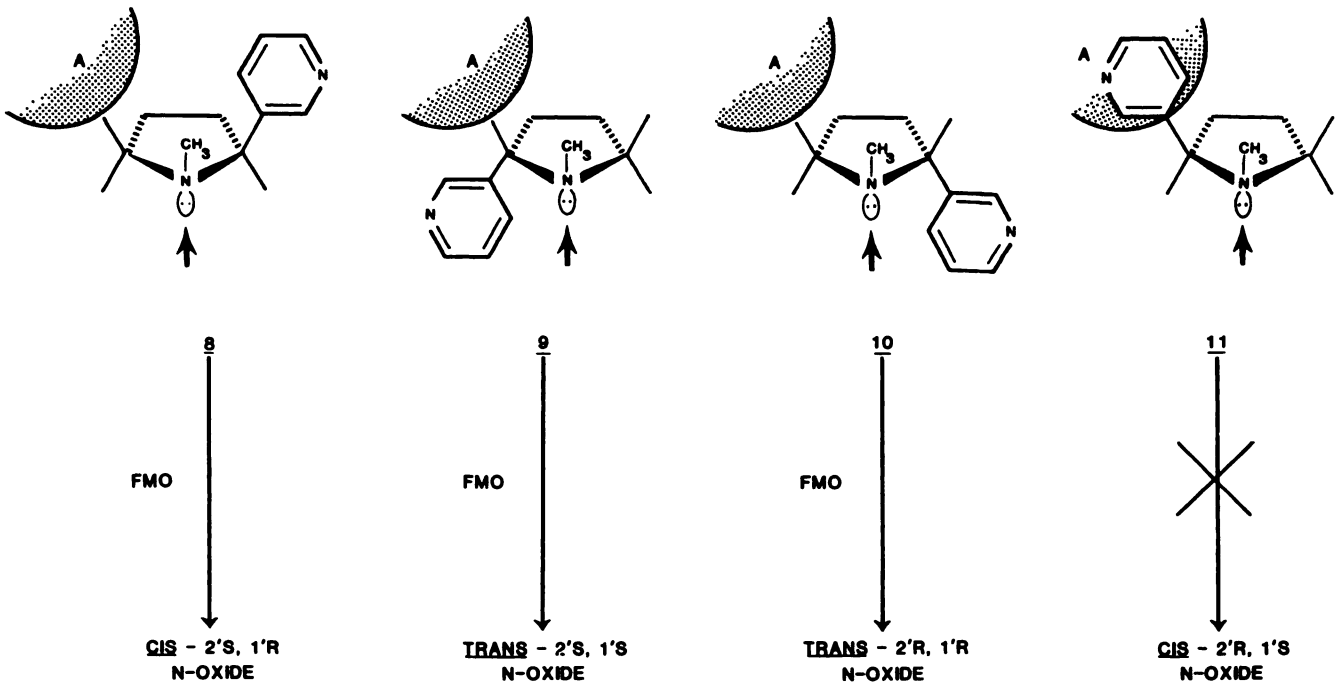


Fig. 1. Stereospecific *N'*-oxidation of nicotine isomers by porcine liver flavin-containing monooxygenase, showing steric hindrance at the active site.

TABLE 2

***In vitro* *N'*-oxidation of (*R*)-(+)- and (*S*)-(−)-nicotine and (*R*)-(+)-*N*-methylnicotinium ion by various oxygenase preparations**

Enzyme system	(*S*)-(−)-Nicotine products[a,b]			(*R*)-(+)-Nicotine products[a,b]			(*R*)-(+)-NMN[c] products
	trans-NNO[d]	*cis*-NNO	*trans*/*cis*	*trans*-NNO	*cis*-NNO	*trans*/*cis*	NMNO[e] (*trans* and *cis*)
Guinea pig liver microsomal preparation	7.5	27.7	0.27	22.8	9.8	2.32	ND[f]
Porcine liver flavin monooxygenase	9.6	9.96	1.0	39.6	ND		ND
Sheep seminal cyclooxygenase	ND	ND	ND	ND	ND		ND

[a] nmol of product (total) recovered.
[b] Values are the mean of duplicate assays that did not vary by more than ± 10%.
[c] NMN, *N*-methylnicotinium ion.
[d] NNO, Nicotine-*N'*-oxide.
[e] NMNO, *N*-methyl-*N'*-oxonicotinium ion.
[f] ND, not detected.

uration. Cyclooxygenase appears to be incapable of catalyzing the *N'*-oxidation of nicotine.

No *N'*-oxide metabolites of the primary *N*-methylated nicotine biotransformation product, *N*-methylnicotinium ion, could be detected in *in vitro* studies with either porcine liver FMO, guinea pig liver microsomes, or ram seminal vesiculer microsomes (cyclooxygenase). The results with FMO are not unexpected, because it is likely that *N*-methylnicotinium ion exists as a dication under the conditions of the incubation, and it has been shown (1) that diamines in which both nitrogens are protonated at physiological pH are not substrates for this enzyme. Cytochrome P-450, by virtue of its reaction mechanism, would not be expected to catalyze the formation of *N*-methyl-*N'*-oxonicotinium ion from *N*-methylnicotinium ion, because the adjacent α-carbons around the *N'* atom are not quaternary in nature (1).

From a structure-activity viewpoint, the distinct product stereospecificity observed with FMO and (*R*)-(+)-nicotine is of obvious interest. The stereochemistry of nicotine *N'*-oxygenation at the catalytic site of FMO is illustrated in Fig. 1. In this model, it is assumed that the *N'*-methyl group and pyrollidine ring bind in a constant manner, and attack by the reactive hydroperoxy flavin occurs as indicated. The generation of specific stereochemistry at the *N*-1' position is dependent upon the relative stereochemistry of the *N'*-methyl group and the adjacent 2'-(3-pyridyl) moiety. Thus, if the 3-pyridyl and *N'*-methyl groups are *cis*, as in **8** and **11**, the resulting *N'*-oxide will have the *cis*-stereochemistry. The converse is true of conformers **9** and **10**. In the above model, exclusive formation of the *trans*-2'*R*, 1'*R* *N*-oxide may indicate that conformer **10** is accommodated by the FMO active site whereas conformer **11** is not. A region near the active site may sterically prohibit binding of (*R*)-(+)-nicotine when the *N'*-methyl and pyridyl groups are *cis*. As suggested in Fig. 1, association of **11** with the enzyme is restricted because the pyridyl moiety is directed into the region of steric hindrance (labeled *A*). Presumably, the binding of **8**, **9**, and **10** at the active site is allowed because the pyridyl group in these conformers is positioned outside of this region.

It is interesting that none of the three major enzymic oxida-

tion systems, FMO, cytochrome P-450, or the peroxidative enzyme cyclooxygenase, were able to catalyze the N'-oxidation of either (*R*)-(+)- or (*S*)-(−)-*N*-methylnicotinium ion to *N*-methyl-N'-oxonicotinium ion. This latter compound is an *in vivo* metabolite of both (*R*)-(+)-nicotine and (*R*)-(+)-*N*-methylnicotinium ion in the guinea pig (13). The enzyme system(s) responsible for its formation thus remain to be established.

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