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Investigations of the Possible Glycosylation of Monoamine Oxidase B from Pig Leucocytes

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Abstract—Monoamine oxidase B (MAO B) from pig liver has been reported to be a sialoglycoprotein. However, when that enzyme from pig lymphocytes and granulocytes was separated by polyacrylamide gel electrophoresis after labelling with the specific irreversible inhibitor [^{3}H]pargyline, staining with 1-ethyl-2-[3-(1-ethyl-naphtho [1,2d] thiazolin-2-ylidene)-2-methylpropenyl] naphtho [1,2d] thiazolium bromide ("Stains-all") failed to detect the presence of sialic acid residues. Treatment of the enzyme in disrupted lymphocytes and granulocytes, or in mitochondrial fractions prepared from them, with neuraminidase resulted in a decrease in MAO activity. However, after the enzyme was rendered soluble by treatment with octylglucoside, treatment with neuraminidase had no effect on the activity. These results indicate that sialic acid residues are not an intrinsic component of MAO B, although associated material containing such groups appears to affect the activity of the membrane-bound enzyme. The activities of membrane-bound preparations of MAO B from pig lymphocytes and granulocytes were unaffected by treatment with trypsin or β-chymotrypsin. After the preparations had been rendered soluble by treatment with octylglucoside there was a decrease in the activity on treatment with β-chymotrypsin, but trypsin treatment had no effect. Thus solubilization resulted in residues sensitive to cleavage by the former enzyme becoming accessible to it. Tryptic and chymotryptic peptides separated from the sodium dodecyl sulphate denatured enzymes by polyacrylamide gel electrophoresis revealed no differences between MAO B prepared from lymphocytes and granulocytes

Monoamine oxidase (MAO) [amine:oxygen oxidoreductase (deaminating)(flavin-containing) EC.1.4.3.4.] is tightly bound to the mitochondrial-outer-membrane and exists as two forms, A and B, which differ in their substrate specificities and inhibitor sensitivities (for review see Tipton 1986). The A form is sensitive to inhibition by low concentrations of clorgyline and is relatively insensitive to inhibition by (−)-selegiline ((−)-deprenyl). It is active towards 5-hydroxytryptamine (5-HT) and has little or no activity towards benzylamine and low concentrations of 2-phenylethylamine (PEA). The B form of the enzyme is active towards these latter two substrates, has little activity towards 5-HT and is sensitive to inhibition by (−)-selegiline but only inhibited by high concentrations of clorgyline. Recent studies involving recombinant DNA have indicated the two enzyme forms to be separate gene products (Bach et al 1988; Hsu et al 1988).

Several lines of evidence have suggested that both MAO isoenzymes may be sialoglycoproteins and that glycosylation may modulate their activities. Oreland (1971) reported the presence of hexosamine and sialic acid in highly purified preparations of MAO B from pig liver. Both forms of MAO in membrane-bound preparations from rat liver bind strongly to concanavalin-A-Sepharose (Tipton & Della Corte 1979) which would be consistent with their being glycoproteins. The observation that the activity of MAO A decreased when membrane-bound preparations of rat liver enzyme were treated with neuraminidase was also interpreted as indicating a role of glycosylation in maintaining the activity of the enzyme (Houslay & Marchmont 1980).

Investigations of the amino acid sequences of the MAO isoenzymes predicted from the DNA sequences have indicated the presence of potential glycosylation sites in both forms (Bach et al 1988). Such studies cannot, of course, reveal whether or not an enzyme is glycosylated after transcription. Neither do the observations that nucleic acid preparations representing the coding sequences of each of the forms may be expressed in mammalian cells as active enzymes that retain the characteristics of MAO A and B activities (Lan et al 1989) resolve the question of post-transcriptional glycosylation.

Clearly if MAO were a glycoprotein and the glycosylation modulated its activity in some way, as suggested by the results described above, a full understanding of its behaviour would have to take this phenomenon into account. The possibility that in-vivo changes in glycosylation might be important in modulating its activity would also have to be considered. Since the only direct analytical data that indicated MAO to be a glycoprotein (Oreland 1971) concern the B form of the enzyme from pig liver, we have investigated the possible glycosylation of the enzyme from pig lymphocytes and granulocytes which have been shown to contain only that form (Balsa et al 1987). In this respect they resemble the enzyme from human leukocytes (Balsa et al 1989). Although the activity of MAO B in human blood platelets has been studied in detail as a marker for mental state or susceptibility to certain mental disorders (for review see Fowler et al 1982), several authors have suggested that the activities associated with leukocytes may be more appropriate in such respects (Baker et al 1977; Bourne 1974; Conolly & Greenacre 1977; Earp et al 1978). In addition to studies on the glycosylation of this enzyme, we have also investigated their sensitivities to proteolytic digestion and used peptide-mapping after limited proteolysis to investigate whether there are any differences in primary structure between the enzymes from these two leukocyte sources.

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Materials and Methods

Methods

Granulocytes and lymphocytes were separated from pig blood by a modification (Balsa et al 1987) of the method of Pegueroles et al (1978). They were resuspended at a concentration of 150–200 × 10^6 cells mL^{-1} ($\simeq$7–9 mg protein mL^{-1}) in 50 mM potassium phosphate buffer, pH 7·2. Cells were disrupted by freezing and sonication at low frequency for 30 s on ice. Mitochondria were obtained by the method of Kohno & Kanno (1985).

Monoamine oxidase activity was determined radiochemically at 37°C by a modification (Fowler & Tipton 1980) of the method of Otsuka & Kobayashi (1964) using 20 μM [^{14}C]2-phenylethylamine (PEA) (2·5 mCi $mmol^{-1}$) as substrate in a final volume of 225 μL of 50 mM potassium phosphate buffer, pH 7·2. Each assay contained 6–8 × 10^6 cells.

Solubilized MAO fractions were obtained using the conditions described by Fritz & Abell (1982), using 0·75% β-octylglucoside followed by dialysis and centrifugation. The final pellets were resuspended in the appropriate volume of 50 mM phosphate buffer, pH 6·2, in order to determine the effect of neuraminidase, trypsin and β-chymotrypsin.

Cellular, mitochondrial and solubilized samples of MAO were digested by addition of a protease solution at a ratio of proteolytic enzyme to protein of 1:5 in the case of trypsin and of 1:1 in the case of β-chymotrypsin. The protease reactions were terminated by the addition of soybean trypsin inhibitor to give a 1:1 weight ratio with respect to the amount of protease present.

Cellular, mitochondrial and solubilized fractions were treated with purified neuraminidase as described by Houslay & Marchmont (1980). Neuraminidase activity was assayed by incubating samples (200 μL) in 50 mM potassium phosphate buffer, pH 6·2, containing 1·5 mg mL^{-1} of neuraminidase and 500 μM *N*-acetylneuramin lactose (sialyllactose) as substrate, at 37°C for 30 min. Samples were then chilled and assayed for the sialic acid by a fluorimetric method (Hammond & Papermaster 1976).

MAO was labelled with [^{3}H]pargyline (13 Ci $mmol^{-1}$), and separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12·5% polyacrylamide)) as previously described (Balsa et al 1987). The precise position of MAO in the preparative gel was determined by fluorography. The bands corresponding to MAO were cut into slices, equilibrated, as described by Cabral et al (1978) and loaded into a second 15% polyacrylamide proteolytic gel. Limited proteolysis and peptide mapping were performed as described by Cleveland et al (1977). Other samples were subjected to SDS-PAGE on 15% polyacrylamide gels and stained for sialic acid as described below.

Staining with the cationic carbocyanine dye "Stains-all" was as described by Campbell et al (1983) with the following modifications. Gels were fixed for 30 min with methanol–acetic acid–water (40:20:40) (v:v:v) and washed exhaustively in isopropanol (25%) at 56°C to remove SDS. The gels were then stained overnight in the dark with 200 mL of "Stains-all" (0·0025%), isopropanol (25%, v/v), formamide (7·5%, v/v) and 30 mM Tris-HCl, pH 8·8. Gels could be stained with Coomassie Blue following "Stains-all" staining if they previously were completely blanched in isopropanol (25% v/v).

Materials

Phenylethylamine hydrochloride-(ethyl-1-^{14}C) ([^{14}C]PEA) and [^{3}H]pargyline-(phenyl-3, benzyl-^{3}H) hydrochloride were obtained from New England Nuclear (Boston, MA). "Stains-all" (1-ethyl-2-[3-(1-ethyl-naphtho[1,2d] thiazolin-2-ylidene)-2-methylpropenyl] naphtho [1,2d] thiazolium bromide) was purchased from Eastman Kodak (Germany). Trypsin (Type XIII from bovine pancreas, TLCK-treated, 10000–13000 benzyl-L-argine ethyl ester units mg^{-1}), β-chymotrypsin (Type VII from bovine pancreas, TLCK-treated, 40–60 benzyl-L-tyrosine ethyl ester units mg^{-1}), trypsin inhibitor (Type II from soybean), neuraminidase (Type VI from *Clostridium perfringens*), *N*-acetylneuramin-lactose (sialyllactose) and β-octylglucoside were obtained from Sigma. All other compounds were standard, analytical-grade, laboratory reagents.

Results

The MAO B activity present in disrupted granulocytes and lymphocytes was not significantly affected by the action of trypsin and β-chymotrypsin (Fig. 1). The activities remaining after 0 and 60 min of incubation with trypsin, were 100% ± 2·1 and 95% ± 3·6 of that of untreated samples (mean ± s.e.m. of four experiments), respectively, in the case of granulocytes, and 100% ± 1·8 and 91% ± 6·3, respectively, in the case of lymphocytes. The activities remaining after 0 and 60 min of β-chymotrypsin treatment were 103% ± 3·5 and 94% ± 5·6, respectively, in the granulocyte fraction, and 100% ± 4·4 and 87% ± 8·7, respectively, in the lymphocyte fraction. Although the protease assays were performed at a high ratio of protease to protein (1:5), there is the possibility that, in the disrupted cell preparations, the presence of so many other proteins might compete with the trypsin and β-chymotrypsin, thus effectively reducing the rate of any proteolysis of MAO B. However, when mitochondria were isolated and digested by these proteases, identical results to those obtained with the whole cell samples were obtained (data not shown). After the enzyme preparations had been solubilized by treatment with β-octylglucoside (Fritz & Abell 1982), digestion with trypsin still failed to cause any significant loss of activity (Fig. 1), the activities of the lymphocyte and granulocyte enzymes being 96·9% ± 6 and 99·4% ± 5, respectively, of those of the untreated samples after 60 min incubation with trypsin. In contrast, digestion with β-chymotrypsin resulted in a 67·6% ± 4 and a 49·7% ± 1 reduction of the activities of the lymphocyte and granulocyte preparations, respectively, after 60 min incubation under the conditions described earlier (Fig. 1).

To investigate possible structural differences of MAO B forms present in granulocytes and lymphocytes, [^{3}H]pargyline-labelled subunits of MAO were prepared (Balsa et al 1987), and the electrophoretic patterns of the products resulting from their partial proteolytic digestion were compared. Incubation of equivalent concentrations of the radiolabelled polypeptide with increasing amounts of trypsin resulted in several fragments (Fig. 2A). The largest peptide bands corresponded to the undigested flavin-containing subunit of MAO, and this diminished in intensity as the amount of trypsin was increased. Nevertheless, it can be seen that the same peptide fragments were present along the

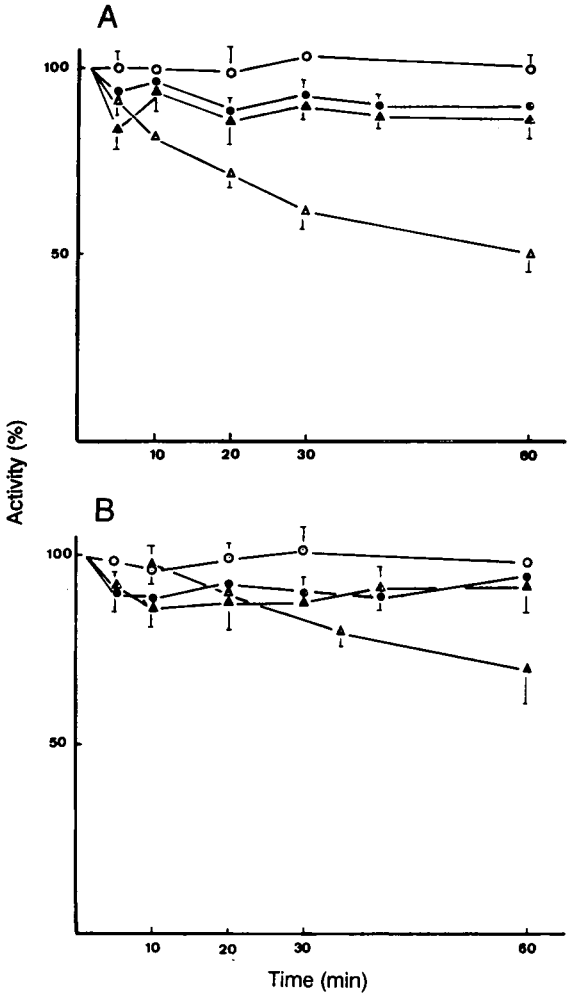

FIG. 1. Digestion of granulocyte and lymphocyte MAO with trypsin and β-chymotrypsin. (A) Whole granulocyte (●, ○) and preparations that had been solubilized by treatment with β-octylglucoside (▲, △) were digested with trypsin (●, ▲) at a proteinase: protein ratio of 1:5, and with β-chymotrypsin (○, △) at a proteinase: protein ratio of 1:1. The digestion was stopped by addition of soybean trypsin inhibitor at a ratio of proteinase:inhibitor 1:1. The activity remaining was determined using [^{14}C]PEA (20 μM) as substrate. Each value is the mean ± range of two experiments. (B) whole lymphocyte (●, ○) and solubilized (▲, △) preparations were digested with trypsin (●, ▲) and β-chymotrypsin (○, △) as described in legend (A).

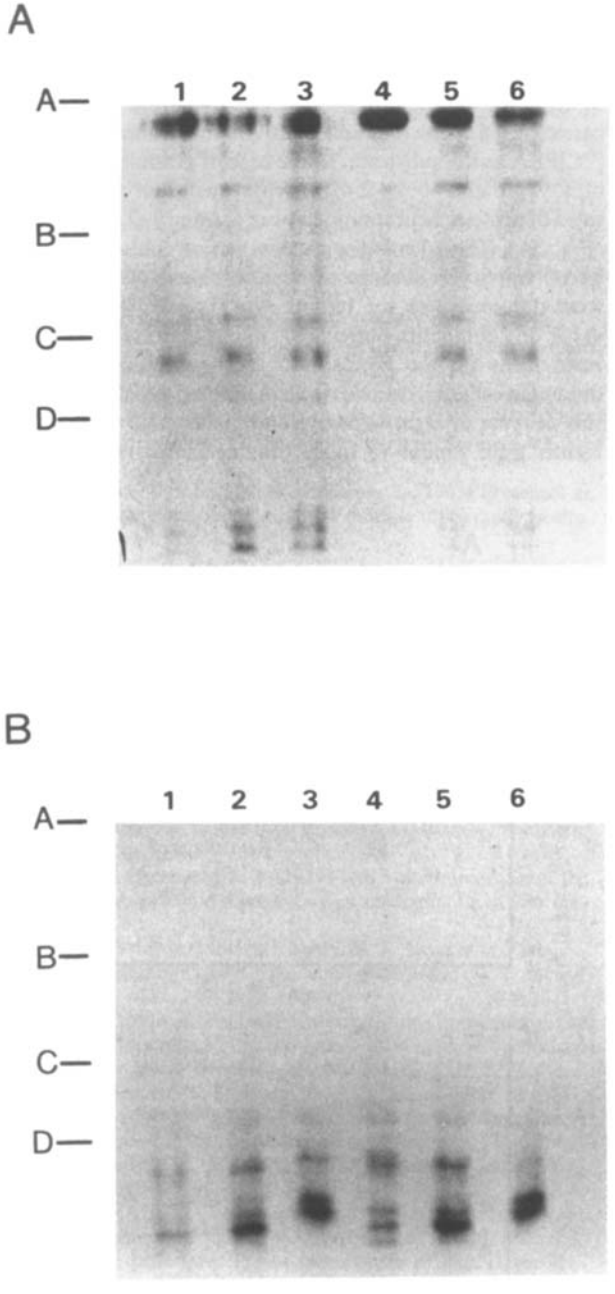

FIG. 2. Study of digestion of granulocyte (G) and lymphocyte (L) MAO from pig blood by peptide mapping. (A) [^{3}H]Pargyline labelled polypeptides from a 12·5% polyacrylamide gel were digested in a second SDS-gel (15% polyacrylamide) with 2 μg/well (track 1), 10 μg/well (track 2) and 20 μg/well (track 3) of trypsin in the case of granulocytes and tracks 4, 5, and 6 were used in the case of lymphocytes. Peptides fragments were then separated by electrophoresis and the peptide bands were detected by autoradiography. Molecular weights were determined by comparison with the mobility of standard proteins: bovine serum albumin (66000) (A), ovalbumin (45000) (B), carbonic anhydrase (31000) (C), trypsin inhibitor (21000) (D) and lysozyme (14400) (not shown). The positions were determined by protein staining of the gel prior to autoradiography. (B) [^{3}H]Pargyline labelled polypeptides were digested with 5 μg/well (tracks 1 and 4), 20 μg/well (tracks 2 and 5) and 100 μg/well (tracks 3 and 6) of β-chymotrypsin for granulocytes and lymphocytes, respectively. Peptide fragments were then separated by SDS-PAGE and peptide bands were detected by autoradiography. The positions a-d are the locations of the molecular weight markers as indicated in the legend to Fig. 1A.

whole time-course of the experiment. Nine peptide fragments were resolved, and their apparent relative molecular masses (M_r) were approximately 63000, 58000, 54000, 33000, 29000, 24000, 17000, 16000 and 15000. There were no detectable differences between the fragments given by the enzymes from lymphocytes and granulocytes.

When MAO from the same source was partially digested with different concentrations of β-chymotrypsin, five peptide fragments (approximate M_r values: 24200, 21000, 18000, 17500, 16400) were obtained (Fig. 2B). As in the case of trypsin digestion, the intensity of the larger peptide bands diminished as the protease concentration was increased. The same peptide fragments were obtained with MAO B from each cellular type.

Neuraminidase specifically removes sialic acid residues

from glycoproteins (see Houslay & Marchmont 1980). To investigate the possible presence of carbohydrate residues attached to MAO B from pig blood leukocytes, incubations of the crude samples, in the presence of neuraminidase, were carried out and the remaining activities were measured using [^{14}C]PEA as the substrate. In the case of granulocytes, a loss of 30% ± 6 and 40% ± 2 of activity, was observed when the ratio of protein : neuraminidase was 1 : 1 and 1 : 2, respectively (Fig. 3A). The 30 min incubation period had no effect on MAO activity in absence of neuraminidase. Similar results were obtained with the lymphocyte fraction (Fig. 3B). To avoid any competitive effects of other substrates or possible restrictions on the accessibility of neuraminidase in the disrupted cells, mitochondria were isolated and digested with this enzyme at a protein : neuraminidase ratio of 1 : 1. The results were similar to those obtained with the disrupted cellular preparations (data not shown). These results suggest that sialic acid residues may modulate the catalytic activity of MAO from granulocytes and lymphocytes. Nevertheless, it is not possible to be sure whether sialic acid is associated with the protein itself or to other material that affects its catalytic function within its membrane-bound environment.

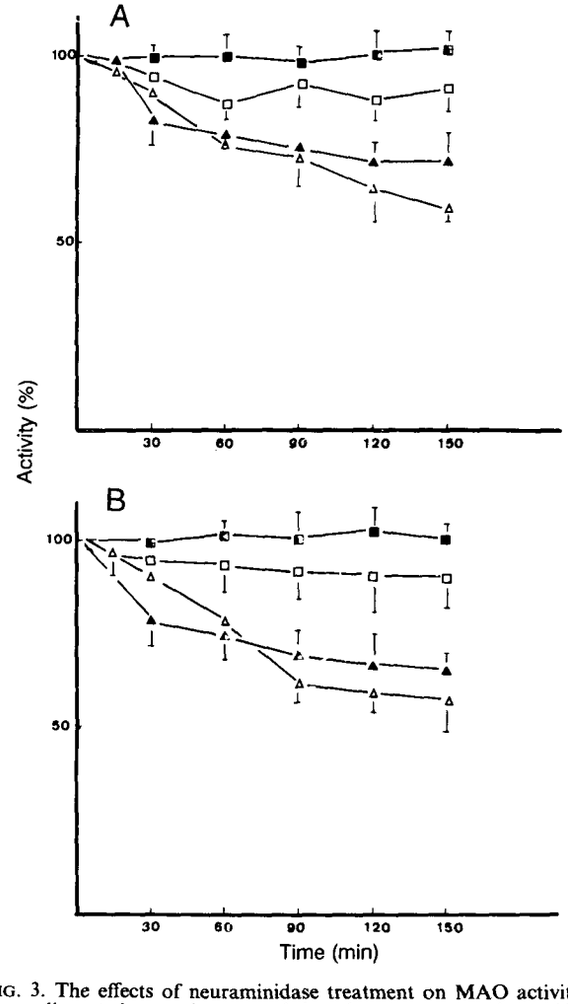

FIG. 3. The effects of neuraminidase treatment on MAO activity from disrupted granulocyte and lymphocyte preparations. (A) Granulocytes (■, □) and solubilized granulocytes (▲, △) were treated with neuraminidase at either 1 : 1 protein : neuraminidase ratio (■, ▲), or a 1 : 2 protein : neuraminidase ratio (□, △) at 37°C in 50 mM phosphate buffer, pH 6·2, and assayed with [^{14}C]PEA (20 μM) as substrate at pH 7·2. Each value is of four denominations. (B) Disrupted (■, □) and solubilized (▲, △) lymphocytes were treated with neuraminidase at either a 1 : 1 protein : neuraminidase ratio (■, ▲) or a 1 : 2 protein : neuraminidase ratio (□, △) and assayed with [^{14}C]PEA (20 μM) as substrate. Each value is the mean ± s.e.m. of four separate determinations.

Solubilization of MAO B with 0·75% β-octylglucoside resulted in a 9-fold increase of the specific activity of the enzyme when [^{14}C]PEA was used as substrate. The effects of neuraminidase on the solubilized enzyme were studied, at protein : neuraminidase ratios of 1 : 1 and 1 : 2 (Fig. 3). No significant loss of activity was observed with preparations from either cell type; the activity remaining being 95% ± 2·3 of the untreated control after incubation at a protein : neuraminidase ratio of 1 : 2 for 150 min. To test whether residual detergent present in solubilized enzyme samples inhibited neuraminidase, a 0·75% solution of β-octylglucoside was dialysed overnight, and then incubated with neuraminidase in the presence of sialyllactose and the liberated sialic acid was determined. The results showed no significant effect of residual β-octylglucoside on the neuraminidase activity (data not shown).

After polyacrylamide gel electrophoresis in the presence of SDS, the gels were stained with the cationic carbocyanine dye, "Stains-all'. This specifically stains sialoglycoproteins and phosphoproteins blue whereas all other proteins are stained in red (Campbell et al 1983). Bovine serum albumin and peroxidase, which contain neutral sugars, and ribonuclease D which is a sialoglycoprotein, were used as markers at concentrations that were the same as that of the MAO present. The first two proteins stained red whereas ribonuclease D stained blue. The band corresponding to the [^{3}H]-radiolabelled FAD-containing polypeptide of MAO from both cellular sources stained red, indicating that this subunit does not contain appreciable amounts of sialic acid or bound phosphate residues. Several bands with lower M_r values were found to stain blue (data not shown). The concentration of material applied to the electrophoresis gels was such as to give 20–30 pmol MAO B in each radioactively-labelled band, as determined by [^{3}H]pargyline binding (Gomez et al 1986). The "Stains-all" procedure should have been capable of detecting any significant presence of sialic acid in the enzyme since the reported sensitivity of that reagent is as low as 1 pmol sialic acid.

Discussion

MAO B activity from both granulocytes and lymphocytes, in whole cells as well as in mitochondria, was resistant to proteolytic digestion by trypsin and β-chymotrypsin, in agreement with the results reported for the enzyme from other sources (Oreland & Ekstedt 1972; Baker & Hemsworth 1978; Buckman et al 1983; White & Stine 1984). Even after the enzymes from the leukocytes had been solubilized by treatment with β-octylglucoside (Fritz & Abell 1982) their activities were not affected by digestion with trypsin. Thus the resistance of the enzyme to the effects of trypsin do not merely result from a protective effect of its membrane-bound environment. In contrast, solubilization rendered the preparations sensitive to digestion with β-chymotrypsin, indicating that process to render susceptible groups accessible to

the action of that proteinase. However, specifically labelling the active-site flavin with the mechanism-based irreversible inhibitor, [^{3}H]pargyline (see Gomez et al 1986), made it possible to obtain peptide maps by digestion with trypsin and β-chymotrypsin under denaturing conditions. Peptide mapping showed the presence of basic and aromatic residues in the MAO polypeptide from both fractions (Fig. 2) but revealed no differences between them. Although the relatively small number of flavin-containing peptides obtained limits the discrimination of this approach, the results obtained provide no evidence for any differences in the primary stuctures of MAO B from these two sources. Since glycosylation often affects the behaviour of proteins on SDS-PAGE these results might also indicate that the enzymes from these two cellular sources do not differ markedly in their degrees of glycosylation.

Some authors have suggested that MAO might be a glycoprotein (Oreland 1971; Tipton & Della Corte 1979; Houslay & Marchmont 1980). Houslay & Marchmont (1980) suggested that sialic acid residues might be associated with glycolipid material, which modulates the enzyme activity in some way, rather than in the enzyme itself. However, the reported presence of sialic acid and hexosamine residues in purified preparations of MAO B from pig liver (Oreland 1971) suggest the enzyme itself to be a sialoglycoprotein. Our results (Fig. 3), demonstrated that the MAO B activity present in granulocytes and lymphocytes is reduced by cleavage of sialic acid residues by neuraminidase. However, these results do not show whether these residues are attached to the protein itself or to associated material that affects its activity. The observation that the activities of the solubilized preparations were not affected by neuraminidase treatment would be consistent with the latter of these two possibilities.

Staining with "Stains-all" also indicated that the flavin-containing polypeptide of MAO B is not a sialoglycoprotein. Thus, the sialic acid could be attached to material, possibly a glycolipid as suggested by Houslay & Marchmont (1980), that affects the activity of the membrane bound enzyme. The possible modulatory role of sialic acid-containing material may simply be one of surface charge affecting the local concentration of substrates in the region of the MAO-active site (see Wojtczak & Nalecz 1979). Until recently, it was believed that MAO was a dimer in which only one of the two subunits contained bound flavine-adenine dinucleotide (FAD) (Collins & Youdim 1975; Russell et al 1979; Oreland et al 1973), and thus the non-flavin-containing subunit might have been regarded as a possible site for glycosylation. However, Weyler (1989) has shown that all subunits of MAO B contain bound FAD. It may thus be concluded that MAO B from pig lymphocytes and granulocytes is not a sialoglycoprotein.

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