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PHOTOAFFINITY LABELING OF BEEF LIVER MONOAMINE OXIDASE-B BY 4-FLUORO-3-NITROPHENYL AZIDE

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Abstract—4-Fluoro-3-nitrophenyl azide (FNPA) competitively inhibited beef liver monoamine oxidase-B (MAO-B) in the dark ($K_i = 2.8\ \mu M$). Upon irradiation in the presence of FNPA, a concentration-dependent photoinactivation of MAO-B was observed. The kinetic analysis showed that the photoinactivation of MAO-B resulted in a decrease in V_{max} but no change in K_m. This result suggests that an irreversible linkage may be formed between the enzyme and the photolyzed FNPA. When [^{3}H]FNPA was photoirradiated with the purified MAO-B, a single radioactive band associated with MAO-B was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The photo-dependent incorporation could be protected by phenylethylamine, the substrate for MAO-B, in a concentration-dependent manner. Complete tryptic–chymotryptic digestion of [^{3}H]FNPA-labeled MAO-B resulted in three radioactive peaks on Sephadex G-25 column chromatography. With the same digestion and separation procedures, only one major radioactive peak was observed for the [^{3}H]pargyline-labeled MAO-B, and its elution volume was different from that of [^{3}H]FNPA-labeled peptides. These results suggest that, upon photolysis, FNPA may incorporate into a region in the active site of MAO-B which may be different from the pargyline binding site—the FAD prosthetic group of the enzyme.

Monoamine oxidase (EC 1.4.3.4) (MAO§) catalyzes the oxidative deamination of a number of biogenic amines. Based on the differences in substrate specificity and inhibitor selectivity, two types of MAO have been described [1–3]. MAO-A preferentially deaminates 5-hydroxytryptamine (5-HT) and norepinephrine and is sensitive to inhibition by clorgyline [2] and LY 51641 [1]. MAO-B preferentially deaminates phenylethylamine (PEA) and benzylamine and is sensitive to inhibition by deprenyl [3].

The molecular natures of these two types of MAO have been studied by many investigators. Immunological studies with beef MAO [4], and more recently with human MAO [5, 6], indicate that MAO-B has an antigenic site not present in MAO-A. Also, the electrophoretic migration of [^{3}H]pargyline-labeled MAO under denaturing conditions reveals that the two types of the enzyme have slightly different molecular weights [7, 8]. Furthermore, the peptides resulting from partial proteolytic digestion of [^{3}H]pargyline-labeled MAO-A and MAO-B migrated differently on SDS gel electrophoresis [9–11]. These studies all suggest that the two types of enzyme exist as different protein entities. However, the structure of the active site of the two types of MAO is not yet known.

We reported previously that, in the dark, 4-fluoro-3-nitrophenyl azide (FNPA) is a potent competitive inhibitor for both types of MAO in rat tissues [12]. However, upon irradiation the photodependent inhibition was found only for MAO-B at low concentrations of FNPA. This finding suggests that FNPA may covalently bind to a region(s) in the active site of MAO-B which may be different from that of MAO-A. Thus, the characterization of the FNPA-labeling site may shed some light on the structural differences between the two types of MAO. Since a purified MAO preparation is essential for such a study, we have performed experiments to demonstrate that FNPA is also a suitable photoaffinity-labeling probe for purified beef liver MAO-B. The results are presented in this paper. Furthermore, our data suggest that [^{3}H]FNPA-labeling sites may be different from the [^{3}H]pargyline binding site on MAO-B.

MATERIALS AND METHODS

Chemicals. FNPA was obtained from the Pierce Chemical Co. [*Phenyl*-3, *benzyl*-^{3}H]Pargyline (15.3 Ci/mmole), 4-[2,6-^{3}H]fluoro-3-notrophenylazide (48 Ci/mmole), β-[*ethyl*-1-^{14}C]phenylethylamine hydrochloride (50 mCi/mmole), and 5-[2-^{14}C]serotonin binoxalate (58.5 mCi/mmole) were from the New England Nuclear Corp.

Purification of beef liver MAO-B. Beef liver was obtained from a local slaughterhouse within 30 min of sacrifice. The liver was transported on ice and used within 1 hr. MAO-B was isolated from beef liver mitochondria according to the procedure of

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§ Abbreviations: MAO, monoamine oxidase; MAO-A, type A monoamine oxidase; MAO-B, type B monoamine oxidase; FNPA, 4-fluoro-3-nitrophenyl azide; PEA, β-phenylethylamine; 5-HT, serotonin; and SDS, sodium dodecyl sulfate.

Salach [13]. Briefly, the mitochondria pellet was washed and resuspended in 0.1 M triethanolamine buffer, pH 7.2, and treated with phospholipase A (1.66 mg/500 mg protein) and phospholipase C (1 mg/500 mg protein) for 1 hr at 30°. The sample was then centrifuged at 41,000 *g* for 15 min, and the pellet was resuspended in 0.1 M triethanolamine, pH 7.2 (10–15 mg protein/ml). To solubilize MAO-B, Triton X-100 (1 mg/3 mg protein) was added to the mixture, stirred for 25 min, and then centrifuged again at the same speed. For each 10 ml of Triton supernatant solution, 1.9 ml water, 1.1 g dextran, 1.2 g Ficoll and 0.8 g polyethylene glycol were added. As soon as all the reagents were dissolved, the emulsion was centrifuged for 20 min at 10,000 *g* in a swinging bucket rotor. The solid material at the interface between the upper (Ficoll–polyethylene glycol) and lower (dextran) layers which contains the MAO was resuspended in 0.1 M triethanolamine buffer to 7–10 mg protein/ml and centrifuged at 41,000 *g* for 20 min. The resulting supernatant fraction was stored overnight at 0–4° and centrifuged again at 41,000 *g* for 20 min on the next day. Purified MAO-B was obtained by a final centrifugation at 252,000 *g* for 90 min at 4°. The MAO-B activity was determined as described previously [12, 14], using [^{14}C]PEA (at 10 μM) or [^{14}C]5-HT (at 100 μM) as substrate. The reaction products were extracted with toluene when PEA was used as the substrate, or extracted with ethyl acetate–benzene (1:1) when 5-HT was used as the substrate. The radioactivity of the reaction product was determined by liquid scintillation spectrometry.

Photolysis. The photoirradiation experiments were carried out as described by Chen *et al.* [12, 14] except that a 1–2 min irradiation was applied in consideration that the purified beef liver MAO-B is sensitive to light. The photolysis was performed by allowing 30 sec for the lamp to cool after each 30 sec of irradiation.

Proteolytic digestion of [^{3}H]*FNPA- or* [^{3}H]*pargyline-labeled MAO-B.* The labeled MAO-B (130 μg) was precipitated by 10% trichloroacetic acid (0°, for 30 min), in order to remove the free ligand. The precipitate was washed once with 1% trichloroacetic acid and once with chloroform–methanol (1:1), and then resuspended in 1–2 ml of 0.1 M Tris buffer, pH 8.0. Digestion of labeled MAO-B was performed at 37° with the use of 0.6 mg trypsin and 0.3 mg chymotrypsinogen by a procedure described by Chuang *et al.* [15]. During the digestion, the pH was maintained at 8 by the addition of 3 N NH_4OH. Two hours later, the same amounts of trypsin and chymotrypsinogen were added, and the incubation was continued for another 2 hr.

Separation of proteolytic digests of labeled MAO-B. The digest was chilled to 4°, and the pH was adjusted to 6 with 1 N HCl. After centrifugation for 10 min at 12,100 *g*, the supernatant fraction was applied to a Sephadex G-25 column (2 × 40 cm) equilibrated with 1% SDS. The peptides were eluted by the same solution.

All other procedures are detailed in the legends of the figures and tables.

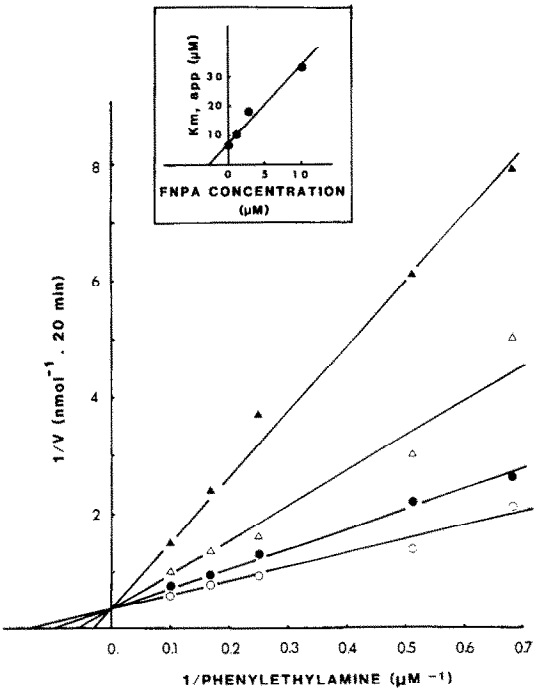

Fig. 1. Competitive inhibition of [^{14}C]phenylethylamine oxidation of purified beef liver MAO-B by FNPA. The assay was performed in the same manner as previously described [12, 14]. The FNPA concentrations were 0 (○), 1 (●), 3 (△), and 10 (▲) μM. K_i determination is shown in the insets.

RESULTS

Photodependent inhibition of crude beef liver MAO-B by FNPA. As shown in Table 1, FNPA inhibited MAO-B from beef liver homogenate photodependently when either PEA or 5-HT was used as substrate. This photodependent inhibition was concentration dependent. In the dark, the concentration-dependent inhibition of 5-HT deamination (10–29%) was observed when FNPA concentration was increased from 1 to 4 μM. It should be noted that, because the MAO assay was performed with 0.2 ml of enzyme–inhibitor mixture and the total volume of assay mixture was 1 ml, the actual FNPA concentration during the assay was from 0.2 to 0.8 μM. Under the same condition, only 4% inhibition of PEA deamination was observed when 4 μM FNPA was incubated with the enzyme. This result is not surprising since PEA is a better substrate for MAO-B than 5-HT. At 4 μM FNPA, 43 and 70% apparent photodependent inhibition (5-min irradiation) of PEA and 5-HT deamination, respectively, was observed. When the inhibition effect of FNPA in the dark was subtracted from the apparent photodependent inhibition, a similar photodependent inhibition was obtained when either PEA (39%) or 5-HT (41%) was used as the substrate.

Inhibition of purified beef liver MAO-B by FNPA. To investigate further the FNPA binding site on MAO-B, purified beef liver MAO-B was used in the following studies. We demonstrated that FNPA is a

Table 1. FNPA inhibition of phenylethylamine and serotonin deamination catalyzed by beef liver MAO-B

FNPA concn (μM)	% Inhibition			
	PEA deamination		5-HT deamination	
	−Light	+Light	−Light	+Light
0	0	0	0	4
1	0	25	10	41
2	0	35	16	57
4	4	43	29	70

* The mixture (2 ml) contained 2.4 mg of beef liver homogenate (100 volume homogenate) in 50 mM phosphate buffer, pH 7.4, and FNPA at the concentrations indicated. Two-tenths milliliter of the mixture was withdrawn for assay prior to, and following, a 5-min photoirradiation. The activities of the sample without FNPA prior to photolysis were taken as 100%, and they were 35.1 nmoles [^{14}C]PEA oxidized/20 min/mg protein at 10 μM [^{14}C]PEA; and 5.1 nmoles [^{14}C]serotonin oxidized/20 min/mg protein at 100 μM [^{14}C]5-HT.

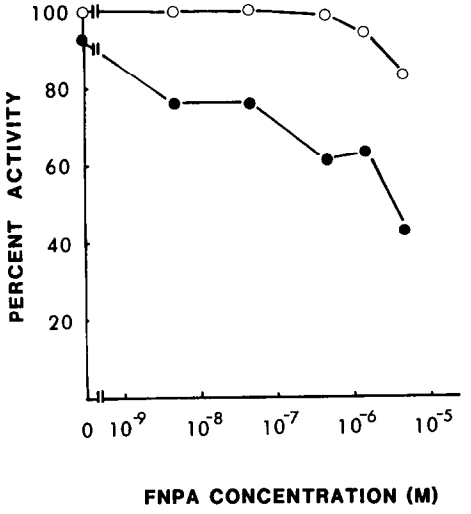

Fig. 2. Photodependent inhibition of the purified MAO-B from beef liver by FNPA. The irradiation mixture (1 ml) contained 7 μg MAO-B and FNPA at concentrations as indicated on the figure. Before (○—○) and after (●—●) 1-min irradiation 0.2 ml of each mixture was taken and assayed for PEA oxidation. The activity of the sample without FNPA prior to photolysis was taken as representing 100% activity. This preparation had a specific activity of 409 nmoles [^{14}C]PEA oxidized/20 min/mg protein at 37°.

competitive inhibitor of the purified beef liver MAO-B with a K_i of 2.8 μM (Fig. 1). Similar to our findings in the crude beef liver homogenate, a light-dependent inhibition of the enzyme by FNPA was also obtained. A 50% inhibition was found when MAO was photolyzed for 1 min in the presence of approximately 3 μM FNPA (Fig. 2). Only 13% inhibition resulted for the same enzyme–FNPA solution prior to the photolysis. Kinetic studies suggest that FNPA may irreversibly label purified MAO-B. In this experiment, purified beef liver MAO-B was irradiated with 5, 15, or 50 μM FNPA at 5° for 1 min. MAO-B activity was determined with various concentrations of PEA, as indicated in Fig. 3. A double-reciprocal plot indicated that the K_m remained the same, whereas the V_{max} was decreased with increasing FNPA concentrations. This result suggests that an irreversible linkage may be formed between the enzyme and the nitrene species of FNPA resulting from its photolysis.

Photodependent labeling of the purified beef liver MAO-B by [^{3}H]*FNPA*. The photodependent incorporation of FNPA into MAO-B has been further demonstrated by a labeling experiment utilizing [^{3}H]FNPA. Two samples were prepared for this experiment. One sample containing 0.3 mg MAO-B and 0.04 nmole [^{3}H]FNPA (48 Ci/mmole) was photolyzed for 1 min at 5°; the second sample contained the same amount of enzyme and [^{3}H]FNPA, but it was kept in the dark throughout the process. Both samples were broiled for 3 min in the presence of 1% SDS and then applied to a Sephadex G-25 column equilibrated with 1% SDS. A radioactive peak associated with the protein peak (fractions 12–16) at the void volume was observed only for the photolyzed sample but not for the sample kept in the dark (Fig. 4). Free [^{3}H]FNPA was eluted in fractions 18–30. [^{3}H]FNPA-photolabeled enzyme was further subjected to 8% polyacrylamide SDS gel electrophoresis. It was found that FNPA indeed incorporated covalently into MAO-B upon photoirradiation (Fig. 5). Moreover, the photodependent [^{3}H]FNPA (0.02 μM) labeling for purified MAO-B could be protected by including PEA (200 μM), the substrate of MAO-B, during photolysis (Fig. 5). The concentration-dependent protection of [^{3}H]FNPA labeling on MAO-B is shown in Table 2. These results suggest that [^{3}H]FNPA may photolabel the active site of the enzyme. The requirement of high concentrations of PEA for the protection of FNPA labeling was expected because FNPA irreversibly bound to the enzyme upon irradiation, whereas PEA was deaminated and dissociated from the active site of the enzyme during those treatments. Also, because the enzyme concentration during the irradiation was 125 times of that present in the regular assay, PEA was oxidized rapidly. Thus, the true concentration of PEA in this experiment was lower than 200 μM.

On the other hand, this result may suggest a certain

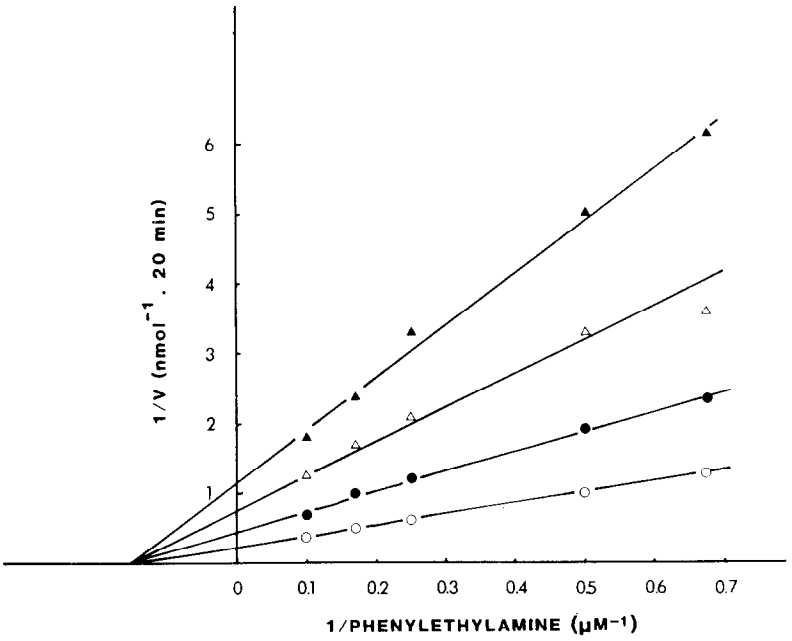

Fig. 3. Decrease in V_{max} of [^{14}C]PEA oxidation following photolysis of purified beef liver MAO-B with FNPA. Fifty-one micrograms of purified MAO-B in 3 ml of 50 mM sodium phosphate buffer was incubated with 5 (●—●), 15 (△—△), or 50 (▲—▲) μM FNPA at 5° and subjected to 1-min irradiation. The assay was initiated by addition of a 0.2-ml aliquot of the mixture. The control sample (○—○) was irradiated for the same period of time in the absence of FNPA.

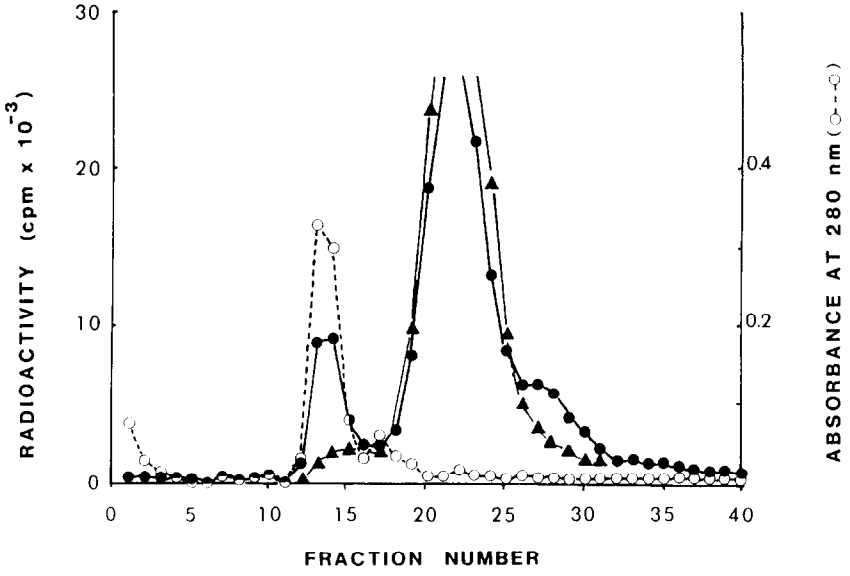

Fig. 4. Covalent labeling of the purified beef liver MAO-B by [^{3}H]FNPA upon photoirradiation. A 2-ml mixture containing 0.32 mg MAO-B and 0.04 nmole [^{3}H]FNPA (48 Ci/mmole) was photoirradiated for 1 min at 5°. The 0.2 ml of 10% SDS was added and boiled for 3 min. This solution was then applied to a Sephadex G-25 column (1.5 × 22 cm) equilibrated with 1% SDS. Eluent (100 μl) from each tube (0.9 ml) was taken to count for radioactivity. The radioactivity separation profile is shown as (●—●). The radioactivity elution pattern of the nonirradiated mixture of MAO-B and [^{3}H]FNPA is shown as (▲—▲). The procedure was the same as described above, except that the enzyme–FNPA mixture was not photolyzed. The protein separation profile is the same for both samples, shown as absorbance at 280 nm (○—○).

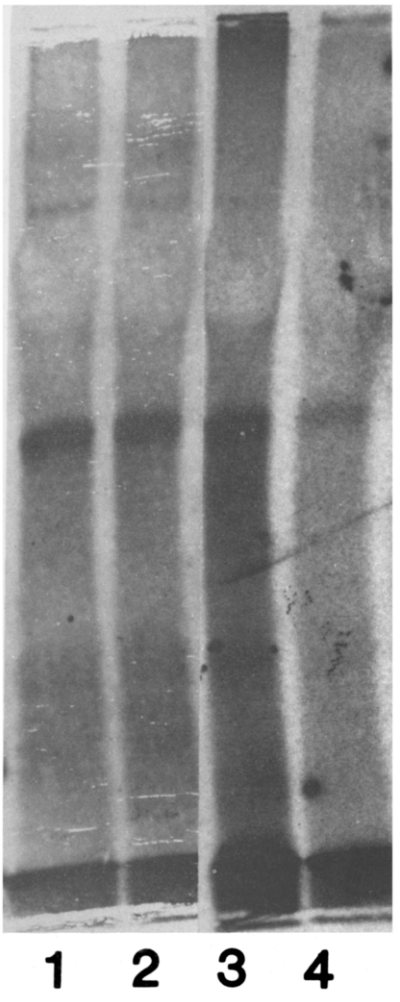

Fig. 5. SDS-polyacrylamide gel electrophoresis of the purified beef MAO-B following irradiation with [^{3}H]FNPA. The labeled enzymes were analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [16] with a 4% stacking gel and 8% separation gel. Proteins were stained with Coomassie brilliant blue. The stained gel was treated with NEN autoradiography enhancer for 1 hr and then washed with cold water for 1 hr. The gel was exposed to Kodak X-ray film for 1 week at −70°. Lane 1: Sixty-five micrograms of beef liver MAO-B which was photoirradiated for 2 min at 4° with 0.02 μM [^{3}H]FNPA. Lane 2: Sixty-five micrograms of enzyme which was photoirradiated for 2 min at 4° with 0.02 μM [^{3}H]FNPA and 200 μM PEA. Lane 3: Autoradiogram of lane 1 gel. Lane 4: Autoradiogram of lane 2 gel.

degree of nonspecific labeling of MAO-B by FNPA because 100% protection of [^{3}H]FNPA labeling was not demonstrated in this experiment. It is not unusual to find nonspecific labeling for photolabeling experiments. The important point of this protection experiment is that PEA did protect FNPA labeling to a significant degree which indicates that FNPA does label the active site of MAO. This result agrees with our earlier results that FNPA competes with substrate binding (competitive inhibition) in the dark (Fig. 1) [12, 17] and PEA protects the photo-dependent inhibition of MAO-B by FNPA [12].

[^{3}H]*FNPA-labeling sites on the purified beef liver MAO-B.* The molecular nature of the FNPA-labeling site(s) on MAO-B was investigated in the following manner. The FAD binding site was identified in a labeling experiment using [^{3}H]pargyline—a suicide substrate of MAO. Sephadex G-25 gel filtration of complete tryptic and chymotryptic digests of [^{3}H]pargyline-labeled MAO-B revealed that the [^{3}H]pargyline-labeled FAD peptide was eluted as a major peak between fractions 60 and 80 (Fig. 6A). There was also a minor peak in fractions 34–49 which may represent an incomplete digest of the labeled enzyme. With the same digestion and separation procedures, three major radioactive peaks were found for the [^{3}H]FNPA-photolabeled MAO-B (Fig. 6B). They are peak I (void volume, fractions 34–49), peak II (fractions 51–60), and peak III (fractions 80–92) (Fig. 6B). This result is not surprising because photoaffinity labeling is three-dimensional and may not be specific for one type of amino acid residue. Therefore, it is possible that FNPA labels several positions in the active site upon irradiation. Also, some of those may be due to nonspecific labeling as we have discussed above. The different radioactivity distribution patterns for tryptic and chymotryptic digests of [^{3}H]FNPA and [^{3}H]pargyline-labeled MAO-B suggests that FNPA-labeling sites may be different from the FAD-binding site. This conclusion is further supported by our recent work in which we have applied an equal amount of tryptic and chymotryptic digest of [^{3}H]pargyline-labeled and [^{3}H]FNPA-labeled MAO-B on the Sephadex column. The separation profile is identical to combined Fig. 6A and 6B (data not shown). However, at the present time, we cannot rule out the possibility that the site of the proteolytic digestion may have been altered when the enzyme was labeled by these compounds. Figure 6B shows that [^{3}H]FNPA labeling could be protected by PEA. Three hundred micromolar PEA was able to protect 36, 67, and 76% of [^{3}H]FNPA peaks, I, II, and III respectively (Fig. 6B). Furthermore, the protection by PEA was concentration dependent (data not shown). Since the resolution of Sephadex G-25 is limited, it is expected that there may be several peptides in each radioactive peak. Some of the peptides may be nonspecifically labeled by [^{3}H]FNPA. Those labeling could not be protected by PEA. Among the three radiolabeled peaks, the percentage of PEA protection of [^{3}H]FNPA incorporation was lowest in peak I. Since this peak is eluted in the void volume of the column, consequently it is more heterogenous than the other peaks.

DISCUSSION

In this study we investigated the interaction of FNPA with a purified beef liver MAO-B. Our data indicate that FNPA was a competitive inhibitor of purified MAO-B in the dark (Fig. 1). Upon irradiation, a concentration-dependent photoinactivation of purified MAO-B was observed when

Table 2. Phenylethylamine protection of the radioactive labeling of the purified beef liver MAO-B by [^{3}H]FNPA

Phenylethylamine concentration (μM)	Bound radioactivity (cpm/1.4 mg protein)	Percent of [^{3}H]FNPA binding
0	4510	100
10	4063	90
20	3806	84
30	3493	77
50	2870	64
100	2445	54

A mixture of 0.5 ml containing 50 μg MAO, [^{3}H]FNPA (20 nM) (6.7 Ci/mmole) and PEA at concentrations as indicated was irradiated for 2 min at 5°. The bound radioactivity was evaluated by syringe column centrifugation [17]. In this procedure a 1-cm tubular syringe, with the plunger removed, was filled to the 1-ml mark with a suspension of Sephadex G-25 pre-equilibrated with 1% SDS. The Sephadex column was supported within the syringe barrel by a small plug of glass wool. The complete syringe column supported within a centrifuge tube holder was first centrifuged for 3 min at 600 rpm with an IEC HN-SII centrifuge. After this initial centrifugation, the enzyme or enzyme–FNPA mixture was denatured by heating in a boiling water bath for 3 min in the presence of 1% SDS, and then applied to the top of the Sephadex column. The syringe column was subjected to a second centrifugation as described above. Radioactivity was determined on an aliquot of the eluate following centrifugation. Protein was determined according to the method described by Lowry *et al.* [18].

either PEA (Fig. 2) or 5-HT was used as a substrate (Table 1). Salach *et al.* [19] have shown that beef liver MAO catalyzes both 5-HT and PEA deamination. However, both deamination reactions were sensitive to deprenyl, a selective inhibitor of MAO-B, and insensitive to clorgyline, a selective inhibitor of MAO-A. Therefore, they concluded that there is only MAO-B in the beef liver. A similar finding was observed in our laboratory. We found that, in beef liver homogenate, 5 μM deprenyl inhibited both 5-HT and PEA deamination 98%, whereas 5 μM clorgyline inhibited only 13% of both deamination reactions (data not shown). The substrate specificity of MAO in beef liver is different from that in rat brain. In rat brain, PEA deamination is catalyzed by MAO-B, whereas 5-HT deamination is catalyzed by MAO-A. Hence, MAO-A and -B are defined by inhibitor sensitivity rather than substrate specificity [19]. The FNPA-photodependent inhibition of both PEA and 5-HT deamination observed in beef liver homogenate agrees with our previous finding that FNPA is more sensitive in photoinactivating MAO-B than MAO-A [12].

Moreover, we used [^{3}H]FNPA to demonstrate a photoinduced binding of FNPA to purified MAO-B (Figs. 4 and 5). This binding could be protected by the presence of PEA during photoirradiation in a concentration-dependent manner (Table 2). This result together with results from kinetic studies strongly suggest that FNPA may be incorporated into the active site of purified MAO-B.

Our recent structure–activity study of FNPA inhibition of MAO-B reveals that the photoactive group, the azido group of FNPA, is essential for its inhibitory effect [17]. This finding further suggests that the [^{3}H]FNPA is incorporated at the active site of MAO-B upon irradiation. To determine whether FNPA labels at the FAD prosthetic group or at a different region of the active site, the following experiments were performed.

The complete tryptic–chymotryptic digestion of [^{3}H]FNPA-labeled beef liver MAO-B produced several [^{3}H]FNPA-labeled peptides. The elution volume of these [^{3}H]FNPA-labeled peptides on the Sephadex G-25 column was different from that of the [^{3}H]pargyline-labeled peptide (Fig. 6A and 6B). [^{3}H]Pargyline is known to bind at FAD of both MAO-A and MAO-B. Since the molecular weight of FNPA (182.1) is similar to that of pargyline (159.2), the difference in their molecular weights cannot account for the difference in the elution volumes of the [^{3}H]pargyline-labeled and the [^{3}H]FNPA-labeled peptides. Therefore, the present data suggest that FNPA-labeling sites may be different from the pargyline-labeled FAD binding site. This conclusion is supported by our recent finding that the flavin spectrum remained unchanged after purified MAO-B was labeled by FNPA (data not shown). Nevertheless, the amino acid sequences of the FNPA-labeled peptides are needed to unequivocally prove this point. This work is currently being carried out in our laboratory.

The active site of MAO has been suggested to be composed of two segments [20]. One segment bears the FAD prosthetic group, and the other segment comprises the amino acid residues of the substrate binding site. The amino acid sequence analysis of the FAD peptide [20–23], isolated after complete proteolytic digestion of purified beef liver MAO-B and human placenta MAO-A, has revealed that the FAD is linked through the cysteine residue to the same penta peptide (Ser-Gly-Gly-Cys-Tyr) for both MAO-A and MAO-B. These results indicate that the substrate and inhibitor selectivity between MAO-A and B may be attributed to the differences in the region(s) other than the FAD binding site; most

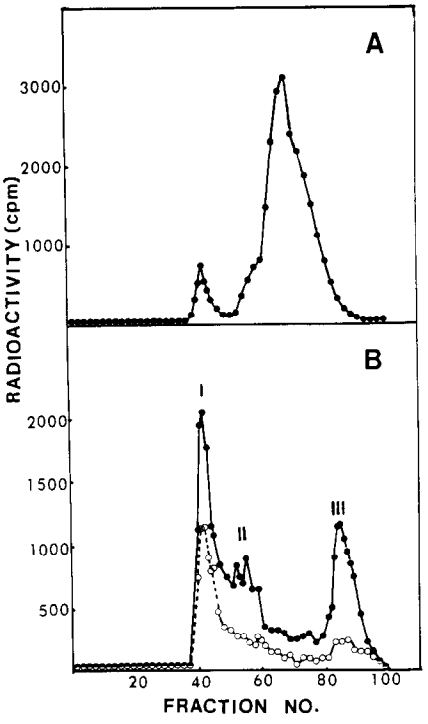

Fig. 6. Separation of tryptic and chymotryptic peptides of beef liver MAO by gel filtration chromatography. (A) One hundred and thirty micrograms of purified beef liver MAO-B in 1 ml of 50 mM phosphate buffer, pH 7.4, was incubated at 37° for 1 hr in the presence of 0.3 μM [^{3}H]pargyline (0.34 Ci/mmole). The treated enzyme was digested with trypsin and chymotrypsin as described in Materials and Methods. Peptides were separated on a Sephadex G-25 column (2 × 40 cm) equilibrated with 1% SDS (●—●). Fractions of 0.9 ml were collected and their radioactivity was determined. (B) One hundred and thirty micrograms of purified beef liver MAO in 1 ml of 50 mM phosphate buffer, pH 7.4, was photoirradiated in the presence of 0.02 μM [^{3}H]FNPA (3.1 Ci/mmole) for 2 min. The labeled enzyme was proteolyzed and applied to the same column (●—●). A second sample contained 130 μg enzyme and 0.02 μM [^{3}H]FNPA was photolyzed in the presence of 300 μM PEA. This sample was digested and applied to the column in the same manner (○—○). This experiment was repeated for more than ten times.

likely there are differences in the substrate binding site. The present study indicates that FNPA labels at regions in the active site of MAO-B which are different from the FAD binding region. Consequently, FNPA may label MAO-B at the substrate binding site. Thus, this compound may be a useful tool to characterize the substrate binding site of MAO-B.

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