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Photoaffinity Labeling of Human Placental Monoamine Oxidase-A by 4-Fluoro-3-nitrophenyl Azide

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SUMMARY

Our previous work has shown that low concentrations of 4-fluoro-3-nitrophenyl azide (FNPA) (0.01–1 μM) photodependently inhibited only the type B monoamine oxidase in rat brain [*Biochem. Pharmacol.* 34:781–785 (1985)]. Evidence is presented in this paper indicating that higher concentrations of FNPA (15 μM) photodependently inhibit type A monoamine oxidase (MAO-A) from human placenta. FNPA acted as a competitive inhibitor for human placental MAO-A in the dark (K_i = 10 μM) when [^{14}C]serotonin was used as the substrate. The inhibition of MAO-A activity by FNPA was concentration dependent and also irradiation time dependent. The specificity of the photodependent incorporation of FNPA to MAO-A was shown by the protective effect of serotonin during the irradiation. The kinetic analysis showed that the V_{max} was decreased whereas the K_m was not changed after FNPA was photolyzed with MAO-A. Furthermore, there was no recovery of MAO-A activity upon washing of the photolyzed FNPA-enzyme mixture. These results suggest that FNPA may be covalently bound to the substrate-binding site. Thus, under the present experimental conditions, FNPA is a suitable photoaffinity labeling probe for human placental MAO-A. This is the first photoaffinity label for MAO-A, which may be useful for characterizing the substrate-binding site of this enzyme.

Monoamine oxidase (EC 1.4.3.4) (MAO) is an FAD-containing enzyme (1, 2), located in the outer membrane of mitochondria (3, 4), that oxidizes amines to their corresponding aldehydes. MAO appears to exist in at least two catalytically distinct forms, which may be called MAO-A and MAO-B. These two forms of MAO are distinguished both by their substrate preferences and their inhibitor specificities (5, 6). MAO-A preferentially deaminates serotonin and is sensitive to clorgyline (5). MAO-B preferentially deaminates phenylethylamine and benylamine and is sensitive to deprenyl (6). Immunological studies with beef MAO (7) and, more recently, with human MAO (8–12) indicate that MAO-B has an antigenic site not present in MAO-A. Also, the two enzymes have slightly different molecular weights based on the electrophoretic migration of MAO that had been covalently labeled with [^{3}H]pargyline in the presence of either clorgyline or deprenyl (13–15). Furthermore, the peptides which resulted from partial proteolytic digestion of [^{3}H]pargyline-labeled MAO-A and MAO-B migrated differently on sodium dodecyl sulfate-gel electrophoresis (16, 17). These studies all suggest that the two types of enzyme have different primary structures. However, these differences may also be caused by different carbohydrates or lipids attached to the enzyme. Furthermore, in order to understand the biochemical basis of the differences in substrate and inhibitor specificity between MAO-A and MAO-B, it is necessary to compare their structures at the active site. The active site of MAO has been suggested to be composed of two segments, one bearing the FAD prosthetic group, and the other comprising the substrate-binding sites (18). The cofactor FAD has been shown to be linked to the same pentapeptide (Ser-Gly-Gly-Cys-Tyr) for both beef liver MAO (a type B MAO) and human placental MAO (a type A MAO) (18–21). These results would indicate that the substrate and inhibitor specificity between the two enzymes may be attributed to the differences at the region(s) other than the flavin-binding site. Such information is not available at the present time.

The photoaffinity labeling technique has been shown to be very useful for investigating specific ligand-binding sites on macromolecules (22–24). Recent studies of the effects of FNPA on both types of MAO in rat brain cortex indicate that FNPA is a potent competitive inhibitor for both types of MAO in the dark (25). However, the photodependent inhibition of FNPA to MAO-B was much more effective than that of MAO-A (25). The effect of FNPA on human placental MAO is presented in this study. This is a first report on a photoaffinity label for MAO-A.

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ABBREVIATIONS: MAO, monoamine oxidase; FAD, flavin-adenine dinucleotide; MAO-A, type A monoamine oxidase; MAO-B, type B monoamine oxidase; FNPA, 4-fluoro-3-nitrophenyl azide; EDTA, ethylenediaminetetraacetate.

Materials and Methods

Chemicals. FNPA was obtained from Pierce Chemical Co. β-Phenylethylamine, and serotonin binoxalate and clorgyline were from Sigma Chemical Co. 5-[2-^{14}C]Serotonin binoxalate (58.5 mCi/mmol) and β-[ethyl-1-^{14}C]phenylethylamine hydrochloride (50 mCi/mmol) were from New England Nuclear Corp. Deprenyl was a gift from Dr. J. Knoll, Semmelweis University of Medicine, Hungary.

Preparation of crude mitochondria from human placenta. Fresh human placenta was chilled on ice as soon as possible after delivery. Blood clots, remnants of amnionic membranes, and the umbilical cord were removed thoroughly. The placenta was washed extensively to remove the blood. Tissue was homogenized with a Polytron homogenizer using buffer containing 0.25 M sucrose, 0.5 mM EDTA, and 0.01 M potassium phosphate, pH 7.2, at 0°. After homogenization, the mixture was diluted with this buffer to give a final concentration of 22% (w/v). The homogenate was neutralized to pH 7.2 with KOH and then centrifuged at 800 × *g* for 15 min. The supernatant solution was centrifuged again at 10,000 × *g* for 15 min. The resulting pellet was suspended and homogenized in the above buffer to give a concentration of 7% (w/v) and stored at −20°.

Protein concentration was measured by the method of Lowry *et al.* (26) using bovine serum albumin as a protein standard.

Determination of MAO activity. The MAO activity was assayed as described previously (27). The assay was performed in screw cap culture tubes. The 1-ml mixture contained 50 mM sodium phosphate buffer, pH 7.4, 100 μM [^{14}C]serotonin (for MAO-A) or 10 μM [^{14}C] phenylethylamine (for MAO-B), and/or an appropriate amount of enzyme. The final specific activities of [^{14}C]serotonin and [^{14}C]-phenylethylamine in the assay mixture were 45 nCi/mmol and 30 nCi/mmol, respectively. After a 20-min incubation at 37°, the reaction was terminated by the addition of 0.1 ml of 6 N HCl. The reaction products were extracted with 6 ml of ethyl acetate/benzene = 1:1 when serotonin was used as substrate or extracted with 6 ml of toluene when phenylethylamine was used as substrate. Each tube was capped and shaken vigorously. The tubes were centrifuged at 2000 rpm for 6 min to separate the two phases. Four ml of organic layer were withdrawn and mixed with 5 ml of scintillation fluid (National Diagnostics). The radioactivity of the reaction product was determined by liquid scintillation spectrometry.

Photolysis. The photoirradiation experiments were carried out as described previously (27). Sample preparations were irradiated at a distance of 5 cm from a tungsten projection lamp (DVY 120V, 650W) at 5°C for 5 min unless otherwise indicated. The dark control samples were kept on ice and covered with aluminum foil to prevent any possibility of photoinactivation. The photolysis was performed by allowing 1 min for the lamp to cool after each min of irradiation.

Results

Inhibitor sensitivity of human placental MAO. MAO from human placenta was examined for sensitivity to the inhibition by clorgyline and deprenyl using the substrates serotonin and phenylethylamine. Fig. 1 shows the inhibition of MAO activity by increasing concentrations of clorgyline and deprenyl. When serotonin was used as substrate, 50% of the MAO activity in human placenta was inhibited by 5 nM clorgyline or 0.5 μM deprenyl. This result indicates that serotonin measures MAO-A activity in human placenta. When phenylethylamine was used as the substrate, 50% of the MAO activity was inhibited by 6 nM clorgyline or 0.55 μM deprenyl. This result indicates that phenylethylamine measures MAO-A activity also. Since clorgyline was considerably more inhibitory than deprenyl even when B substrate, phenylethylamine, was oxidized, the result suggests that human placental MAO is an exclusive source of the MAO-A.

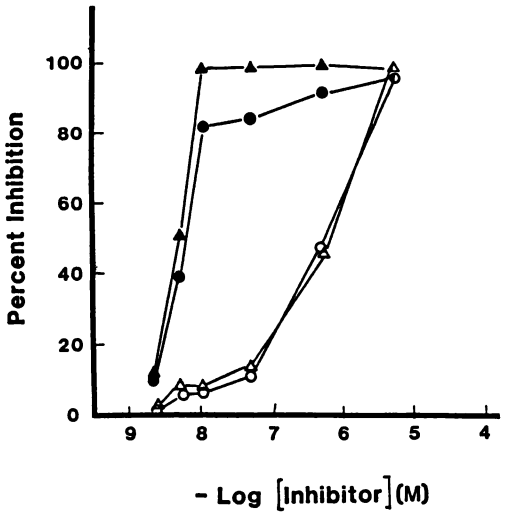

Fig. 1. Effect of clorgyline and deprenyl inhibition of human placental MAO-A. MAO activity in crude mitochondria from human placenta was determined by the oxidation of 100 μM serotonin (▲, △) and 10 μM phenylethylamine (●, ○) after a 1-hr preincubation with different concentrations of clorgyline (▲, ●) and deprenyl (△, ○). Each point is the mean of duplicate assays at each inhibitor concentration. The MAO activity in the absence of inhibitors was 300 nmol of serotonin oxidized/20 min/mg of protein or 15.2 nmol of phenylethylamine oxidized/20 min/mg of protein.

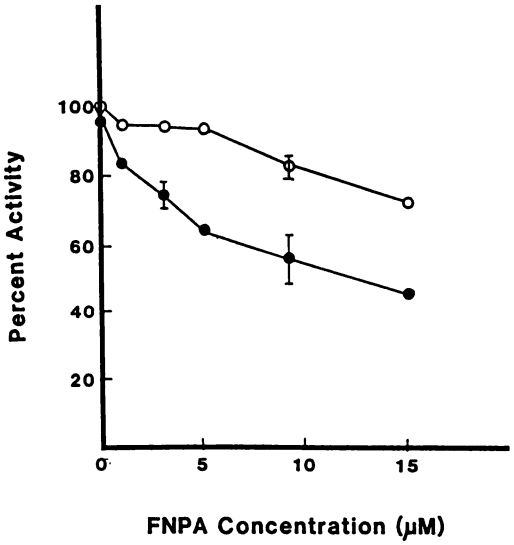

Fig. 2. Concentration dependency of the inhibition of human placental MAO-A activity by FNPA. Two-ml mixtures contained 50 mM sodium phosphate buffer, pH 7.4, 0.43 mg of crude mitochondria from human placenta, and varying FNPA concentrations as indicated in the figure. An aliquot of 0.2 ml was taken to assay for 100 μM [^{14}C]serotonin oxidation in the dark (○) and following a 5-min photoirradiation (●). The reaction was started by bringing the reaction mixture to 37° and incubating for 20 min, and the reaction was stopped by the addition of 0.1 ml of 6 N HCl. The products were extracted as described under Materials and Methods. The activity of the sample without FNPA before photolysis was 337 nmol of serotonin oxidized/20 min/mg of protein and was taken as 100%. All points represent the mean of two determinations [standard error is shown (*bars*) when points exceed symbol size).

Irradiation time and concentration-dependent inhibition of human placental MAO-A by FNPA. Fig. 2 shows the concentration dependency of the FNPA inhibition of MAO-A activity before and after photolysis when [^{14}C]serotonin was used as the substrate. In the absence of FNPA, only a 7% inhibition of MAO-A activity was obtained after 5 min of

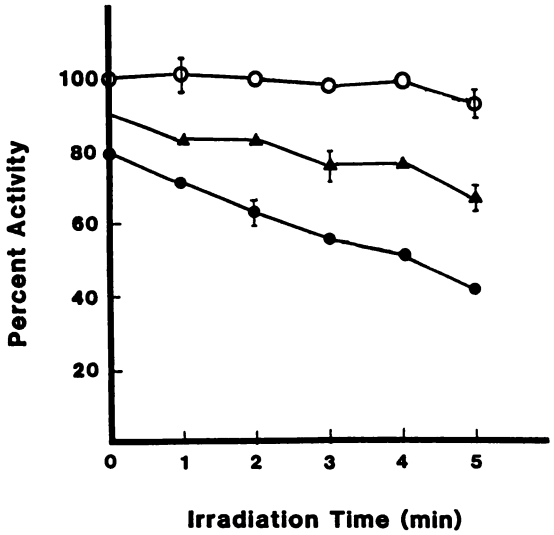

Fig. 3. Irradiation time-dependent inhibition of human placental MAO-A activity by FNPA. A 3-ml mixture contained 0.43 mg of human placental MAO-A and 5 (▲) or 15 μM (●) FNPA in 50 mM phosphate buffer, pH 7.4. After 0, 1, 2, 3, 4, or 5 min of irradiation, an aliquot of 0.2 ml was withdrawn and assayed for serotonin oxidation. A light control (○) was prepared in the same manner, except for the omission of FNPA from the mixture. The activity of the nonirradiated control (0 time) was 322 nmol/20 min/mg of protein at 100 μM serotonin and was taken as 100%. All points represent the mean of two determinations (standard error is shown (*bars*) when points exceed symbol size).

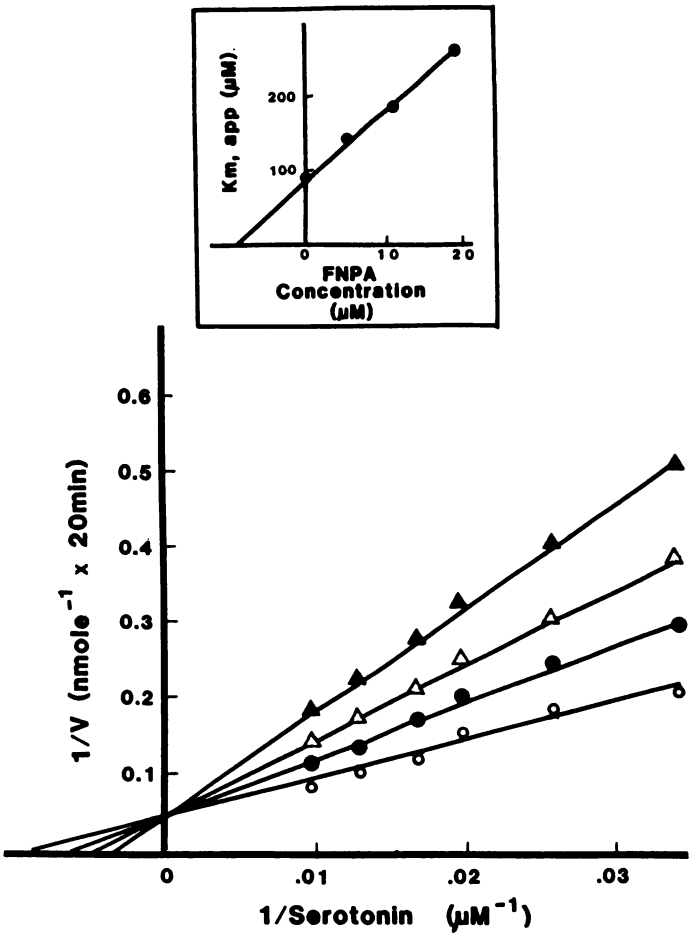

Fig. 4. Competitive inhibition of the [^{14}C]serotonin oxidation of human placental MAO-A activity by FNPA. The assay was performed in the dark as described under Materials and Methods. The FNPA concentrations were 0 (○), 6 (●), 12 (△), and 20 μM (▲). K_i determination is shown in the *inset*. Each point represents the mean of duplicate determinations from a typical experiment. The *lines* were drawn by linear regression.

photolysis. A concentration-dependent inhibition was observed when FNPA concentrations were increased from 1, 3, 5, 10, to 15 μM. At 15 μM FNPA, a 55% inhibition of MAO-A activity resulted. This result indicates that, upon photoirradiation, a concentration-dependent inhibition of human placental MAO-A activity by FNPA results.

The effect of the irradiation time on the MAO-A activity is shown in Fig. 3. A 17% inhibition was obtained after 1 min of photolysis of 0.43 mg of crude mitochondria pellet from human placenta (in 4 ml) in the presence of 5 μM FNPA. Further photolysis (5 min) resulted in a 34% inhibition of MAO-A activity under the same experimental conditions. Moreover, at 15 μM FNPA, a 29% inhibition was obtained after 1 min of photolysis of the same amount of the enzyme. Further photolysis (5 min) resulted in a 58% inhibition of MAO-A activity. For the light control, crude mitochondria from human placenta were photolyzed in the absence of FNPA. Only a 7% inhibition was found after 5 min of irradiation. Thus, the degree of human placental MAO-A inhibition increased with increasing photolysis time in the presence of FNPA, and the inhibition could not be attributed to the effect of the light alone.

Kinetics of dark and light inhibition of human placental MAO-A by FNPA. Human placental MAO-A is competitively inhibited by FNPA in the dark. As shown in the double reciprocal plot in Fig. 4, the slope obtained in the presence of 0, 6, 12, or 20 μM FNPA indicates an increase in K_m, whereas the V_{max} remains unchanged. The K_i value for FNPA inhibition of serotonin deamination is 10 μM (Fig. 4, *inset*). Upon photolysis, FNPA irreversibly labeled MAO-A, as shown by the following experiment. Human placental MAO-A was first irradiated in the presence of 0, 5, 10, or 15 μM FNPA and then MAO-A activity was determined with various concentrations of [^{14}C]serotonin as shown in Fig. 5. A double reciprocal plot indicated that the K_m remained the same, whereas the V_{max} was decreased by increasing FNPA concentrations. This result suggests that a covalent linkage is formed between the enzyme and the nitrene species resulting from the photolysis of FNPA, and that the FNPA-labeling site may be the same as the serotonin-binding site.

Photodependent irreversible labeling of human placental MAO-A by FNPA. In order to demonstrate that photoinduced labeling of MAO-A by FNPA was indeed irreversible, four samples were prepared: 1) a control sample containing only human placental MAO-A, 2) a dark control sample containing human placental MAO-A and 15 μM FNPA but not photolyzed, 3) a sample containing human placental MAO-A and FNPA, and subjected to photoirradiation, and 4) a sample containing human placental MAO-A and prephotolyzed FNPA. The MAO-A activity in these samples was measured before and after removing the unbound FNPA by washing and centrifugation. The control activity (sample 1, Table 1) was taken as 100%. As shown in Table 1, before the washing and centrifugation procedure, an inhibition was observed for the dark control (sample 2, 23% inhibition), the photolyzed sample (sample 3, 56% inhibition), and also the prephotolyzed sample (sample 4, 8% inhibition). However, upon washing and centrifugation, the FNPA inhibition of MAO-A activity in the dark control was reversed, indicating that FNPA is a reversible

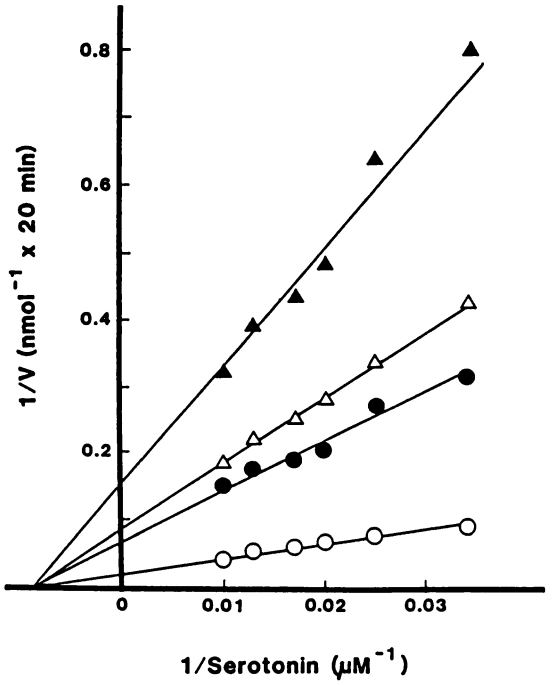

Fig. 5. Decrease in V_{max} of [^{14}C]serotonin oxidation following photolysis of human placental MAO-A with FNPA. One mg of crude mitochondria from human placenta in 3 ml of 50 mM sodium phosphate buffer was incubated with 0 (○), 5 (●), 10 (△) or 20 μM (▲) FNPA at 4° and subjected to 5 min or irradiation. The assay was then performed in the same manner as described under Materials and Methods. Each point represents the mean of duplicate determinations from a typical experiment.

TABLE 1

FNPA inhibition of human placental MAO-A

Four experiments were performed: experiment 1, crude mitochondria from human placenta (0.63 mg) suspended in 2 ml of 50 mM phosphate buffer, pH 7.4; experiment 2, the same enzyme suspension as in experiment 1, but also containing 15 μM FNPA, and the mixture kept in the dark; experiment 3, 15 μM FNPA in 2 ml of enzyme suspension as in experiment 2, but subjected to a 5-min photoirradiation; experiment 4, identical to experiment 3, except that FNPA was photoirradiated in the absence of enzyme which was subsequently added to the incubation mixture. A 0.2-ml aliquot of each of the mixtures was taken for the determination of MAO-A activity; the remaining portion of the preparation was subjected to centrifugation at 50,000 ×*g* for 10 min. The pellet was washed once more by the same centrifugation procedure and was finally suspended in 1 ml of 50 mM phosphate buffer, pH 7.4. MAO-A activity was then assayed using 100 μM serotonin. Control activity was 222 nmol of serotonin oxidized/20 min/mg of protein. Each point is the mean of duplicate determinations.

Experiment	Condition	Percentage of MAO-A activity	
		Before centrifugation	After centrifugation
1	Control	100	100
2	Dark control with FNPA	77	94
3	Photoirradiated in the presence of FNPA	44	57
4	Prephotoirradiation of NPA	92	111

inhibitor for the MAO-A in the dark (sample 2, Table 1). The inhibition of MAO-A activity in the prephotolyzed sample (sample 4, Table 1) was also reversed after washing. This observation eliminates the possibility of noncovalent interactions between the enzyme and light-induced FNPA derivative(s). The only sample which remained inhibited after extensive washing was the MAO-A photolyzed in the presence of FNPA (sample 3, Table 1). (Compare inhibition before and after washing.) This observation indicates a clearly photoinduced irreversible binding of FNPA to human placental MAO-A.

Protection of photoinactivation of human placental MAO-A by serotonin. It was of interest to determine whether the substrate of MAO-A, serotonin, could protect the enzyme against photoinactivation. Since FNPA can irreversibly bind to the enzyme upon irradiation while serotonin is deaminated and released from the enzyme, it was necessary to use a high concentration of serotonin in order to provide complete protection against photoinactivation by FNPA. In this experiment, the concentration of FNPA was held constant (15 μM), and the amount of serotonin was varied. As shown in Fig. 6, 1.12 mM serotonin was able to provide almost 100% protection against the photoinactivation of MAO-A by FNPA. This result suggests that FNPA interacts at the active site of human placental MAO-A.

Discussion

MAO-A and MAO-B can be distinguished by their different sensitivities to the inhibitors clorgyline (5) and deprenyl (6). Fig. 1 shows that human placental MAO activity is highly sensitive to clorgyline when either serotonin (Type A substrate) or phenylethylamine (type B substrate) was used as the substrate. Human placenta MAO was inhibited at a concentration of clorgyline 2 orders of magnitude lower than that of deprenyl (Fig. 1). This finding was consistent with previous reports that human placental MAO contained only the A form (28, 29). The effect of FNPA on human placental MAO-A is presented in this report. FNPA acted as a competitive inhibitor for human placental MAO-A in the dark (K_i = 10 μM) when serotonin was used as the substrate (Fig. 4). The kinetic analysis showed that

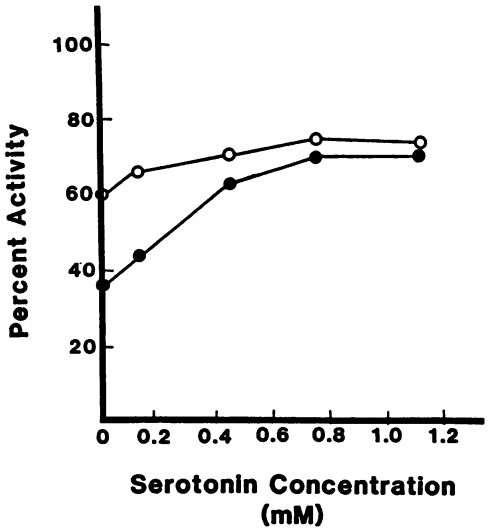

Fig. 6. Serotonin protection of photodependent inhibition of human placental MAO-A activity by FNPA. A 1-ml mixture contained 0.22 mg of crude mitochondria from human placenta, 15 μM FNPA, and varying serotonin concentrations as indicated in the figure. The mitochondria were washed by centrifugation to remove FNPA and serotonin. An aliquot of 0.2 ml was taken to assay for [^{14}C]serotonin oxidation before (○) and after (●) photoirradiation for 5 min. A control containing only enzyme and serotonin was assayed before and after photolysis. The latter results were taken as 100% control activity for the photolyzed samples containing FNPA. Each point is the mean of duplicate determinations.

the V_{max} was decreased whereas the K_m was not changed after increasing concentrations of FNPA were photolyzed with MAO-A (Fig. 5). Furthermore, the photoinduced inhibition by FNPA cannot be eliminated by extensive washing (Table 1). These results suggest that FNPA may be covalently bound to the active site of MAO-A.

The inhibition of MAO-A activity by FNPA was concentration dependent (Fig. 2) and also irradiation time dependent (Fig. 3). A 55% inhibition of MAO-A activity was obtained after 5 min photolysis of 0.43 mg of human placental mitochondrial MAO with 15 μM FNPA (Fig. 2). The specificity of FNPA labeling was shown by the fact that the presence of serotonin during photolysis could effectively prevent the photodependent inhibitory effect of FNPA (Fig. 6).

These results indicate that FNPA may be a suitable photoaffinity labeling probe for characterization of the substrate-binding site of human placental MAO-A.

Our previous work has shown that low concentrations (0.01–1 μM) of a number of phenylazides (30) and *N*-(2-nitro-4-azidophenyl)serotonin (27) photodependently inhibited only MAO-B effectively. Furthermore, we have photolabeled [^{3}H]FNPA to purified beef liver MAO-B (31). This result indicates that the photodependent incorporation of [^{3}H]FNPA to purified MAO-B was protected by the substrates and inhibitors of MAO. This result indicates that [^{3}H]FNPA labels the active site of MAO (32). The active site of MAO has been suggested to be composed of two segments: one bearing the cofactor FAD, and the other comprising the substrate-binding site (18). It has been shown that pargyline binds to the FAD in MAO, and that the pentapeptide linked to pargyline was identical for both types of MAO (21). Therefore, the different catalytic activities of two types of MAO may be attributed to the differences at the substrate-binding site. Recently, we have photolabeled the purified beef liver MAO-B with [^{3}H]FNPA and subjected this sample to tryptic-chymotryptic digestion. The separation profiles of [^{3}H]FNPA-labeled and [^{3}H]pargyline-labeled tryptic-chymotryptic peptides after Sephadex G-25 column chromatography are distinctly different (32). This result suggests strongly that the [^{3}H]FNPA-labeling sites are different from the pargyline-binding site. This finding supports further the idea that [^{3}H]FNPA labels the substrate-binding site of MAO-B.

This report provides evidence to suggest that higher concentrations of FNPA (15 μM) photolabeled the substrate-binding site of human placental MAO-A. Since crude mitochondrial preparations were used in this study, it may be difficult to define precisely the concentration required for MAO-A inactivation, because it is not clear whether FNPA may react with other proteins or may be metabolized by other enzymes. These factors may affect the true concentration of FNPA required to inactivate MAO-A. Nevertheless, the data presented in this paper clearly demonstrated that FNPA is a photoaffinity-labeling probe for MAO-A. Thus, FNPA becomes a useful tool for characterizing the substrate-binding site for both MAO-A and MAO-B. The structure of the substrate-binding site will be able to explain the different catalytic activities of two types of MAO.

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