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PURIFICATION AND COMPARISON OF LIVER MICROSOMAL FLAVIN-CONTAINING MONOOXYGENASE FROM NORMAL AND STREPTOZOTOCIN-DIABETIC RATS

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Abstract—Liver microsomal flavin-containing monooxygenase (FMO) activity towards thiobenzamide is two-fold increased in streptozotocin diabetic (insulin deficient) rats and mice and, to a lesser degree in congenital insulin resistant Ob/Ob mice. No difference in thermal stability appears between microsomal FMOs from both normal and diabetic rats. FMO has been purified to homogeneity from these two sources, with a 50-fold increase of specific activity. Their apparent molecular weight is respectively 50,000 and 49,000 and a discrete modification appears in the HPLC profiles of tryptic peptides from purified FMOs. They appear immunochemically very similar and present in equal quantity in microsomal membranes from both normal and diabetic rats, so that the increased activity cannot be ascribed to an increased concentration of the enzyme protein.

Biotransformation of xenobiotics in liver of diabetic (insulin deficient) animals has been shown to be profoundly modified [1–4] and in some cases it has been possible to ascribe some of these modifications to variations of the isozymic pattern of cytochrome P-450 [5], the terminal oxidase of the mixed-function oxidase system (MFO). However, in spite of the existence of numerous isozymes, which exhibit different substrate specificity [6], the cytochrome P-450 system is not the only enzyme system involved in drug metabolism. Indeed the oxidation of many nitrogen- and sulfur-containing compounds involves another enzyme system: the flavin-containing monooxygenase (FMO, EC.1.14.13.8) [7]. In contrast with cytochrome P-450, the FMO is not inducible by exogenous substrates [8] and variations of FMO activity were observed only in modified physiological states: gonadectomy [9], pregnancy [10] and starvation [11]. The FMO activity seems also to undergo a diurnal variation [11]. In these last cases, a modification of the enzyme stability might explain the results [11]; in the first two cases it appears clearly that steroid hormones exert a regulatory role.

On the other hand we have previously reported that FMO significantly contributes to imipramine (antidepressant) metabolism via an important imipramine-*N*-oxide formation [12]). We have demonstrated the exclusive involvement of FMO in this metabolite formation and its strong increase under the diabetic state [12]. These previous data fit well with the peculiar feature of FMO to be only increased by endogenous stimuli and prompted us to study FMO activity in liver microsomes from both insulin-deficient (produced by one unique streptozotocin injection) and insulin-resistant (congenital Ob/Ob mice) diabetic animals, then to purify the enzyme. Here we report its activity and some of its properties.

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MATERIALS AND METHODS

Blue Sepharose CL 6B and 2′, 5′-ADP Sepharose 4B were obtained from Pharmacia Fine Chem. (Sweden). Reactive Red 120 agarose, Lubrol PX and streptozotocin were purchased from Sigma Chemical Co. (St Louis, MO). Thiobenzamide was obtained from Aldrich Chemical Co (Belgium). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were from Boehringer (Manheim, F.R.G.).

Male Sprague–Dawley rats (7 weeks old) (IFFA CREDO, France) were made diabetic by a single intrafemoral injection of streptozotocin (STZ) (70 mg/kg) freshly dissolved in saline solution pH 4.5. Normal and congenital (Ob/Ob) male mice of the C57BL/6 strain, 10 weeks old, were purchased from the "Centre d'Elevage du C.N.R.S.", Orléans, France. STZ-diabetic mice were obtained by a single intraperitonal injection (150 mg/kg). Glucosuric rats and mice (detected by means of glukotest strips) and corresponding control animals were killed two weeks later. Microsomal membranes were prepared as previously described [12].

Flavin-containing monooxygenase (FMO) activity was measured by monitoring the conversion of thiobenzamide to thiobenzamide-*S*-oxide at 370 nm [13]. Assays were conducted at 35° using an Acta MVI Beckman double beam spectrophotometer, in a final volume of 1.0 ml containing 0.1 M Tris pH 8.5, 0.1 mM EDTA, 1.0 mM thiobenzamide (added in 5 μl of acetonitrile), an NADPH regenerating system (0.15 mM $NADP^+$, 0.25 mM glucose-6-phosphate and 0.5 unit/ml glucose-6-phosphate dehydrogenase) and about 0.2 mg of microsomal protein. Purification of flavin containing monooxygenase was performed according to the procedure described by

Sabourin *et al.* [14] except that Emulgen 911 was substituted by Lubrol PX.

Protein concentration was determined by the method of Lowry *et al.* [15].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [16]. Gels were stained with Coomassie Blue R250 as described by Fairbanks *et al.* [17].

Tryptic digestion of each purified FMO (25–50 μg) was realized overnight at 37°, in 0.025 M ammonium carbonate pH 8.8, with 1 μg of TCPK treated trypsine (Sigma) dissolved in 1 mM HCl (Schroeder) [18]. Tryptic peptides were resolved by reverse phase high-performance liquid chromatography in an LC-6A Liquid Chromatograph (Shimadzu) equipped with a SPD-6AV spectrophotometer and a C-R3A integrator, using an Aquapore RP 300 column (24 × 0.4 cm) (7 μm dia. spherical beads) (Brownlee Labs). Column was equilibrated in 0.1% trifluoroacetic acid (TFA) (buffer A) and elution was performed by a linear gradient: 0–100% of Buffer B in 60 min (flow rate 1.5 ml/min) containing 99.9% acetonitrile plus 0.1% TFA. Eluent of the column was monitored at 214 nm.

Antibodies to normal rat liver FMO and to diabetic rat liver FMO were raised in New Zealand female rabbits as indicated by Williams *et al.* [19]. The immunoglobulin fraction was isolated from antisera by ammonium sulfate precipitation and DEAE sepharose chromatography [20]. Control immunoglobulins were isolated from non-immunized rabbit serum.

Ouchterlony double immunodiffusion was performed as previously reported [20].

Western blots of SDS-PAGE gels were performed as described by Guengerich *et al.* [21] and quantitation was performed by densitometry after immunostaining. Immunoinhibition of the thiobenzamide-*S*-oxidation was performed by preincubation at 4° for 10 min of microsomal membranes with different amounts of control or specific antibodies, in the presence of the NADPH-generating system.

RESULTS AND DISCUSSION

The liver microsomal FMO activity (tested with thiobenzamide as substrate) in both diabetic rats and mice is reported in Table 1. In STZ-diabetic (insulin deficient) rats, the basal activity is two-fold higher than in normal rats. In spite of a higher basal activity in liver of normal mice as compared with that in normal rats, the diabetic state produced by STZ in mice also leads to a two-fold increase of the FMO activity. In congenital diabetic (insulin-resistant) Ob/Ob mice, the FMO activity is also increased but at a lesser degree (50%). The residual activity after thermal inactivation of the FMO (2 min at 45° as recommended by Ziegler [7]) is not significantly different in normal and diabetic animals. In mouse liver microsomes the residual activity is about 50%, indicating an equal involvement of both FMO and MFO systems, in agreement with previous observations by Tynes and Hodgson [22]. The lower residual activity in rats does not appear significantly different from that in mice in Student's *t*-test analysis. On the other hand, we observe that the two types of diabetes (insulin-deficient produced by STZ and insulin-resistant in genetically Ob/Ob mice) do not similarly affect the activity of the FMO system: the increase of FMO activity is lesser in Ob/Ob mice than in STZ-mice. In a previous study [3] concerning the MFO system, we had also noted the influence of the type of diabetes on the extent of enzyme activity variations. Such an increase of enzyme activity, especially in STZ-diabetic animals, may result either from an increase of the amount of the enzyme in liver microsomes or from the presence of a different isozyme. In order to clarify the involved mechanism, we have chosen to purify the enzyme from both normal and diabetic rats.

After solubilization of microsomal membranes and three affinity chromatographies as described by Sabourin *et al.* [14] we have obtained, from both sources, a homogeneous protein as judged from denaturating polyacrylamide gel electrophoresis (Fig. 1). The flavin monooxygenases migrate very close to each other, with apparent molecular weights of 50,000 and 49,000 respectively for the enzyme from normal and diabetic rats. The respective specific activities are 139 and 261 nmol of thiobenzamide oxidized min^{-1} mg^{-1}. The purity is thus about 50-fold over that of the microsomal enzyme for both proteins, which is in the same order of magnitude as purification from pig liver (60-fold) reported by Sabourin *et al.* [14]. But it is much less than puri-

Table 1. Thiobenzamide-*S*-oxidation by liver microsomes from normal and diabetic rats and mice

	Basal activity nmol × min^{-1} × mg^{-1} microsomal proteins	Residual activity %
Normal rats	2.85 ± 0.56	34 ± 6
STZ-diabetic rats	5.86 ± 0.60	25 ± 7
Normal mice	8.85 ± 0.17	51 ± 4
STZ-diabetic mice	16.93 ± 0.44	43 ± 5
Ob/Ob diabetic mice	13.49 ± 0.61	46 ± 3

Results are the mean ± SEM of respectively six and four individual determinations for rats and mice. Thiobenzamide-*S*-oxidation was assayed either directly on liver microsomes or after a short preincubation (2 min) at 45° in buffer alone. Then tubes were immersed into an ice bath and completed for the assay. Residual activity is expressed in percentage of basal activity (without preincubation).

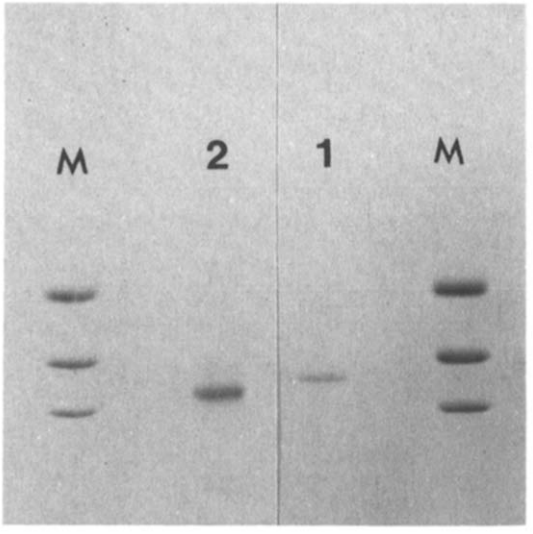

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified FMO from normal and STZ-diabetic rat livers. M: Molecular weight markers were: bovine serum albumin (67,000); yeast hexokinase (50,000) and rabbit muscle enolase (41,000). (1) 3 μg of FMO from normal rat; (2) 3 μg of FMO from diabetic rat.

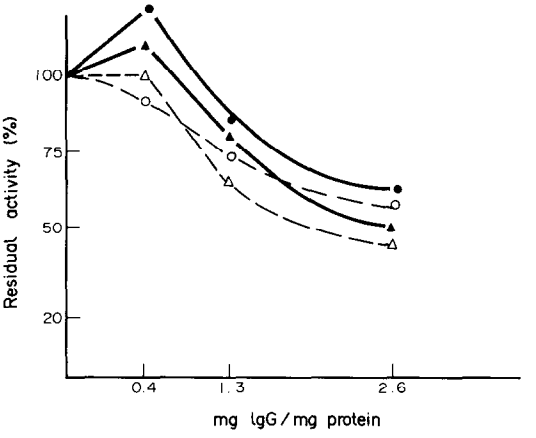

Fig. 2. Inhibition of thiobenzamide-*S*-oxidation in liver microsomes from normal (△ ▲) or diabetic (○ ●) rats by anti-FMO antibodies raised against either the FMO from normal rat (▲ ●) or the FMO from diabetic rat (△ ○). Each point was determined in duplicate; the residual activity is expressed as a percentage of activity assayed in presence of the same amount of control immunoglobulins.

fication from rat liver reported by Kimura *et al.* [23]. However, in this case, the comparison is difficult since the substrate used in the assay (dimethylaniline) is different; the accessibility to the active center of the enzyme by different substrates may not be identical.

Immunological properties of each purified FMO have been compared in Ouchterlony immunodiffusion, immunoquantitation and immunoinhibition of thiobenzamide-*S*-oxide activity experiments. Antibodies raised against each FMO cross-react with the homologous antigen but also with the other one, forming a continuous precipitin line between them (data not shown). Thus both enzymes share common epitopes. The absolute level of each enzyme in liver microsomal membranes has been measured by densitometry of stained bands after the specific recognition of the FMO by its homologous antibody in Western blots experiments. The protein band revealed by the antibody in microsomal membranes migrates as the pure antigen laid on the same electrophoretic gel, at different concentrations to allow quantitation in microsomal membranes. The amount of FMO in liver microsomes from normal and diabetic rats is respectively 1.04 ± 0.05, and 1.05 ± 0.17 nmol mg^{-1} microsomal protein (mean SEM of 3 individual determinations). Thus the enzyme protein is present in similar amounts in normal as in diabetic rats. Figure 2 shows the ability of both antibodies to inhibit oxidation of thiobenzamide in liver microsomes from both normal and diabetic rats. Both antibodies affect similarly the enzyme activity. The residual activity is about 45% and 60% in liver microsomes from respectively normal and diabetic rats; a higher immunoglobulin concentration (5.2 mg IgG/mg microsomal protein) does not lead to a further significant decrease of the activity (data not shown). This residual activity is slightly higher than that expected from results of the FMO heat inactivation reported in Table 1. This may be explained either by difficulties of immunoglobulin molecules to access to the enzyme located inside the microsomal membrane and/or by an overestimation of the FMO involvement in the thiobenzamide oxidation in thermal inactivation experiments since assumption is made (by us and others) that none of the isozymes of cytochrome P-450 are inactivated.

Structural comparison of the purified FMOs was achieved by means of their tryptic peptide profiles obtained by HPLC (Fig. 3). They appear very similar except in two places indicated by arrows. Considering the faint accuracy of molecular weight determination by electrophoretic migration in denaturing gels, the slight molecular weight difference between the purified FMOs may not be significant; nevertheless it is reproducible. So, this result may indicate a slight structural difference, as supported by analysis of the tryptic peptide profiles. Post-translational modifications such as phosphorylation and glycosylation might account for that. In fact it has been shown that phosphorylation modifies the electrophoretic migration of proteins [24] and that glycation (non-enzymatic glycosylation) of ε-amino group of lysine alters the trypsin cleavage [25]. Whatever the reason of the increased FMO activity in liver of diabetic animals, it appears that the two enzymes are structurally very close since they are immunochemically similar.

In conclusion, we have shown that the activity of the FMO system, as that of the MFO system, is well increased in liver microsomes of insulin deficient diabetic animals and to a lesser degree in that of insulin-resistant diabetic animals. This higher FMO activity might be due to (i) the presence of an endogenous effector [26], (ii) a post-translational activation, or (iii) a different conformation of the enzyme in the membrane due to changes in lipid composition in diabetic animals [27, 28]. On the

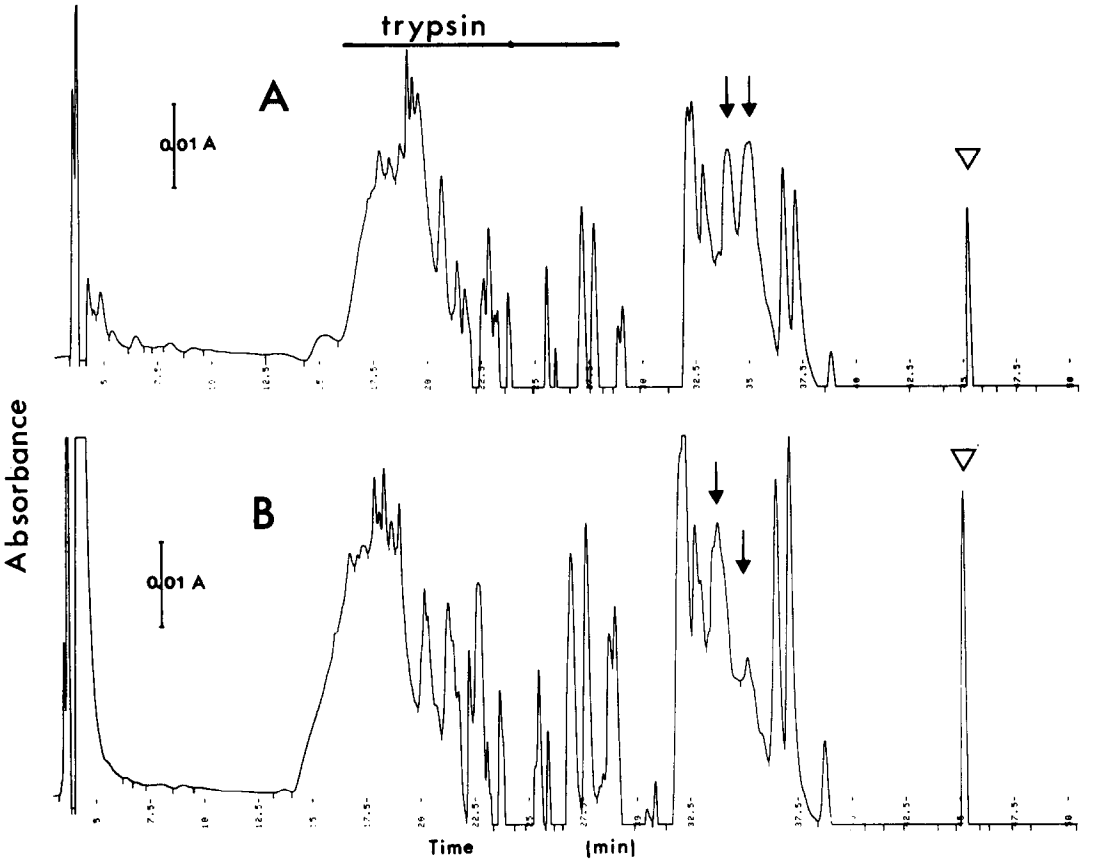

Fig. 3. HPLC profiles of the tryptic peptides of purified FMO from normal (A) and diabetic (B) rat livers. Purified FMOs (25 μg in A and 50 μg B) were treated with trypsin, applied to a reverse phase column and eluted by a linear gradient (0–100%) of acetonitrile in 0.1% TFA as indicated in Material and Methods. ▽ indicates the residual undigested FMO. Peaks between 15 and 30 min correspond to the trypsin. Similar patterns were observed in two different tryptic digestions of the purified FMOs.

other hand, our results indicate that there are no differences either in the amount of the enzyme or in its heat-lability. Although unlikely, our results cannot exclude the possibility of the synthesis of an isozyme very similar to the normal form, induced by the profound hormonal changes present in diabetic animals.

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