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Accumulation of Mitochondrial P450MT2, NH_2-terminal Truncated Cytochrome P4501A1 in Rat Brain during Chronic Treatment with β-Naphthoflavone:

A ROLE IN THE METABOLISM OF NEUROACTIVE DRUGS*

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Abstract

The biochemical and molecular characteristics of cytochrome P4501A1 targeted to rat brain mitochondria was studied to determine the generality of the targeting mechanism previously described for mitochondrial cytochrome P450MT2 (P450MT2) from rat liver. In rat brain and C6 glioma cells chronically exposed to β-na-phoflavone (BNF), P450MT2 content reached 50 and 95% of the total cellular pool, respectively. P450MT2 from 10 days of BNF-treated rat brain was purified to over 85% purity using hydrophobic chromatography followed by adrenodoxin affinity binding. Purified brain P450MT2 consisted of two distinct molecular species with NH_2 termini identical to liver mitochondrial forms. These results confirm the specificity of endoprotease-processing sites. The purified P450MT2 showed a preference for adrenodoxin + adrenodoxin reductase electron donor system and exhibited high erythromycin *N*-demethylation activity. Brain mitoplasts from 10-day BNF-treated rats and also purified P450MT2 exhibited high *N*-de-methylation activities for a number of neuroactive drugs, including trycyclic anti-depressants, anti-convulsants, and opiates. At 10 days of BNF treatment, the mitochondrial metabolism of these neuroactive drugs represented about 85% of the total tissue activity. These results provide new insights on the role of P450MT2 in modulating the pharmacological potencies of different neuroactive drugs in chronically exposed individuals.

Cytochrome P450 (P450)[1] enzymes play a critical role in the metabolism of an array of endogenous as well as exogenous substrates (1–3). The xenobiotic inducible forms with roles in the metabolism of carcinogens, pollutants, and drugs were thought to be exclusively associated with the endoplasmic reticulum (hereafter referred to as microsomes) of liver, brain, and other tissues. In contrast to this general belief, recent reports from our laboratory showed that the BNF-inducible P4501A1 and phenobarbital-inducible P4502B1 are also

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[1]The abbreviations used are: P450, cytochrome P450; P450 reductase, NADPH cytochrome P450 reductase; Adx, adrenodoxin; Adr, adrenodoxin reductase; BNF, β-naphthoflavone; COX I, cytochome c oxidase subunit I; ERND, erythromycin *N*-demethylase; OAA, ω-oc-tylamine-agarose; PEG, polyethyleneglycol (average molecular weight 8000); EROD, ethoxyresorufin *O*-deethylase; DTT, dithiothreitol.

targeted to mitochondria under both *in vitro* and *in vivo* cell transfection conditions. These results are consistent with previous studies from our, as well as, other laboratories showing the presence of P450 proteins cross-reacting with antibodies to the major microsomal forms, in liver and brain mitochondria from inducer treated and untreated rats, and also insects (4–10). Direct sequencing of hepatic mitochondrial P450 proteins purified from BNF-treated rats suggested the occurrence of two forms of P450MT2, both of which were NH_2-terminal cleaved versions of P4501A1 (11). The molecular form cleaved past the 4th amino acid residue (+5/1A1) represents a minor component while that cleaved past residue 32 (+ 33/1A1) represents the major component in mitochondria from BNF-treated rat liver. This major form will be routinely referred to as P450MT2 throughout this paper.

The mitochondrial targeted P450MT2 exhibited many molecular and biochemical properties distinct from the parent microsomal P4501A1 (12): 1) P450MT2 interacted with Adx with an affinity of 0.6 μM K_d, 2) functionally productive interaction of Adx with P450MT2 occurred through the same COOH-terminal basic domain and the same positively charged residues that are known to be involved in interaction with *bona fide* mitochondrial P450s, such as P450scc and P450c27 (13, 14), 3) P450MT2 bound to erythromycin (K_d of 50 μM) as determined by spectral analysis, and 4) P450MT2 exhibited high ERND activity, but vastly reduced CYP1A1 specific marker, EROD activity, in an Adx supported system. In contrast, the microsomal P4501A1 with intact NH_2-terminal end showed very low ERND, but high EROD activity, in a P450 reductase-supported system. The ERND activity of the mitochondrial targeted P4501A1 was further supported by experiments showing that mitochondrial P450MT2 rendered protection against erythromycin mediated inhibition of mitochondrial translation (15). Thus, the NH_2-terminal truncated mitochondrial P450MT2 may be conformationally different from the microsomal P4501A1.

We hypothesized that the NH_2-terminal signals of a number of microsomal P450s, including 1A1, are chimeric in that they carry signals for targeting proteins to both the endoplasmic reticulum and mitochondria. The chimeric nature of the amino-terminal 45-amino acid stretch of P4501A1 was further supported by the ability of this signal to target heterologous proteins such as the cytosolic dihydrofolate reductase and mature portion of rat P450c27 to endoplasmic reticulum and mitochondria (16). Our studies on the mechanism of mitochondrial targeting of P4501A1 suggested the activation of a cryptic mitochondrial targeting signal at residues 33–44 by sequence specific cleavage past the 4th and 32nd residues of the protein by a cytosolic endoprotease (11, 15, 16). In the present study, the sequence specificity of 1A1 chain cleavage by endoprotease and the generality of the mechanism of P450MT2 biogenesis was further tested by purifying and characterizing the 1A1-like P450 from BNF-treated rat brain mitochondria. Our results show that the brain mitochondrial P450MT2 is truncated at the same position as the major liver mitochondrial forms. Furthermore, under chronic BNF treatment conditions both in rat brain and cultured C6 glioma cells, the mitochondrial P450MT2 continues to accumulate and become a major part of the total 1A1 pool. The brain mitochondrial form exhibited high *N*-demethylation activity with a variety of antidepressant and anticonvulsant drugs suggesting a role in modulating the pharmacological potencies of neuroactive drugs.

MATERIALS AND METHODS

Treatment of Animals and Subcellular Fractionation of Tissues

Maintenance of male Harlan Sprague-Dawley rats (100–150 g, Harlan Sprague-Dawley Inc., Indianapolis, IN) received intraperitoneal administration of BNF (80 mg/kg body weight in corn oil) once daily for either 4 or 10 days, as described (4). The control rats were administered with equivalent amounts of corn oil.

Animals were killed 24 h after the last injection and overnight fasting by CO_2 asphyxiation, and perfused transcardially with ice-cold saline. The livers and brains were rapidly removed, rinsed with saline excised in sucrose-mannitol buffer (2 mM HESES, pH 7.4, 70 mM sucrose, 220 mM mannitol, and 2 mM EDTA). Liver mitochondria were isolated as described (17). Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at 120,000 × *g* for 1 h at 4 °C. Brain mitochondria were isolated using discontinuous Percoll density gradient banding as described by Sims (18). Briefly, brains were homogenized in 10 volumes of isolation medium containing 0.32 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1 mM DTT, and 0.1 M Tris-HCl buffer, pH 7.4, and centrifuged at 1,300 × *g* for 3 min. The supernatant was centrifuged at 21,000 × *g* for 10 min. The resultant pellet was resuspended in isolation medium and purified by banding through a discontinuous Percoll gradient. Mitochondria were suspended in sucrose/mannitol buffer (50 mg of protein/ml) and treated with digitonin (Wako Chemicals) at a final concentration of 75 μg/mg protein. The mitochondrial suspension was shaken on ice for 5 min, and the mitoplasts were pelleted by centrifugation at 12,000 × *g* for 10 min. The mitoplast pellet was washed 4 times with the sucrose/mannitol buffer, and used for further analysis. The purity of the mitochondrial preparation was checked routinely by assaying the marker enzyme activities and by immunoblot analysis as described before (10, 19). The purity of digitonin was found to be a critical factor in the yield as well as quantity of mitoplasts from brain tissue. In our hands, digitonin from Wako Chemicals yielded satisfactory results. The P450 content of mitoplasts was measured by the sodium dithionite-reduced carbon monoxide binding difference spectra (20) in a buffer system containing 100 mM KH_2PO_4, pH 7.4, 20% glycerol (v/v), 0.5% sodium cholate (w/v), and 0.4% Triton N-101 (v/v) for measuring the P450 content.

Solubilization and Purification of Brain Mitochondrial P450MT2

Mitoplasts from 10-day BNF-treated rat brain were suspended in 100 mM KH_2PO_4 buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 1 mM DTT, at a final concentration of 20 mg of protein/ml and sonicated for 3 min (30 s pulse followed by 60 s of standing on ice) at setting 6 of a Branson sonifier. The P450 was solubilized by adding sodium cholate to a final concentration of 0.8% and fractionated with PEG as described previously (6). The 0–15% PEG precipitate was collected by centrifugation at 100,000 × *g* for 1 h and the pellet was suspended in 100 mM KH_2PO_4, pH 7.4, containing 20% glycerol, 1.0 mM EDTA, 1.0 mM DTT, and 0.5% sodium cholate. The proteins were dissolved by homogenization with a glass homogenizer followed by stirring the suspension for 30 min at 4 °C. The solubilized protein fraction (10 mg of protein/ml) was subjected to ω-octylamine-agarose column chromatography as described before (7, 8). The column was first washed until the A_{280} of the eluant reached <0.05, and the protein bound to the column was eluted successively with 10 column volumes of buffer A containing 0.2 and 0.5% emulgen, respectively. Fractions rich in P450 (>0.1 Á at 417 nm) were pooled and concentrated by ultrafiltration through Amicon filters. The concentrated samples were dialyzed overnight against three changes of buffer B (10 mM KH_2PO_4 buffer, pH 7.7, 20% glycerol, 0.2% sodium cholate, and 0.2% emulgen), and subjected to further purification by Adx-Sepharose chromatography (12).

Coupling of purified Adx to Sepharose matrix was carried out as described by Honukoglu *et al*. (21). Briefly, human recombinant Adx (0.1 μmol) was conjugated to 15 ml of CNBr-activated Sepharose 4B (7 ml bed volume) by incubating at 4 °C for 24 h. The P450 purified by ω-octylamine-agarose chromatography (10 mg/ml suspension of 40–60% purity) in 10 mM K_2HPO_4 buffer, pH 7.4, containing 10% glycerol, 0.1% sodium cholate, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT (buffer C) was loaded on to Adx-Sepharose column (5 ml bed volume), pre-equilibrated with buffer C. The column was washed with 10 volumes of the same buffer and the bound proteins were eluted with a step

gradient of 5 ml each containing 50,100,150, and 200 mM KCl in buffer C. The fractions were monitored for their absorbence at 417 nm.

Growth and Treatment of C6 Glioma Cells

Rat glioma C6 cells were grown as described before (22) in Ham's F-10 medium with 15% horse serum and 2.5% fetal bovine serum as described in the ATCC manual. BNF was dissolved in Me_2SO and added in fresh medium alternate days for 4 days beginning from the 4th day of the passage. BNF in 0.1% Me_2SO was added to a final concentration of 50 μM. Control plates were treated with an equivalent volume of 0.1% Me_2SO alone. In chronic BNF treatment experiments, after 72 h of initial treatment, cells were replated and continuously exposed to BNF for up to 192 h. Cells were homogenized in 10 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.4, 150 mM KCl, 10 mM EDTA, 1 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride), with a Potter-Elvehjem Teflon-glass homogenizer. Microsomes and mitochondria were isolated by differential centrifugation as described (17). Mitochondria were further purified on discontinuous Percoll density gradient as described earlier (18) and washed twice with the homogenization buffer to eliminate contaminating Percoll and used for analysis.

Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Proteins were analyzed on 12% SDS-polyacrylamide gels and visualized by staining with Coomassie Brilliant Blue (24) or subjected to immunoblot analysis. In some experiments proteins were also subjected to highresolution gradient (14–16%) SDS-polyacrylamide gels for resolving different P450MT2 forms as described (15). The conditions of protein blotting and immunodetection of protein bands were as described (15, 25). Rabbit polyclonal antibodis raised against purified rat liver mitochondrial P450MT2 (10) or intact P4501A1 (Amersham Pharmacia Biotech, Piscataway, NJ) were used.

NH_2-terminal Sequencing

The Adx-Sepharose affinity purified cytochrome P450 (∼100 pmol) was run on a gradient SDS-polyacrylamide gel (14–16%) and transblotted onto Sequi-blot polyvinylidene difluoride membrane (Bio-Rad). The amino acid sequence of the two differently migrating protein components was determined by the phenylthiohydantion procedure in a Beckman LH 3600 gas-phase sequencer.

Metabolism of Xenobiotic Substrates

The *N*-demethylation of various substrates was measured by assaying the rate of formaldehyde formation (26, 27). Reactions with mitoplasts and microsomes were run in a 500-μl final volume in a buffer containing 50 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 200 μg of mitochondrial or microsomal protein, and 100 μM substrate. Purified Adx (0.1–0.2 nmol) + Adr (0.01–0.02 nmol) were added in some reactions with mitoplast preparations as indicated. Reconstitutions with purified P4501A1 and P450MT2 were carried out in dilaurylphosphatidyl choline vesicles (7) using the same buffer system described above for intact membranes. The final reaction volume was 200 μl, and contained 50 pmol of P450, 0.2 nmol of Adx, and 0.02 nmol of Adr or 0.1 nmol of P450 reductase and 100 μM substrate. After 3 min of preincubation at 37 °C the reaction was initiated by the addition of 1 mM NADPH and the reaction was continued at 37 °C for another 20 min in a shaking water bath. Reactions were terminated by adding 0.5 volume of ice-cold trichloroacetic acid (10% w/v). The insolubles were pelleted by centrifugation at 10,000 × *g* at room temperature. An aliquot of the supernatant was mixed with an equal volume of Nash reagent (250 μl or 100 μl) further incubated at 55 °C or 15 min. The reaction product, formaldehyde, was measured according to the method of Nash (27). A notable drawback of this assay is a generally high

background value, which can be controlled by using freshly prepared Nash reagent and controlling the heating step during color development to 55 °C for 15 min. Typically, we obtained A_{412} values in the range of 0.01 to 0.014 for control tubes without added enzyme, and all significant *N*-demethylation activities reported here correspond to >3.5 fold of background reading.

Confocal Immunofluorescence Microscopy

Rat C6 glioma cells were grown on coverslips and treated with BNF (50 μM) for 72 or 96 h and processed for antibody staining essentially as described before (11, 28). After permeabilization with 0.1% Triton X-100, cells were blocked with 5% goat serum for 1 h at room temperature. Cells were double immunostained with 1:100 dilution of rabbit polyclonal antibody to P4501A1 and 1:50 dilution of mouse monoclonal antibody to the mitochondrial genome encoded human COX I (Molecular Probes, Eugene, OR) as the mitochondrial-specific marker. Cells were washed repeatedly with phosphate-buffered saline (10 mM Na_2HPO_4, pH 7.0, and 150 mM NaCl), and incubated with fluorasceine 5-isothocyanate-conjugated anti-rabbit donkey IgG for the detection of P4501A1 and Texas Red-conjugated anti-mouse donkey IgG for the detection of COX I protein. Incubation with both the secondary antibodies (Jackson ImmunoResearch Laboratories) were carried out for 1 h at 37 °C at 1:100 dilution. Unbound secondary antibodies were removed by repeated washing with phosphate-buffered saline. Fluorescence microscopy was carried out under a TCS laser scanning microscope (Leica Inc.). 0.5-μm optical sections were scanned at the *z* axis with both fluoresceine 5-isothocyanate and Texas Red channels fully open to prevent any shifting or distortion of the images.

Protease Protection and Alkaline Extraction

Freshly isolated mitochondria and microsomes were subjected to trypsin digestion as described (10). Mitochondria and microsomes were suspended in 50 mM potassium phosphate buffer containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT and subjected to trypsin digestion (30 μg of trypsin/mg protein) at room temperature for 30 min. The reaction was stopped by adding trypsin inhibitor (300 μg/mg protein) and an equal volume of 2 × Laemmli's sample buffer (24). The samples were incubated in a boiling water bath for 5 min and loaded onto a 12% SDS-polyacrylamide gel for immunoblot analysis.

Extraction of mitochondrial and microsomal proteins with 0.1 M Na_2CO_3 (pH 11), separation of soluble and insoluble protein fractions, and Western blot analysis were essentially as described (9, 10), using freshly isolated mitochondria and microsomes. Except for the protease protection and alkaline extraction, in all other experiments digitonin-treated mitoplasts were used.

RESULTS

Purity of Mitochondrial Preparations

The purity of rat brain mitochondrial preparations was routinely tested by immunoblot analysis for mitochondrial-specific COX I protein and microsome-specific P450 reductase. As shown in Fig. 1 (*left panel*), proteins solubilized from mitoplast preparations contained 39-kDa protein cross-reacting with antibody to COX I, while the microsomal fraction contained negligible COX I protein. As expected, the microsomal protein contained high levels of P450 reductase (Fig. 1, *right panel*), while the mitochondrial preparations contained negligible 78-kDa protein cross-reacting with antibody to P450 reductase. Although not shown, rat brain mitoplast preparations contained less than 2% micro-some-specific marker enzyme, rotenone-insensitive NADPH cytochrome c reductase, but nearly 90% of cytochrome c oxidase activity. As previously shown (4, 7) the rat liver mitoplast

preparations used in this study contained less than 1% microsomal contamination based on marker enzyme assays.

Extent of P450MT2 Induction in Liver and Brain Mitochondria

To understand the possible functional significance of microsomal P4501A1 and mitochondrial P450MT2 in the rat liver and brain, we determined their relative abundance at moderate 4 days of BNF treatment, and chronic 10 days of BNF treatment. The immunoblot analysis of protein in Fig. 2*A* shows that the 1A1 antibody-reactive protein in the rat brain mitochondria increased steadily at 4 and 10 days of BNF treatment. The level of antibody-reactive protein in the brain microsomal fraction, on the other hand, was significantly reduced at 10 days of BNF treatment after reaching a peak at 4 days. As seen from Fig. 2*B*, the induction pattern is nearly similar in the liver; the level increases steadily in the mitochondrial compartment, while that of the microsomal compartment peaks at 4 days of treatment followed by a significant reduction by 10 days of treatment.

The total tissue levels (per gram basis) of mitochondrial and microsomal 1A1 antibody-reactive proteins in the liver and brain are presented in Fig. 2, *C* and *D*, respectively. These values were calculated based on the recovery of different organelles (mg of proteins)/g of tissue. Results show that the rate of accumulation of 1A1-like protein in mitochondria and microsomes follow different kinetic patterns. Interestingly both in the liver and brain, at 10 days of chronic BNF treatment, the mitochondrial P450MT2 preferentially accumulates to reach about 45–57% of the total tissue pools. The preferential increase of mitochondrial P450MT2 during chronic exposure to BNF was also investigated in C6 glioma cells, in which the expression of endogenous *P4501A1* gene is induced in response to chemical inducers. Results of immunoblot analysis (Fig. 3*A*), and quantitation of subcellular contents of 1A1 antibody-reactive protein in Fig. 3*B*, show that similar to that seen with rat brain, the level of mitochondrial P450MT2 at 96 h of treatment was about 4-fold of that detected in microsomes. Additionally, at chronic 192 h of treatment, the mitochondrial P450MT2 represented over 95% of the total tissue pool, while the level of antibody reactive protein in the microsomal fraction was drastically reduced.

Intramitochondrial Location of 1A1-like Protein in BNF-induced Rat Brain

The intramitochondrial location of the antibody-reactive P450 protein associated with brain mitochondria was investigated using protease digestion of freshly prepared mitochondria and microsomes from 10-day BNF-treated rat brains. The immunoblot (Fig. 4*A*) developed with antibody to P4501A1 shows that trypsin digestion did not affect the level of antibody-reactive protein in intact mitochondria, suggesting intramitochondrial location. Similar treatment of brain microsomal preparations, however, nearly quantitatively eliminated the antibody reactive protein. Furthermore, as shown in Fig. 4*B*, the antibody reactive protein from the mitochondrial fraction was nearly quantitatively extracted with 0.1 M Na_2CO_3, suggesting the membrane extrinsic nature of the mitochondrial form. The microsome-associated protein, on the other hand, was completely resistant to alkaline extraction suggesting a transmembrane orientation. Similar resistance to limited proteolysis and solubility in alkaline Na_2O_3 extraction were reported for the rat liver mitochondrial P450MT2 (15).

The mitochondrial targeting of P4501Al-like protein in inducertreated C6 glioma cells was ascertained by immunohistochemical colocalization of P450 with mitochondria-specific COX I protein, which is encoded by the mitochondrial genome. As seen from Fig. 5*A* (*upper panel*), control uninduced cells were stained insignificantly with 1A1 antibody, while mitochondria-like punctate structures were stained with COX I antibody. In 72-h BNF-treated cells (*middle panel*), however, 1A1 antibody yielded intense staining with

cytoplasmic organelles, a majority of which colocalized with structures stained with COX I antibody, suggesting mitochondrial localization. Some of the 1A1 antibody-stained structures in 96-h BNF-treated cells also colocalized with organelles stained with bCOP antibody (Fig. 5*B*). The latter may represent proteins targeted to plasmamembrane (29, 30).

Characterization of Brain Mitochondrial P450MT2

Use of high resolution gradient gel, followed by immunoblot analysis (Fig. 6), resulted in the resolution of two distinctly migrating P4501A1 antibody interacting species from both liver and brain mitochondria of BNF-induced rats. The two brain forms resolved in this study resembled the previously identified liver forms, the latter designated as P450MT2a and MT2b (11, 12). The faster migrating species identified from the brain mitochondria co-migrated with bacterially expressed +33/1A1 as well as purified liver mitochondrial P450MT2b, while the slow migrating protein co-migrated with microsomal P4501A1, or bacterially expressed +5/1A1 (latter result not shown). These results suggest the P4501A1 molecular forms targeted to brain mitochondria are similar to the liver mitochondrial forms.

With a view to determine the molecular characteristics of the brain mitochondrial P450MT2 and to identify site(s) of proteolytic processing we purified P450MT2 from 10-day BNF-treated rat brain mitochondria. The purification scheme was modified from the one used for the purification of the liver mitochondrial forms (6), and consisted of a combination of PEG fractionation, chromatography on OAA column (7, 8), and affinity binding to Adx-Sepharose resin (12). The purification steps and the recovery of total proteins and P450 content at individual steps are listed in Table I. It is seen that nearly 72% of the mitochondrial P450 was solubilized with sodium cholate and nearly 60% of the solubilized P450 was recovered in the 15% PEG precipitate. A significant portion of P450 (15% of input) loaded on the OAA column was eluted with 0.2% Emulgen, while nearly half of input P450 was eluted as brown-colored material with 0.5% Emulgen. The former fraction, however, did not contain proteins in the size range of ∼50 kDa on SDS-polyacrylamide gels (results not shown). We therefore used the 0.5% Emulgen-eluted fraction for further purification by Adx affinity chromatography. A small fraction (about 2%) of input P450 was eluted with 50 mM KCl-containing buffer from the Adx column, while about 20% of input P450 was eluted with 100 mM KCl-containing buffer. Although not shown, no significant P450 was eluted with 150 and 200 mM KCl containing buffers. The 100 mM KCl eluate showing highest specific activity of 13.7 nmol of P450/mg of protein represents about 35–40-fold purification.

The SDS-polyacrylamide gel pattern in Fig. 7*A* shows that both sodium cholate solubilized and PEG-precipitated fractions contained a number of protein species detected in the total mitochondrial protein. The P450-rich fraction eluted from the OAA column at 0.5% Emulgen contained a prominent band of about 54 kDa in addition to a number of less prominent bands, suggesting a 50–60% purity. The fraction eluted from the Adx column with 50 mM KCl-containing buffer (*lane* 5) resolved as a 54–55 kDa species. Although this fraction showed a reduced CO spectrum suggesting the presence of P450, the protein recovery was too low for further analysis. The protein eluted from the Adx column with 100 mM KCl-containing buffer with a specific activity of 13.7 nmol of P450/mg of protein resolved as a major band with apparent molecular mass of 54 kDa (*lane 6*), and appeared about 85% pure. SDS-polyacrylamide gel pattern of the purified fraction on a 14–16% gradient gel (Fig. 7*B*) resulted in the resolution of two differently migrating protein species similar to that observed with purified liver mitochondrial P450MT2. The faster migrating MT2b is the major component representing about 60–70% of the protein fraction and the slower migrating MT2a is a minor component. Although not shown, both of these proteins cross-reacted with antibody to P4501A1 in immunoblot blot analysis.

As shown in Fig. 7*C*, NH_2-terminal sequence analysis of the faster migrating MT2b protein yielded sequence identical to the liver mitochondrial P450MT2b, starting with the 33rd residue of the microsomal P4501A1. The slower migrating band yielded sequence similar to the liver mitochondrial MT2a. These results suggest the specificity of the endoprotease processing in different tissues.

Catalytic Activities of the Brain Mitochondrial P450MT2

To gain further insight into the functional and molecular differences between the mitochondrial P450MT2 and microsomal P4501A1, we tested ERND activity of the two membrane isolates. We also tested a number of neuroactive drugs, amitriptilyne and imipramine, diazapam, morphine, and lidocaine. These substrates have been previously shown to be metabolized by various microsomal P450s, other than P4501A1 (32). Mitoplasts from untreated brain showed marginal activities of 0.8–1.4 nmol/mg of protein with all six substrates, which was marginally increased by adding Adx + Adr proteins (Fig. 8, *A* and *B*). Mitoplasts from 4-day BNF-treated rats showed activities of about 1.6–2 nmol/mg of protein for all six substrates, and the activities were increases 2-fold by adding Adx + Adr proteins. Consistent with the increased P450MT2 content in Fig. 2, mitoplasts from 10-day BNF-treated brain showed even higher activities of 8–9 nmol/mg of protein/min with all six substrates in the presence of added Adx and Adr proteins. The microsomal fractions from untreated rat brain reconstituted with endogenous P450 reductase, on the other hand, showed marginal activity of 1.0–1.5 nmol/mg/min. The microsomal activity for none of the substrates increased both at 4 and 10 days of BNF treatment. Thus, while the increase of *N*-demethylation activities with all six substrates in BNF-induced mitochondria was nearly proportional to the extent of steady state increase in mitochondrial P450MT2, the microsomal activities did not change in response to both increase of P4501A1 at 4 days of BNF treatment or a drastic reduction of P4501A1 at 10 days of treatment. These results suggest that the observed *N*-demethylation activities in the microsomal fraction may be due to other constitutively expressed P450 forms.

To further ascertain the role of P450MT2 in the metabolism of the various neuroactive drugs described above, we carried out reconstitution of purified P450MT2 with these substrates. Table II shows the results of ERND activity reconstituted with purified P4501A1 and brain mitochondrial P450MT2. P4501A1 in the presence of P450 reductase system yielded very low activity of 0.05 nmol/nmol of P450/min. As expected, P450MT2 in the presence of NADPH and Adx + Adr yielded the highest activity of 2.5 nmol/nmol of P450/min. The activity was highly dependent on the addition of electron transfer proteins, as well as NADPH. Furthermore, the ERND activity was inhibited by both CO and a P450 inhibitor, SKF525-A, confirming that the observed ERND activity is catalyzed by P450. Finally, as shown for the liver mitochondrial enzyme, the purified brain P450MT2 yielded 1.3 nmol/nmol of P450/min activity when reconstituted with P450 reductase. As seen from Fig. 9, the brain P450MT2 also exhibited high activities in the range of 2.5–2.6 nmol/nmol of P450/min with all of the other substrates in Adx + Adr-supported systems, and nearly half the activities in P450 reductase-supported systems. Although not shown, purified microsomal P4501A1 yielded uniformly low activities (0.02–0.03 nmol/nmol of P450/min) with all of the substrates in a P450 reductase-supported system. However, as expected (33), purified P4501A1 yielded high EROD (6 nmol/nmol of P450/ min) activity in a P450 reductase-supported system. These results suggest the possibility that P450MT2 may play an important role in the metabolism/inactivation of an array of neuroactive drugs and pain suppressants. These results also support the possibility that different electron donor proteins or mode of electron channeling may influence substrate oxidation.

DISCUSSION

The molecular characteristics of P450MT2 (SWISS-PORT P10085) H_2-terminal truncated P4501A1 from rat liver mitochondria has been extensively studied, although the nature of its counterparts from other rat tissues remain unclear. Recent studies from our laboratory showed that 10 days of chronic BNF administration resulted in a significant induction of 1A1 antibody-reactive protein in lung mitochondria (16), although a low abundance of the protein in the lung even under induced conditions prevented its detailed analysis. In the present study, we have therefore investigated the nature of P4501A1 targeted to rat brain mitochondria under moderate 4 days of treatment and 10 days of chronic treatment with BNF. A number of studies have shown the constitutive expression of *P4501A1* in the rat brain (34–40). Similarly, induction of *P4501A1* gene expression in the brain and also C6 glioma cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and different polycyclicaromatichydrocarbons has been extensively studied (17, 18, 34, 36–40). However, the relative abundance and distribution of this P450 in different cytoplasmic organelles, particularly microsomes and mitochondria of inducer-treated rat brains, remains unclear. Using a prototype *CYP1A1* inducer, BNF, we show that the accumulation of antibody-reactive protein in the microsomes and mitochondria follow different kinetic patterns. The microsomal P450 content peaks at 4 days of treatment, followed by a sharp decline by 10 days of chronic treatment. The mitochondrial content, on the other hand, shows a steady time-dependent increase to reach nearly half of the total tissue pool by 10 days of BNF treatment. To avoid possible pain and suffering due to longer BNF treatment regimens in animals, we used rat glial cell line to investigate the effects of prolonged treatment with BNF. It is noteworthy that, by 192 h exposure of cells to BNF, the mitochondrial P450 pool reaches over 95% of the total cellular pool, suggesting an important physiological function for mitochondrial P450MT2. The 50 μ_M BNF used in this study neither affected cell morphology nor caused cell death (results not shown). Although reasons for the steady accumulation of mitochondrial form are not clear, an increased cytoplasmic endoprotease activity during chronic BNF treatment could be a contributing factor. Different protein turnover rates in these two membrane compartments may also be an important factor. Unpublished results[2] in our laboratory show a dramatic increase in cytosolic endoprotease activity in both rat brain and C6 glioma cells chronically treated with BNF. Currently efforts are underway to purify this protease.

Because of the problems of cross-contamination generally inherent in most mitochondrial isolation procedures, we have used the gradient banding method (18) followed by digitonin treatment for the isolation of mitochondria. The resultant mitoplasts contain no detectable microsomal specific marker protein, P450 reductase (Fig. 1), and less than 0.6% microsomal specific NADPH cytochrome c reductase (rotenone insensitive). Furthermore, mitochondrial location of the antibody reactive P450, and the similarity of brain mitochondria-associated P450 with the well characterized liver mitochondrial P450MT2 were established using multiple criteria: 1) the mitochondrial associated P450 was resistant to limited protease digestion, while that associated with the microsomal membrane was highly sensitive to similar treatment conditions (Fig. 4*A*). 2) Immuno-histochemical analysis showed that in BNF-treated C6 glioma cells, 1A1 antibody stained a large population of punctate membrane structures, which were co-localized with membrane structures stained with COX I antibody, suggesting mitochondrial location (Fig. 5). Notably, some of the 1A1 antibody-stained structures also co-localized with more rounded vesicular structures stained with antibody to Golgi-specific protein, bCOP. However, both in terms of abundance and shape these latter membrane structures appeared distinct from structures overlapping with

[2]E. Boopathi, H. K. Anandatheerthavarada, S. V. Bhagwat, G. Biswas, J-K. Fang, and N. G. Avadhani, unpublished results.

mitochondrial membranes in Fig. 5*A*. 3) Similar to that demonstrated for the liver P450MT2, the brain mitochondria-associated P450 resolved as two distinctly migrating components on gradient polyacrylamide gels (Fig. 6). 4) The 1A1 antibody reactive protein associated with brain mitochondria was readily extractable with alkaline Na_2CO_3 buffer suggesting membrane extrinsic orientation, as opposed to insolubility in alkaline buffer and transmembrane orientation of the microsome-associated P450 (Fig. 4*B*). These results are consistent with the properties previously described for liver mitochondrial P450MT2 and suggest a true mitochondrial targeted counterpart in the brain.

To further ascertain the molecular characteristics and also to study its catalytic properties, we purified the mitochondria-associated P450 using a combination of PEG fractionation, ω-octylamine-agarose chromatography, and Adx affinity binding methods. We believe that a superinducibility of the mitochondrial P450 by chronic BNF treatment coupled with the effective solubilization of P450 were important factors toward the successful purification of the brain P450MT2. It should be pointed out that our previous attempts at purifying this form using intact brain mitochondrial preparations were unsuccessful mainly due to poor solubilization of P450. Thus, use of digitonin-stripped mitoplast was critical for the effective solubilization by sonic disruption and cholate treatment as used in this study. Although a partial purification of P4501A1 from induced rat brain microsome was reported previously (41), this is probably the first successful isolation of the brain mitochondrial form to near homogeneity. The purified P450MT2 resolved as two distinct components similar to that shown for the liver mitochondrial counterpart, and yielded NH_2-terminal sequence identical to P450MT2a, starting with residue 5, and MT2b, starting with residue 33 of P4501A1. These results confirm the specificity of the cytoplasmic endoprotease, which may be an important regulatory factor in modulating the cellular level of P450MT2. Although not shown, both human and mouse counterparts of P4501A1 show similar cytoplasmic protease processing and mitochondrial targeting, confirming the generality of the targeting mechanism described for the rat liver P450MT2 (11).

Studies on the xenobiotic substrate metabolism by brain P450 have largely focused on the microsomal fraction (23, 34–36, 39, 40, 42). Microsomes from both inducer-treated rat brain and C6 glioma cells show high EROD activity (34–36), a marker for P4501A1. Although not shown, consistent with these reported results we also observed high EROD (0.4 nmol/mg of protein/min) activity with microsomes from BNF-treated brain and also C6 glioma cells. Interestingly, both intact mitoplasts from BNF-treated rat brain and purified brain P450MT2 showed ERND activity, which is not a marker for parent P4501A1. The ERND activity of mitoplast preparations increased 3-fold by adding Adx + Adr. Similarly, the purified P450MT2 showed nearly 2-fold higher activity (Table II) in an Adx + Adr supported system as compared with the activity in a P450 reductase-supported system, suggesting a preference for the former electron donor system. The ERND activity of the purified enzyme represents a true monooxygenase activity based on inhibition by CO and SKF525-A, and dependence on NADPH, as well as requirements for electron transfer proteins (Table II). Although not shown, P450MT2 both as part of intact mitoplasts or in purified form showed relatively low EROD activity when reconstituted with both electron donor protein systems. These results confirm and extend our previous observations with liver P450MT2 (11, 12, 15) that NH_2-terminal truncation and the new mitochondrial environment affect both substrate binding specificity and electron transfer protein binding ability of the enzyme.

An important finding of this study is that mitochondrial targeted rat brain P450MT2 exhibited *N*-demethylation activity for a number of neuroactive drugs. Amitriptyline and imipramine are widely used tricyclic anti-depressants. These MAO inhibitors are also known to affect serotonin uptake (43, 44). Diazepam is an anticonvulsant drug, which

facilitates GABA uptake acting through benzodizapene receptors (44). These compounds are known to be metabolized by the 2C and 3A family P450s (44). We also tested an opiate, morphine, and an anesthetic lidocaine. Microsomal 2B family enzymes are implicated in the metabolism of these substrates (3). It was surprising that mitochondria from BNF-induced brain and also purified P450MT2 showed high *N*-demethylation activities for all five compounds tested. Interestingly, mitoplasts from 4- and 10-day BNF-treated brains showed activities consistent with the extent of MT2 induction. The dependence of *N*-demethylation activities in all cases on added Adx is most likely due to the loss of this soluble protein from the organelle during isolation and gradient banding. Based on the mitochondrial P450 content of 0.3 nmol/mg of protein at 10 days of BNF treatment, the mitochondrial P450MT2 in its native membrane setting yielded nearly 10-fold higher activity as compared with the purified enzyme. This marked difference in activity is consistent with a previous observation that purified/reconstituted P450c27 yielded considerably lower activity than the native mitochondrial enzyme (16) and point to the possible involvement of yet unidentified mitochondrial cofactor(s) for activity. It should be noted that the microsomal fraction from control brain showed only about 1.3–1.5 nmol/mg of protein/min activity that did not show any increase by both 4 and 10 days of BNF treatment. It is likely that the basal level of microsomal *N*-demethylation activity is due to endogenous P450s other than P4501A1. The activity with isolated mitoplasts and purified enzyme, on the other hand, appear to be due to P450MT2, as they were effectively inhibited by P4501A1 antibody (results not shown). Based on the relative activities and mitochondrial and microsomal contents we estimate that at the chronic 10-day induction, the mitochondrial activity for the metabolism of these neuroactive substrates represents about 85% total tissue activity.

Results reported in this study suggest an important physiological function for mitochondrial P450MT2 in modulating the pharmacokinetics and pharmacodynamics of a family of antidepressants, anticonvulsants, opiates, and other neuroactive drugs. We believe that the 10-day BNF treatment used in this study mimics the human and animal population chronically exposed to polycyclicaromatichydrocarbons, cigarette smoke, and related pollutants. For these reasons, the individual's P450MT2 level should be an important consideration in the proper selection of antidepressant and anticonvulsant drugs, as well as in determining the drug dose.

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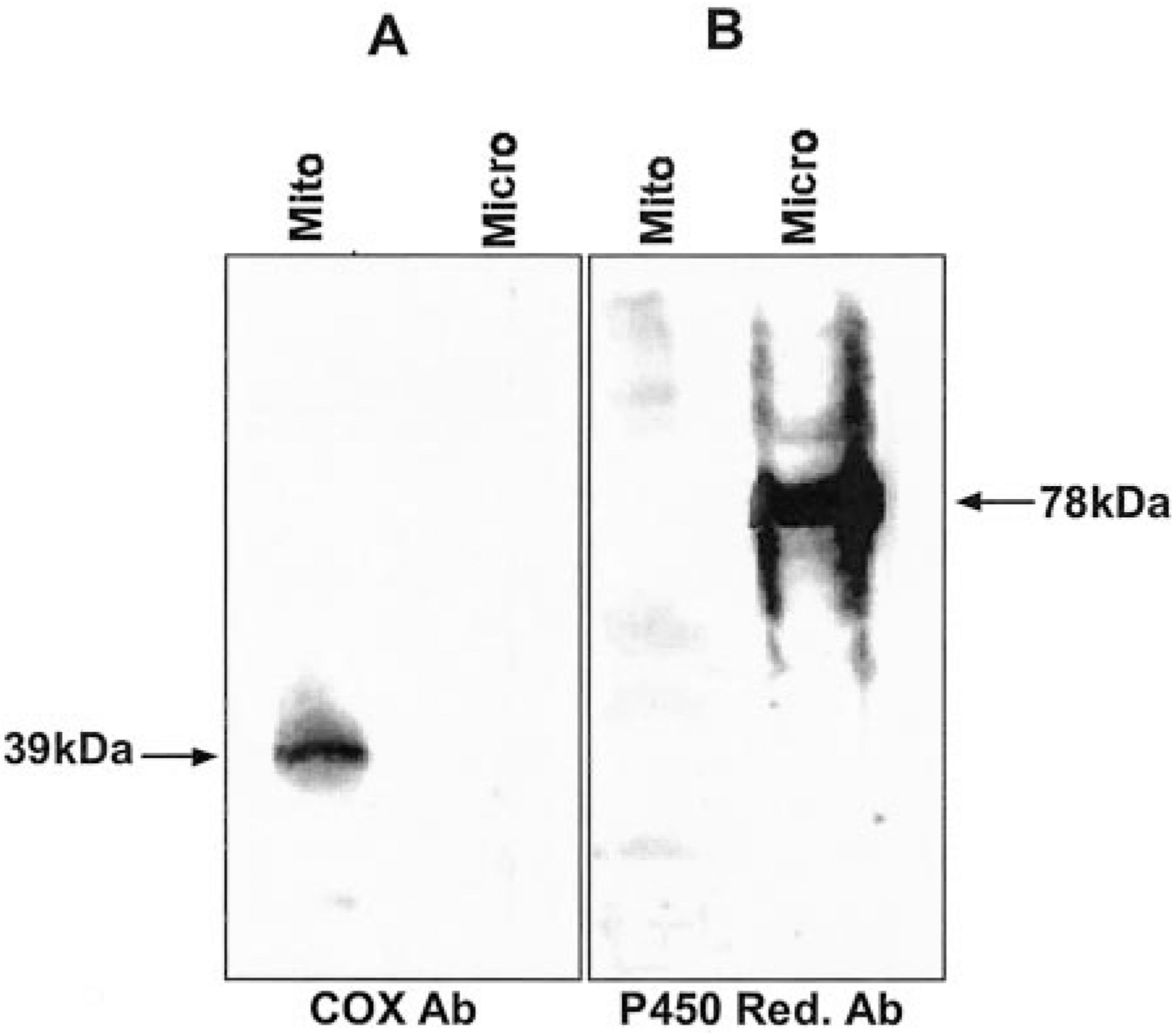

Fig. 1. Purity of brain mitochondrial preparations

Proteins solubilized from a representative microsomal and digitonin-treated mitochondrial preparations from rat brain (75 μg each) were subjected to immunoblot analysis. *A*, immunoblot with monoclonal antibody to COX I (1:3000 dilution); and *B*, polyclonal antibody against rat P450 reductase (1:3000 dilution). The blots were developed using horseradish peroxidase-conjugated secondary antibodies (1:30,000 dilution) as described under “Materials and Methods.”

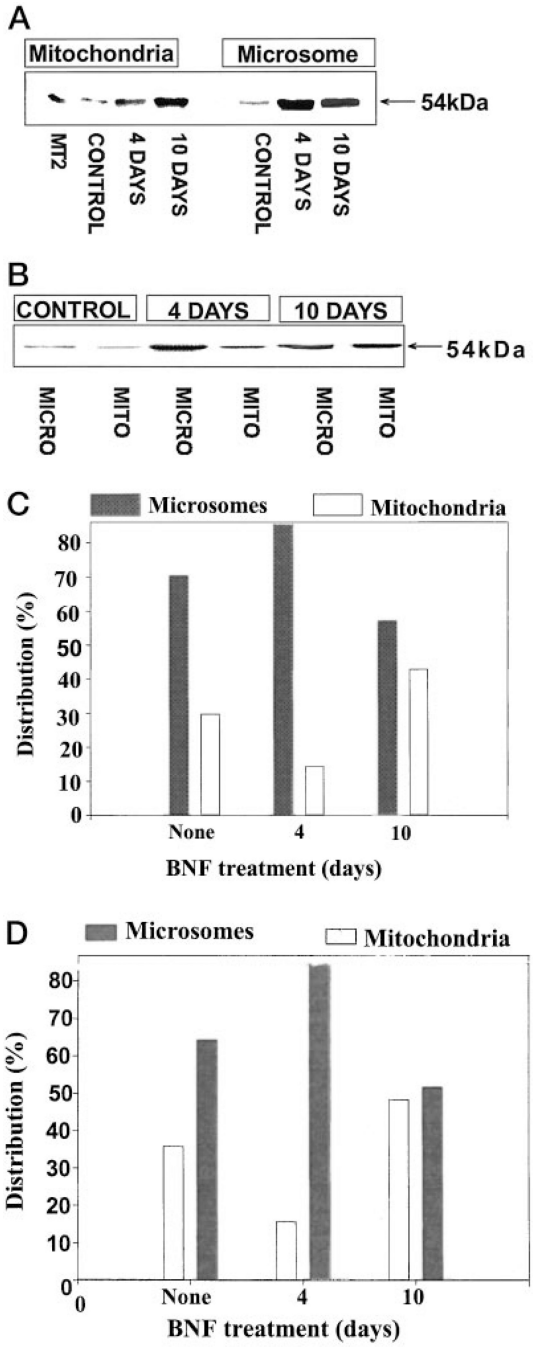

Fig. 2. Different patterns of induction of P4501A1 antibody-interacting protein in the mitochondrial and microsomal fractions

Mitochondrial and microsomal isolates (75 μg of protein in each case) from control, 4- and 10-day BNF-treated rats were subjected to immunoblot analysis using polyclonal antibody against P4501A1 (1: 3000 dilution) and horseradish peroxidase-conjugated secondary antibody as described in the legend to Fig. 1 and under “Materials and Methods.” *A*, subcellular fractions from brain; and *B*, subcellular fractions from liver. Percent tissue distribution in figures. *C*, liver; and *D*, brain were calculated based on the band densities and yield of mitochondrial and microsomal membranes. Typically we obtained 4.8–5.0 mg of mitoplast and 7.2–7.5 mg of microsomal protein/g (wet weight) liver tissue, and 3.6–3.8 mg

of mitoplast and 5.6–6.1 mg of microsomal proteins/g (wet weight) brain tissue. The combined mitochondrial and microsomal antibody reactive protein content, which amounted to more than 90% of the total tissue content was considered to be 100% for calculating % distribution.

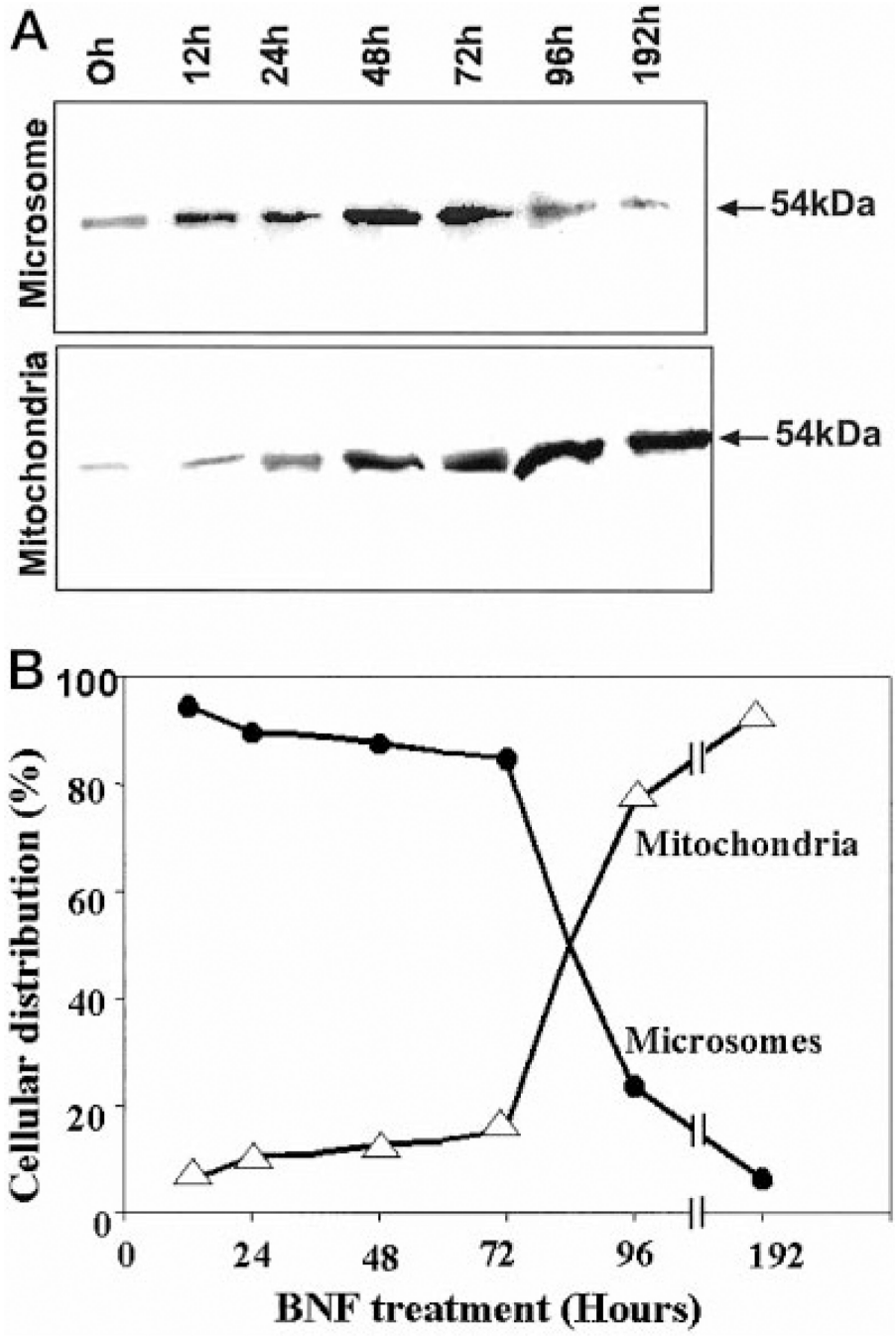

Fig. 3. Relative levels of mitochondrial and microsomal P450 in BNF-treated C6 glioma cells

Cells were exposed to BNF for varied lengths of time, mitoplasts, and mitochondria were isolated and subjected to immunoblot analysis (75 μg of protein each) as described in the legend to Fig. 2. Cellular distribution in *B* was calculated also as described in the legend to Fig 2. The overall recovery in terms of protein, mitoplasts (3.4 mg/g cells, wet weight), and microsome (5.6 mg/g cells, wet weight) did not vary significantly during the 192 h of treatment. Δ, represents mitochondria, and ♦, represents microsomes. There was no detectable cell death or growth inhibition by BNF treatment up to 192 h.

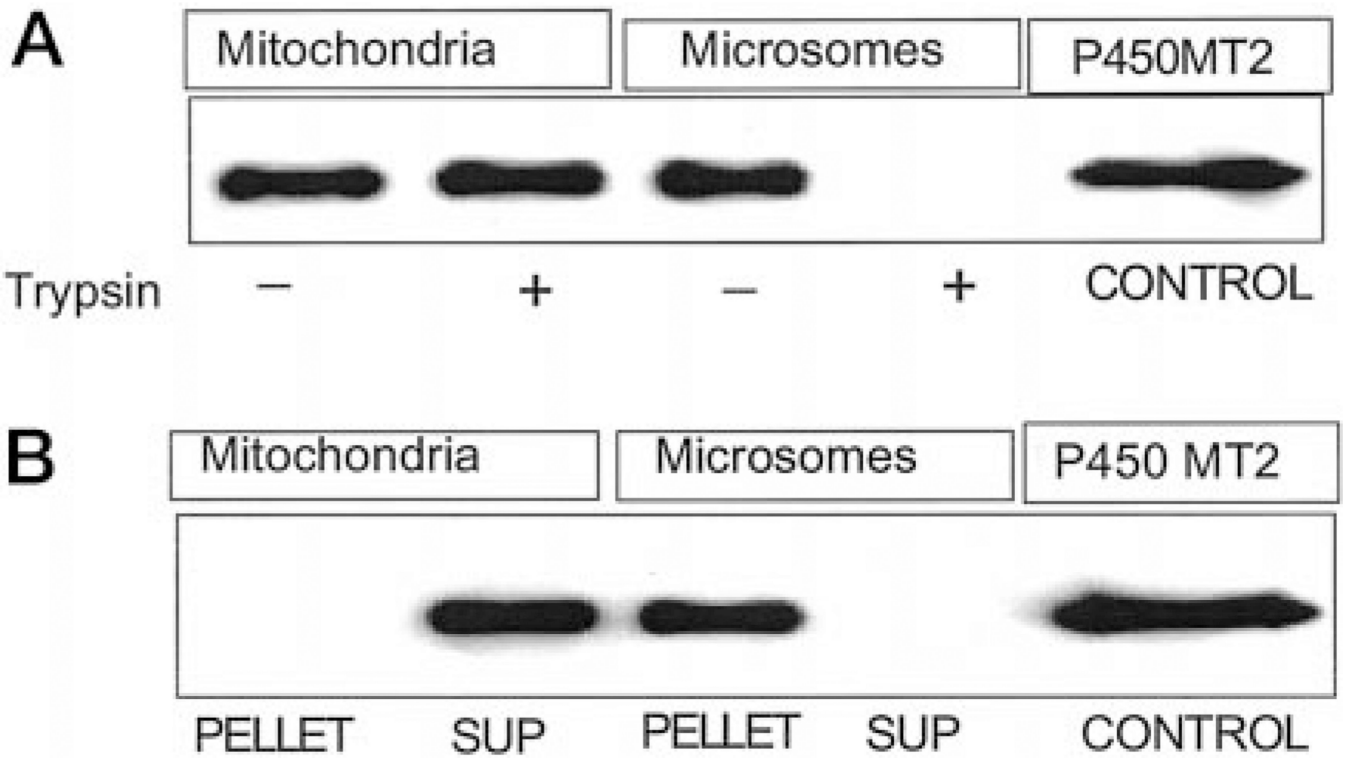

Fig. 4. Distinctive features of brain mitochondrial P450

In *A*, freshly isolated mitochondria and microsomes from 10-day BNF-treated rat brain were subjected to trypsin digestion as described under "Materials and Methods," and 75 μg of protein each was subjected to immunoblot analysis using polyclonal antibody to liver P450MT2. In *B*, mitoplasts and microsomes from 10-day BNF-treated rat brain were subjected to alkaline Na_2CO_3 extraction as described under "Materials and Methods." Both soluble and insoluble fractions equivalent of 100 μg of starting mitochondrial and microsomal proteins were subjected to immunoblot blot analysis as in *A*. In *A* and *B*, 2 μg of purified P450MT2 was run as a control.

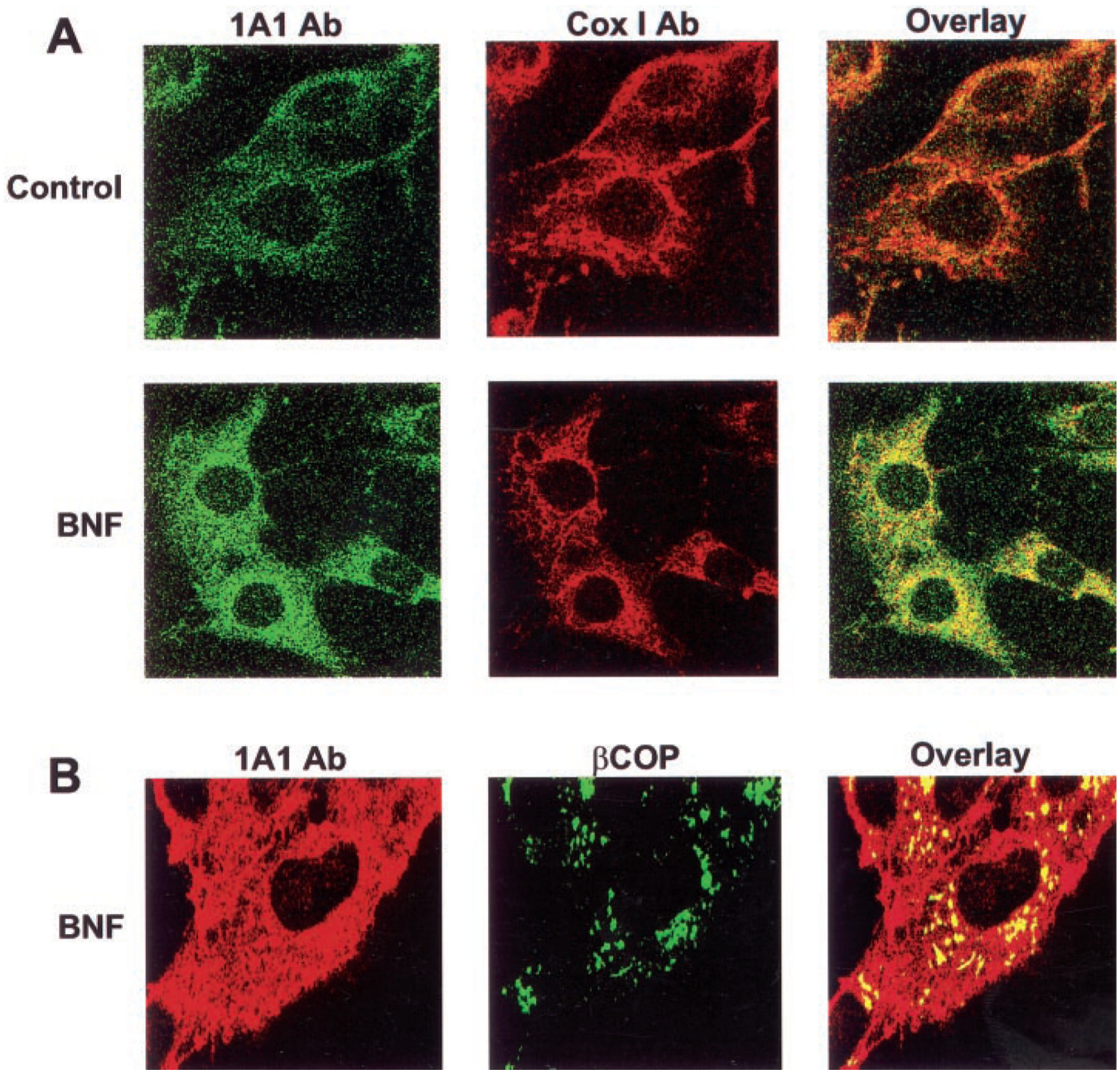

Fig. 5. Mitochondrial localization of P450MT2 in C6 glioma cells treated with BNF

In *A*, control cells or cells treated with BNF for 72 h were co-immunostained with 1A1 antibody (*left most panel*) or COX I antibody (*middle panel*). In *B*, cells treated with BNF for 96 h were co-immunostained with 1A1 antibody (*left most panel*) and bCOP antibody (*middle panel*). The *right most panels* in *A* and *B* represent overlay of double immunostained patterns. Details of immuno-staining and microscopy were as described under “Materials and Methods.”

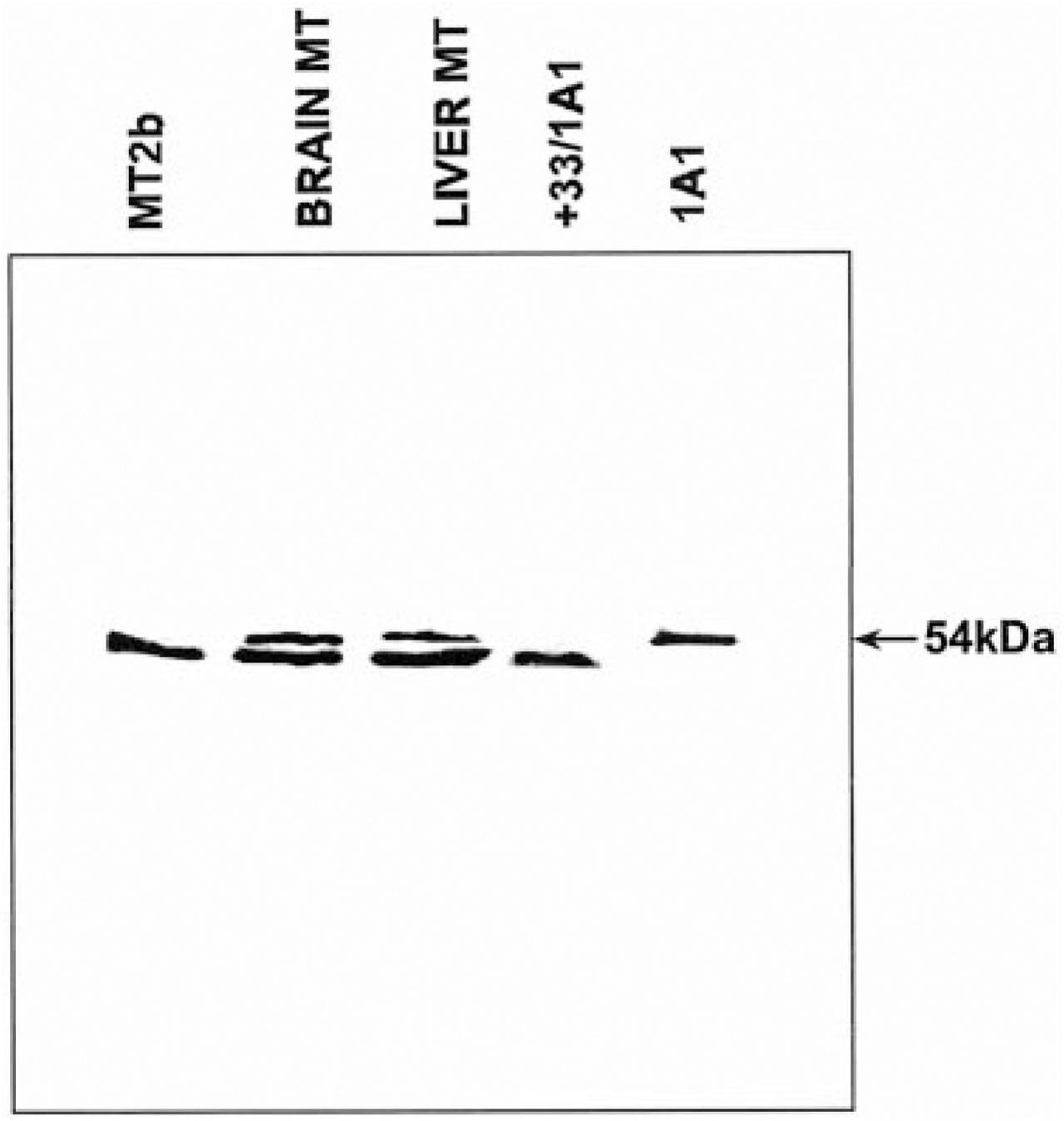

Fig. 6. Resolution of brain mitochondrial P450MT2 on gradient polyacrylamide gels

Liver and brain mitoplasts, and brain microsome from 10-day BNF-treated rats (75 μg each) were subjected to immunoblot analysis on a 14–16% gradient polyacrylamide gel as described under "Materials and Methods." Purified liver MT2b, liver microsomal P4501A1, and bacterially expressed/purified +33/1A1 (2 μg each) were run alongside as controls. The bands were visualized by staining with Coomassie Brilliant Blue.

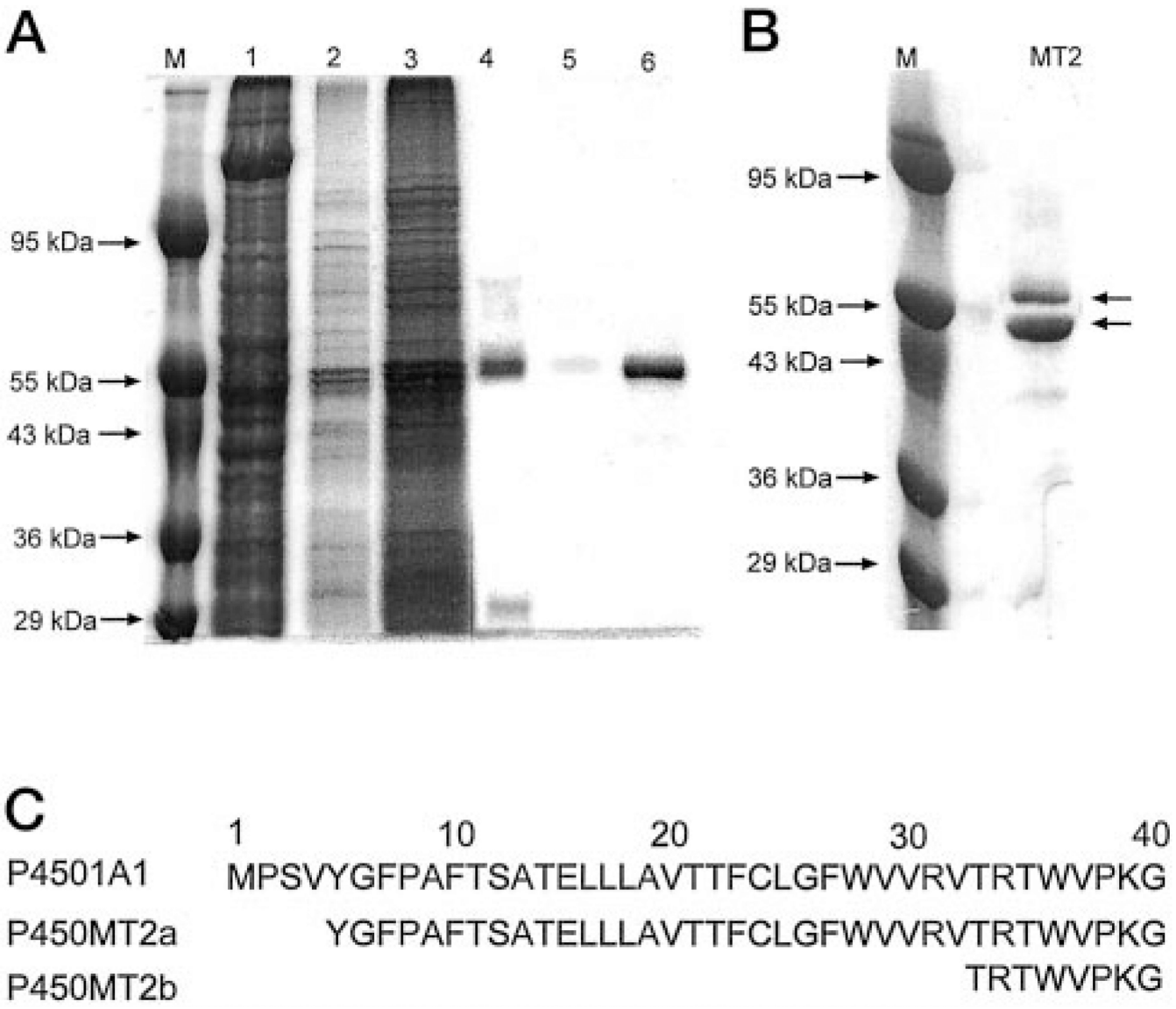

Fig. 7. Electrophoretic resolution and NH_2-terminal sequence of purified brain P450MT2

In *A*, protein fractions at various stages of purification from Table I were subjected to electrophoresis on a 12% polyacrylamide gel. *Lane M*, molecular weight marker; *lane 1*, 75 μg of total mitoplast protein; *lane 2*, 30 μg of cholate solubilized fraction; *lane 3*, 75 μg of 15% PEG precipitate; *lane 4*, 20 μg of ω-octylamine-agarose column fraction eluted with 0.5% Emulgen; *lane* 5, 1 μg of protein eluted from Adx-Sephraose column with 50 ml KCl-containing buffer; and *lane 6*, 2 μg of protein eluted from Adx-Sepharose column with 100 mM KCl-containing buffer. In *B*, 2 μg of protein eluted from Adx-Sephraose column with 100 mM KCl-containing buffer was resolved on a 14–16% gradient polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue. *C* shows the NH_2-terminal sequence of P450MT2a (slower migrating band) and MT2b (faster migrating band) from *B*, compared with microsomal P4501A1.

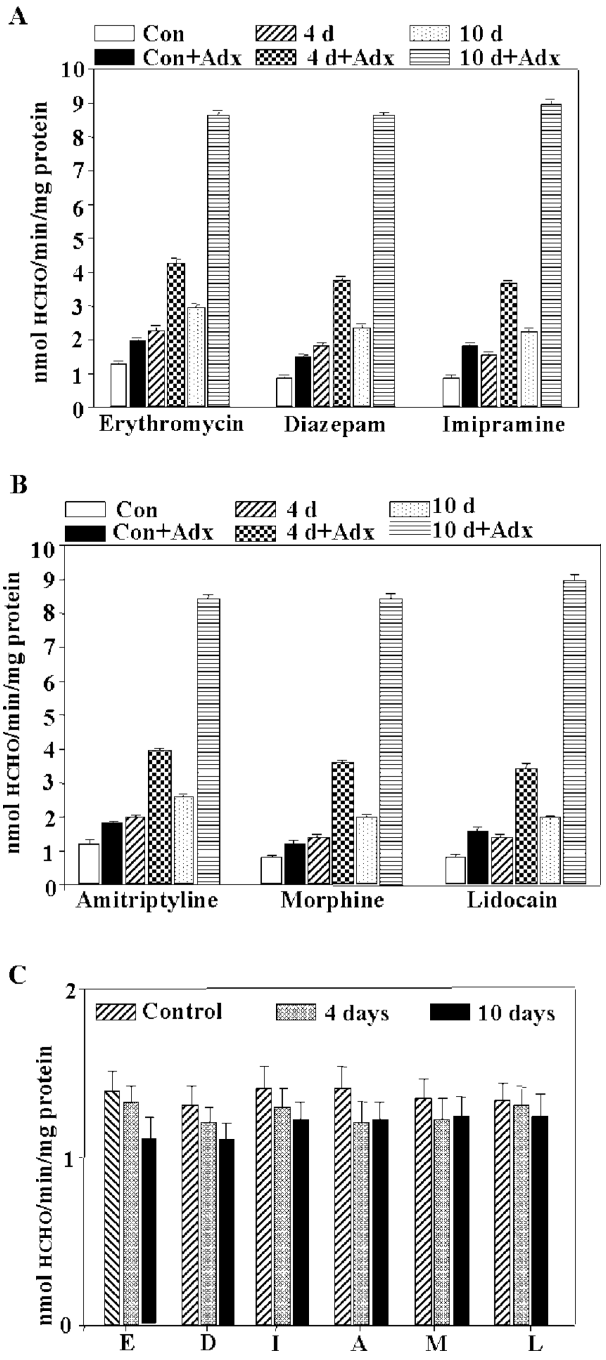

Fig. 8. *N*-Demethylation activities of mitoplasts and microsomes from BNF-induced rat brains

Mitoplasts (*A* and *B*) and microsomes (*C*) were isolated from control, 4- and 10-day BNF-treated rat brains and assayed for *N*-demethylation activities using indicated substrates (100 μM each), as described under "Materials and Methods." Assays with mitoplast preparations were carried out with or without added Adx and Adr (0.2 nM Adx and 0.02 nM Adr) with a view to counter the possible loss of these soluble proteins during isolation and gradient banding. In the case of microsomal fraction the endogenous P450 reductase served as electron donor protein. The values represent average ± S.E. (n = 4). In *C*, *A* = amitriptyline, *E* = erythromycin, *D* = dizapam, *I* = imipramine, *L* = lidocaine, *M* = morphine.

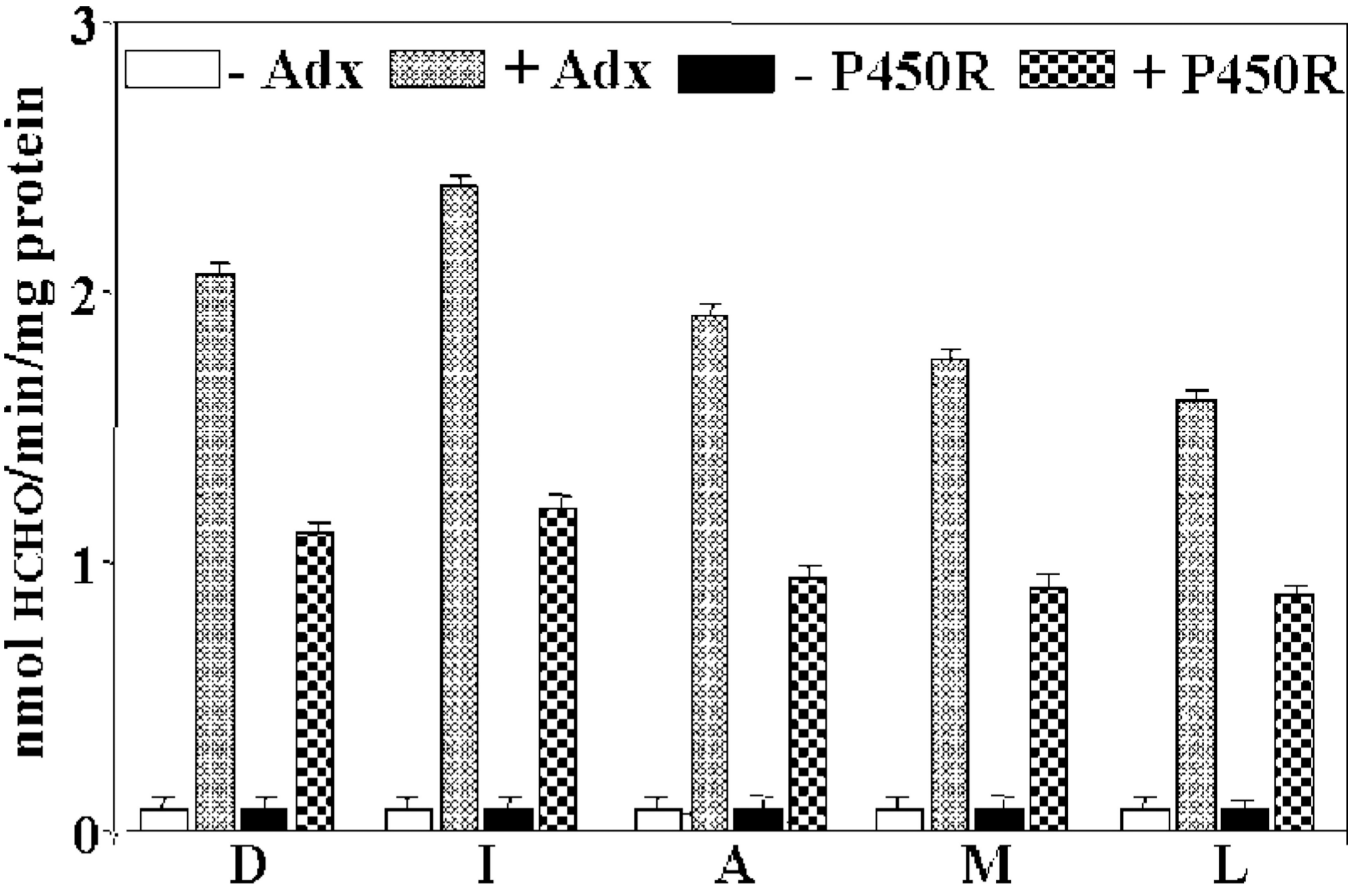

Fig. 9. Metabolism of neuroactive substrates by purified brain P450MT2

Reconstitution of enzyme activity was carried out in dilau-rylphosphatidyl choline liposome vesicles in the presence or absence of added 0.2 nM Adx + 0.02 nM Adr or 0.1 nM P450 reductase as described under "Materials and Methods." *N*-Demethylation activities were assayed using 100 μM each of the indicated substrates also as described under "Materials and Methods." The values represent average ± S.E. (n = 4). *A* = amitriptyline,*D* = dizapam, *I* = imipramine, *L* = lidocaine, *M* = morphine.

Table I

Purification of rat brain mitochondrial P450MT2

Protein content was measured by the method of Bradford (31), and P450 content was assayed by CO-reduced spectra as described under “Materials and Methods.”

Fractions	Protein content	P450	Specific Activity	Purification	Yield
	mg	*nmol*	*nmol /mg*	*-fold*	*% of input*
Mitoplasts	424	165	0.30		100
Cholate solubilized fraction	239	119	0.5	1.2	72
15% PEG fraction	84	71.4	0.8	2.2	43
OAA column fractions					
0.2% Emulgen eluate	5.4	10.8	2.1		
0.5% Emulgen eluate	12.6	30.9	2.4	6.2	19
Adx-Sepharose column fractions					
50 ml KCl eluate	0.2	0.5	3.2		
100 mM KCl eluate	0.43	5.9	13.7	35	3.5

Table II

Reconstitution of erythromycin N-demethylation activity with purified P450s

Reconstitution was carried out in the presence of dilarylphosphatidylcholine liposomes as described under "Materials and Methods." CO was gently bubbled through the enzyme suspension for 45 s (@60 bubbles/min) and control enzyme was bubbled through N_2 at the same rate. SKF525-A was added at a final concentration of 1 ml. The values represent average mean ± S.E. (n = 4).

P450 form	Electron transfer system	Inhibitor	Activity
			nmol/nmol P450
P4501A1	P450 reductase + NADPH	None	0.05 ± 0.01
P450MT2	None	None	0.03 ± 0.003[a]
P450MT2	Adx + Adr + NADPH	None	2.5 ± 0.1
P450MT2	Adx + Adr (−NADPH)	None	0.03 ± 0.005[a]
P450MT2	Adx + Adr + NADPH	CO	0.35 ± 0.01
P450MT2	Adx + Adr + NADPH	SKF 525-A	0.41 ± 0.02
P450MT2	P450 reductase + NADPH	None	1.3 ± 0.05

[a]The A412 values of these assay mixtures were 3.0−3.5-fold of the control tubes without added enzyme.