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SHORT COMMUNICATIONS

Neurotoxins: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1,2,3,4-tetrahydroisoquinoline and 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline as substrates for FAD-containing monooxygenase of porcine liver microsomes

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Abstract—The activity of FAD-containing monooxygenase (FMO) (EC 1.14.13.8) of porcine liver microsomes was examined with the neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1,2,3,4-tetrahydroisoquinoline (TIQ) and 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline (MDTIQ), as substrates. FMO catalyses these neurotoxins. The kinetic parameters of FMO for the neurotoxins and electron donors were determined. K_m values for MPTP, TIQ and MDTIQ were determined to be 47 μM, 6.9 mM and 5.6 mM, respectively. The K_m for the electron donor, NADPH, was variable from 31 to 200 μM depending on the substrate used. The activities of FMO for these neurotoxins were comparable with that for dimethylaniline.

It is well known that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP*) and 1,2,3,4-tetrahydroisoquinoline (TIQ) effectively produce Parkinsonism in humans, monkeys and mice [1], which is alleviated by levodopa treatment. This finding led to the working hypothesis that similar chemical compounds accumulate in the human brain, causing Parkinson's disease to appear during aging. We found that TIQ and 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline (MDTIQ) as well as MPTP act as substrates for FAD-containing monooxygenase (FMO) of porcine liver microsomes. Recently, some laboratories reported that MPTP is an effective substrate for FMO [2, 3]. The known roles of FMO are N- and S-oxygenation of xenobiotics, and this enzyme is labile and widely distributed in the endoplasmic reticula of tissues, being present at high concentrations in the liver, kidney and lung [4–6].

In this study, the kinetic properties of FMO of porcine liver microsomes, which exhibit higher activity than those of other mammals, were investigated using MPTP, TIQ and MDTIQ as substrates.

Materials and Methods

Animals. Female porcine (Landrace, 1-year-old) liver was obtained within 1 hr of killing from a local slaughterhouse and was transported to the laboratory on cracked ice.

Microsomal preparation. Liver microsomes were prepared from fresh liver perfused with an ice-cold 0.15 M potassium chloride solution, by the method of Mitoma *et al.* [7], to remove as many contaminants such as blood and other subcellular fractions as possible. The purity of the microsomes was examined by electron microscopy. Contamination with mitochondria was determined and the activity of succinate dehydrogenase was found to be less than 2% of total protein.

Enzyme assay. The activity of FMO of liver microsomes was determined optically by measuring the decrease in absorbance at 340 nm due to oxidation of NADPH under aerobic conditions and in the presence of 3 mM *n*-octylamine as an inhibitor of cytochrome P450. The reaction mixture contained 184 μg microsomal protein, 0.2 M potassium phosphate buffer (pH 8.0), 1 mM EDTA, 0.1% (v/v) Triton N-101, 3 mM *n*-octylamine, and appropriate amounts of NADPH and substrate, in a final volume of 1 mL. The reaction mixture was put into a cuvette of 1 cm light path and the endogenous rate of the absorbance change at 340 nm was recorded for 5 min at 32° before addition of the substrate. After the addition of the substrate, the rate of absorbance change was measured for 5 min, the endogenous rate being subtracted.

Other analytical methods. Protein concentrations were determined optically by the biuret reaction method [8], using bovine serum albumin as a standard. The extinction coefficient of bovine serum albumin used was 0.68 g^{-1} L cm^{-1} at 280 nm [9]. The molar extinction coefficient of NADPH used was 6300 M^{-1} cm^{-1} at 340 nm, pH 7.0 [10].

Chemicals. MPTP and MDTIQ were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TIQ, *N,N'*-dimethylaniline (DMA) and *n*-octylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan); NADPH and NAD^+ were from the Oriental Yeast Co. (Tokyo, Japan). Other chemicals were of the highest quality commercially available.

Kinetics of FMO. FMO catalyses the following reactions:

$$\text{NADPH} + \text{enzyme (E)} \rightarrow \text{EH}_2 + \text{NADP}^+$$

$$\text{EH}_2 + \text{substrate} + \text{O}_2 \rightarrow \text{substrate-O} + \text{H}_2\text{O}$$

which can be studied kinetically by measuring the decrease in the absorbance at 340 nm due to the oxidation of NADPH. On kinetic investigation of the reaction of FMO, the K_m values for NADPH, NADH, MPTP, TIQ, MDTIQ and DMA can be obtained by means of Lineweaver–Burk plotting. The steady-state can be derived for a two-substrate system, if an excess concentration of molecular oxygen is present in the reaction system.

Results and Discussion

The kinetic properties of the reaction mechanism of FMO were examined with respect to MPTP, TIQ and MDTIQ as substrates, by means of Lineweaver–Burk plots. Fig. 1A shows reciprocal plots of the activity of FMO at various concentrations of NADPH against a series of fixed concentrations of MPTP. As shown in this Figure, the slope of the line relating to the velocity of oxidation of

* Abbreviations: FMO, FAD-containing monooxygenase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TIQ, 1,2,3,4-tetrahydroisoquinoline; MDTIQ, 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline; DMA, dimethylaniline.

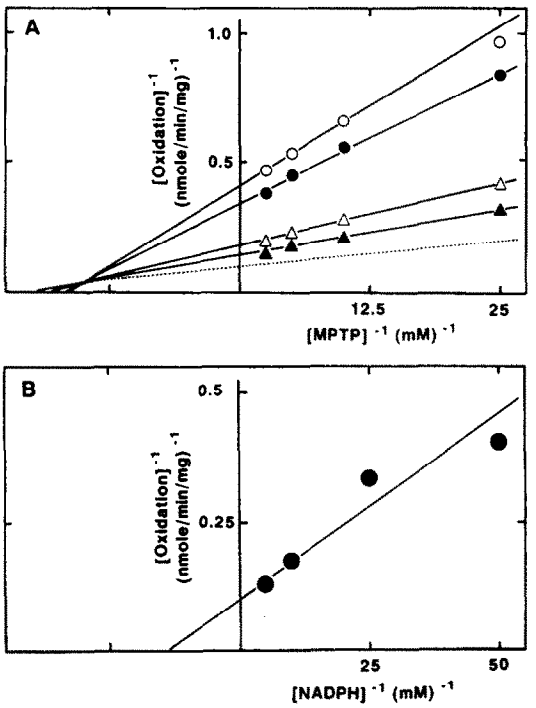

Fig. 1. Double reciprocal plots of the initial velocity of FMO against MPTP concentrations at a series of fixed concentrations of NADPH. The reaction mixture contained 184 μg microsomal protein and NADPH, as indicated in Materials and Methods, in 0.2 M potassium phosphate buffer at pH 8.0 and 32°. (A) Open circles, 20 μM NADPH; closed circles, 40 μM NADPH; open triangles, 100 μM NADPH; closed triangles, 200 μM NADPH. The broken line shows the values extrapolated to an infinite concentration of NADPH. (B) Reciprocal plots of the V_{max} values obtained in (A) vs NADPH concentrations. This replot shows the reciprocals of V_{max} at infinite concentrations of MPTP and NADPH at the intercept on the ordinate.

NADPH is affected by changes in the NADPH concentration. The four straight lines at various concentrations of NADPH have a common intercept point in the upper-left quadrant. On the basis of the data (V_{max} and slope) presented in Fig. 1B, the K_m of FMO for MPTP was obtained, as shown by the dotted line in Fig. 1A, from the value of the intercept point on the abscissa as a negative reciprocal K_m value. The K_m for MPTP was 47 μM.

The V_{max} values at various concentrations of NADPH were obtained from the values of the intercept points of the reciprocal velocities on the ordinate in Fig. 1A. These values were plotted secondarily against a series of fixed concentrations of NADPH, to obtain the K_m for NADPH. The secondary plots of the V_{max} values are shown in Fig. 1B. The K_m for NADPH was 74 μM.

The K_m and V_{max} of FMO for TIQ and MPTIQ, like the K_m and V_{max} for MPTP, were calculated from reciprocal plots of the activity of FMO at various concentrations of NADPH against a series of fixed concentrations of TIQ and MPTIQ. When TIQ or MDTIQ was used as the substrate for FMO, the K_m value for NADPH was 111 or 200 μM. Table 1 summarises and compares the kinetic parameters, K_m and V_{max}, of FMO for various neurotoxins and DMA. Although theoretically the K_m of FMO for NADPH must stay the same even when various substrates are used in the experiments, the experimental K_m differed.

The electron donor for FMO is NADPH. However, NADH is also a weak electron donor and a little activity could be observed with NADH near the spectrophotometric limit. Accordingly, to examine the affinities of NADPH and NADH for FMO, the inhibitory effect of NAD^+ on the activity of FMO was examined using NADPH as an electron donor. The reciprocals of DMA or MPTP concentrations against the reciprocals of the reaction velocities were investigated in the absence and presence of NAD^+ as an inhibitor. A series of intercept lines on the ordinate was obtained at varying concentrations of NAD^+. The inhibition type was typically competitive, and the K_i values for DMA and MPTP were 0.83 and 0.31 mM, respectively. This demonstrates that the affinity of NADPH for FMO is about 4–25 times higher than that of NAD^+.

FMO, of which two isozymes, forms I and II, were demonstrated by Ozols [4, 11] and Yamada *et al.* [12], is localized in various tissue microsomes and is a labile enzyme [13]. FMO also exists as the two isozymes in the microsomes of rat, rabbit and human liver but not porcine liver. It has been reported that its activity is much greater in porcine liver microsomes than in other animal tissue microsomes. Thus, we used porcine liver microsomes as the source of FMO, to examine the metabolism of neurotoxins. In this study, the activity of FMO was measured spectrophotometrically, in the presence of a substrate, as to the oxidation of NADPH. Accordingly, this activity measurement was not affected by monoamine oxidases A and B [13, 14], even if the microsomes used were contaminated by mitochondria. In addition, the measurement was also not affected by NADPH-cytochrome P450 reductase, because it was confirmed that this enzymatic activity is blocked by 3 mM *n*-octylamine [15] or 0.1 mM phenylisocyanide [16].

FMO of mammalian tissue microsomes catalyses the N- and S-oxygenation of various compounds. We found that neurotoxins such as MPTP, TIQ and MDTIQ, which cause

Table 1. Kinetic parameters of FMO for various neurotoxins

Substrate	K_m (μM)	V_{max} (nmol/min/mg protein)	K_m for NADPH (μM)
DMA	17	7	31
MPTP	47	10	74
TIQ	6900	35	111
MDTIQ	5600	16	200

The reaction mixture contained 184 μg microsomal protein, 0.2 M potassium phosphate buffer (pH 8.0), 1 mM EDTA, 0.1% (v/v) Triton N-101, 3 mM *n*-octylamine, and appropriate amounts of NADPH and substrate, in a final volume of 1 mL. After the addition of substrate, the rate of absorbance change at 340 nm was measured for 5 min.

neuropathy, are relatively effective substrates for FMO. The specific activities of FMO of porcine liver microsomes for MPTP, TIQ and MDTIQ were in the range of 10–35 nmol/min/mg protein. These activities of the FMO were comparable with its activity toward DMA, which has been recognized as the most effective substrate for FMO [11].

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