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Substrate specificity of guinea pig liver flavin-containing monooxygenase for morphine, tropane and strychnos alkaloids

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Flavin-containing monooxygenase (FAD-monooxygenase) localized in many tissues of mammals catalyses oxidation of hetero atoms of a wide variety of sulfur and nitrogen containing xenobiotics [1, 2]. This enzyme has been purified from the liver of hogs [3], rats [4], mice [5, 6] and rabbits [6, 7] and the lung of mice [6] and rabbits [6, 8]. A current interest of many workers in the field of FAD-monooxygenase enzymology is the multiplicity of this enzyme. Recently, we have purified and characterized two distinct forms of FAD-monooxygenase from guinea pig liver [9].

N-Oxidation is a common metabolic pathway of alkaloids. For example, morphine [10], oxycodone [11], atropine [12] and strychnine [13] are metabolized to the *N*-oxide in the animal bodies. FAD-monooxygenase is ar important candidate of the enzyme responsible for the *N*-oxidation of these alkaloids. Although substrate specificity of FAD-monooxygenase has been widely studied especially for sulfur-containing compounds, the information for FAD-monooxygenase-dependent oxygenation of alkaloids is very limited. In the present study, we determined catalytic parameters of a purified guinea pig liver FAD-monooxygenase for oxidation of morphine, tropane and strychnos alkaloids. These results showed interesting structural requirements for the substrate.

Materials and Methods

Chemicals. Nalorphine hydrochloride and naloxone hydrochloride were donated by the Ministry of Health and Welfare of Japan and the National Institute on Drug Abuse, U.S.A., respectively. Other materials used were of the highest quality commercially available. All drugs examined as the substrate were *l*-form.

Purification of guinea pig liver FAD-monooxygenase. FAD-monooxygenase was purified from guinea pig liver microsomes by the method described elsewhere [9]. This method gave two distinct forms (FMO-I and FMO-II), which are distinguishable from the molecular weights, peptide mappings, amino terminal sequences, immunochemical natures and substrate specificities [9]. FMO-II was shown to exist in larger amounts than did FMO-I in the guinea pig liver microsomes [9]. Because of the small quantity of FMO-I isolated, this isozyme was unavailable for enzyme assays in the present study.

Assays. FAD-monooxygenase activity was assayed by measuring absorbance decrease at 340 nm due to substrate-dependent NADPH oxidation. The reaction was carried out at 37° in a final volume of 1 mL of mixture containing a substrate, 0.125 mM NADPH, enzyme (FMO-II 8.9 μg

Table 1. Kinetic constants of guinea pig liver flavin-containing monooxygenase for morphine congeners

Substrate	K_m* (μM)	V_{max}* (nmol/min/mg protein)	V_{max}/K_m
Morphine	3390 ± 790	257 ± 33	0.06
Codeine	460 ± 54	386 ± 20	0.86
Ethylmorphine	331 ± 41	418 ± 19	1.34
Thebaine	234 ± 18	422 ± 10	1.78
Oxymorphone	UD	>154†	—
Oxycodone	7100 ± 3450	665 ± 263	0.11
Nalorphine	UD	>138†	—
Naloxone	UD	>164†	—
Thiourea	54 ± 1	365 ± 5	7.4

UD, unable to determine.

* The values represent optimal values ± SD which were calculated by a curve fitting program of Michaelis–Menten equation.

† These values were obtained at a substrate concentration of 10 mM.

protein) and 50 mM Tris–HCl (pH 8.4). In the absence of substrate, the endogenous rate of NADPH oxidation was determined. Then, substrate was added and the substrate-dependent rate of NADPH oxidation was measured. FAD-monooxygenase activities were obtained by duplicate determinations at three different substrate concentrations from about 0.2 to 4 times the K_m value. The kinetic parameters with standard deviations were calculated using a non-linear least square program "MULTI" [14]. In this procedure the data were fitted to the Michaelis–Menten equation. Protein was determined by the method of Bensadoun and Weinstein [15] using bovine serum albumin as the standard.

Results and Discussion

Chemical structures of substrates used in this study are shown in Fig. 1. Table 1 shows kinetic constants of morphine congeners for guinea pig liver FAD-monooxygenase. The K_m value of thiourea, an excellent substrate of FAD-monooxygenase, for FMO-II was found to be 54 μM (Table 1) while it was reported to show 30 and 10 μM, respectively, for purified mouse and pig liver FAD-monooxygenases [16]. The V_{max} values for oxidation of morphine congeners by FAD-monooxygenase did not vary so much (257–665 nmol/min/mg protein) except oxymorphone, nalorphine and naloxone. The K_m as well as the V_{max} of the latter three compounds were not determined because of their insolubility at the higher concentrations in the assay conditions (pH 8.4). Morphine was a poor substrate, however, K_m for codeine and ethylmorphine were seven and ten times lower than that of morphine. The same modification effect of the alkylation of the 3-hydroxy group on K_m values was suggested between oxymorphone and oxycodone. Further, thebaine which has two methoxy groups at the 3- and 6-position showed the lowest K_m value among the substrates tested. These findings indicate that alkylation of the 3-hydroxy group in the morphine structure enhances the affinity for FAD-monooxygenase as a substrate. Modification of the alcoholic hydroxy group (6-position) was seemed to be less effective than that of the phenolic hydroxy group (3-position) since the K_m of thebaine was not so low in comparison with those of codeine and ethylmorphine.

Kinetic constants of tropane and strychnos alkaloids are shown in Table 2. The K_m value for atropine was relatively high. Scopolamine has a similar structure to atropine; that is, the only difference between the alkaloids is an epoxide on the tropane ring (Fig. 1). However, it is interesting to note that the K_m value of scopolamine was 4.5-fold lower than that of atropine. Cocaine, another type of tropane alkaloid, was indicated to be a poor substrate. However, N-hydroxylation of norcocaine, one of the metabolites of cocaine, has been reported to be well catalysed by purified FAD-monooxygenase [17]. This reaction was assumed to be the important process to convert cocaine to a hepatotoxic metabolite [17]. Norcocaine may be, therefore, a far better substrate than the parent cocaine. It is known that strychnine is N-oxidized by liver enzyme [18, 19]. Although guinea pig liver was shown to have the highest strychnine *N*-oxidase activity than the other species [19], the affinity of strychnine for purified guinea pig FAD-monooxygenase was very low. Brucine, a dimethoxy derivative of strychnine, was also found to be a poor substrate. This result suggests that *N*-oxide formation of strychnos alkaloids are mainly catalysed by cytochrome P450 but not by FAD-monooxygenase. Our previous study in which the inhibitory effect of SKF-525A and methimazole on microsomal metabolism of strychnine was examined well supported the above consideration [18]. Furthermore, we confirmed that seven purified cytochrome P450s from liver microsomes of

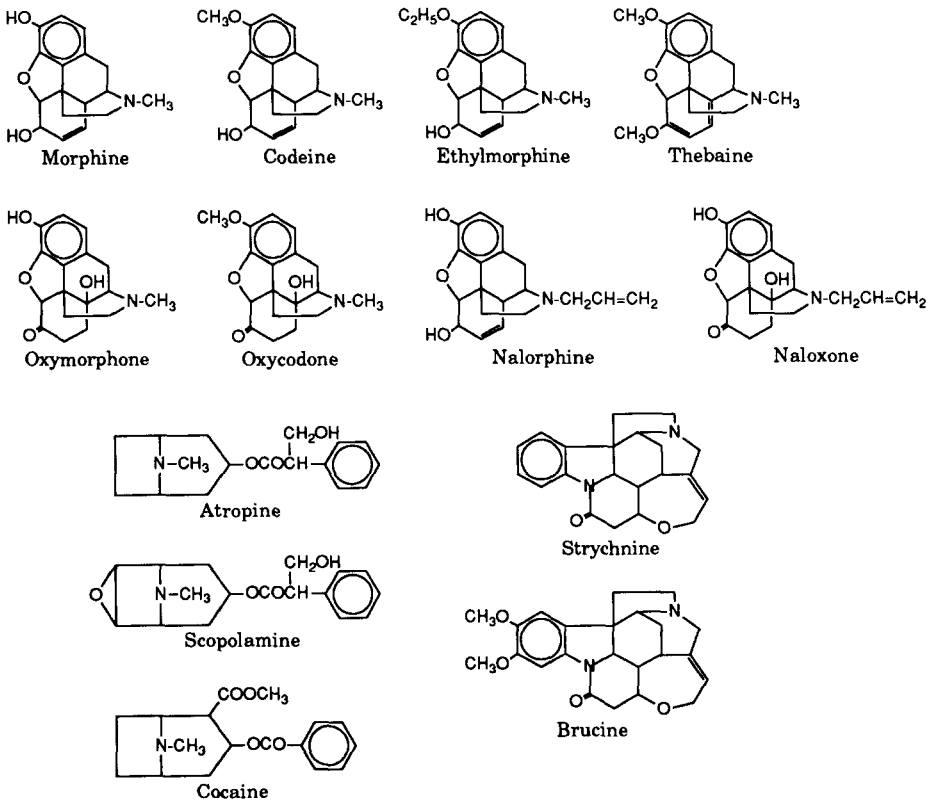

Fig. 1. Chemical structures of substrates used in the present study.

Table 2. Kinetic constants of guinea pig liver flavin-containing monooxygenase for tropane and strychnos alkaloids

Substrate	K_m* (μM)	V_{max}* (nmol/min/mg protein)	V_{max}/K_m
Atropine	3270 ± 550	500 ± 58	0.15
Scopolamine	731 ± 85	367 ± 17	0.50
Cocaine	2820 ± 450	174 ± 11	0.06
Strychnine	5180 ± 1400	191 ± 30	0.04
Brucine	UD	>19†	—

UD, unable to determine.

* The values represent optimal values ± SD which were calculated by a curve fitting program of Michaelis–Menten equation.

† These values were obtained at a substrate concentration of 10 mM.

untreated and phenobarbital- and 3-methylcholanthrene-pretreated rats show significant strychnine *N*-oxidase activity in the reconstituted system (unpublished observation).

In conclusion, the present study showed that (i) morphine, tropane and strychnos alkaloids can be oxidized by guinea pig liver FAD-monooxygenase, although some of them are poor substrates; (ii) both alkylation of the 3-hydroxy group of morphine congeners and epoxidation of the tropane ring enhance affinity for FAD-monooxygenase; and (iii) alkylation of the 6-hydroxy group of morphine congeners seems to be less effective than the 3-hydroxy modification for increasing the affinity to FAD-monooxygenase.

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