# Purification of a dichlorophenol-indophenol oxidoreductase from rat and bovine synaptic membranes: tight complex association of a glyceraldehyde-3-phosphate dehydrogenase isoform, TOAD64, enolase-γ and aldolase C

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NADH–dichlorophenol-indophenol oxidoreductases (PMOs) were purified from synaptic plasma membranes or synaptic vesicles (small recycling vesicles) from both bovine and rat brains and from a neuroblastoma cell line, NB41A3. Several isoforms could be identified in purified plasma membranes and vesicles. Purification of the enzyme activity involved protein extraction with detergents, $(NH_4)_2SO_4$ precipitation, chromatography under stringent conditions and native PAGE. PMO activity could be attributed to a very tight complex of several proteins that could not be separated except by SDS/PAGE. SDS/PAGE resolved the purified complex into at least five proteins, which could be micro-sequenced and identified unambiguously as hsc70, TOAD64 and glyceraldehyde-3-phosphate dehydrogenase tightly associated with the brain-specific proteins aldolase C and enolase-γ. Enzyme activity could be purified from both synaptic plasma membranes and recycling vesicles, yields being much greater from the latter source. Highly purified plasma membranes (prepared from a neuroblastoma cell line NB41A3 by iminobiotinylation of intact cells and affinity purification with avidin and anti-avidin antibodies under very stringent conditions) also displayed PMO activity tightly associated with TOAD64. The association of PMO in a tight complex was confirmed by its immunoprecipitation from cellular and membrane extracts of NB41A3 using antibodies directed against any component protein of the complex followed by immunodetection with antibodies directed against the other members. Antibodies also inhibited the enzyme activity synergistically. In addition, induction of the different components of the complex during dichlorophenol-indophenol stress was demonstrated by the S1 RNase-protection assay in synchronized NB41A3 cells. The role of the complex in membrane fusion and cellular response to extracellular oxidative stress during growth and development is discussed.

## INTRODUCTION

Several oxidoreductase systems have been identified in animal and plant plasma membranes [1–3], including NADH oxidase (controlled by growth hormones), iron reductases, NADH–cytochrome $b_5$ reductase, peroxidase, superoxide-generating NADPH oxidase, semi-dehydroascorbate reductase, xanthine oxidase and thioredoxin reductase. These *trans*-plasma-membrane oxidoreductases (PMOs) have been investigated in various animal cell types including erythrocytes, hepatocytes, thymocytes and neurons. Their activation is related to the redox control of receptor function and receptor-mediated signal-transduction pathways [4,5] and to exocytosis of clathrin-coated vesicles as well as to receptor-mediated and adsorptive endocytosis [6]. Rapid vesicle fusion with synaptic plasma membrane during exocytosis has been recently shown to be catalysed by an isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with very fast plasmenylethanolamine-dependent fusion activity [7]. In addition, there is growing evidence that PMOs are involved in the control of cell growth and development, acting as redox sensors to modulate cell proliferation in response to external pro- or anti-oxidants [3].

PMOs probably constitute the extracellular redox sensor for signalling external oxidative stress to the cell and inducing appropriate cellular response. They allow cells to react to radicals and reactive oxygen intermediates generated extracellularly, which damage cell membranes because of their high reactivity. Such radicals are formed by redox cycling agents (mainly aromatic polycyclic compounds and drugs) formed as by-products of enzymic reactions as a consequence of exposure to radiation or redox-active substances or by the Fenton reaction in the presence of metals. In addition, in the nervous system a number of neurotransmitters may act as redox cycling agents (catecholamines) or induce cell activation via oxygen activation (glutamate via the glutamate receptors) [8]. Therefore mechanisms are necessary to detoxify radicals and repair the damage caused by them. Antioxidant enzymes (superoxide dismutases, peroxidases, catalases, PMOs) are an important part of this cellular defence response. Induction of the genes encoding the antioxidant enzymes and repair activities is a crucial step in the cellular response to oxidative stress and requires that the cell senses increased levels of reactive oxygen species or radicals and transduces the signal into changes in gene expression.

Owing to the heterogeneity of PMOs within the cell surface, their nature and subunit composition have remained unknown so far. We have previously identified and characterized a PMO in neuronal synaptic plasma membranes and in a neuroblastoma cell line, NB41A3 [9–12]. In the present paper, we report on the

Abbreviations used: DCIP, dichlorophenol-indophenol; SV, synaptic vesicle; SPM, synaptic plasma membrane; PMO, *trans*-plasma-membrane oxidoreductase; TNBT, Tetranitro Blue Tetrazolium; MAP, microtubule-associated protein.

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purification of this NADH–dichlorophenol-indophenol (DCIP) reductase from various sources in neural tissue. The enzyme is located in synaptic plasma membranes (SPMs) and recycling vesicles. PMOs from bovine and rat SPMs and synaptic vesicles (SVs) have similar properties and in all cases were purified in the form of a very tightly associated protein complex. Five of its major components could be identified unambiguously. The identification of these components has allowed the possible functions of this PMO to be better understood.

## MATERIALS AND METHODS

### Chemicals

Aprotinin, avidin, biotin, pepstatin, sodium nitrite, insoluble Protein A, Triton X-100, ferricyanide and NAD(P)(H) were purchased from Fluka, and Superose S12 was from Pharmacia. Anti-avidin antibody, DCIP, DT diaphorase (EC 1.8.1.4), 2-iminobiotin hydrazide and PMSF were from Sigma, and growth media including Dulbecco's modified Eagle's medium, fetal calf serum, trypsin and PBS were from Gibco.

### Primers for PCR amplification

These were synthesized by Amplimum®. The following primers were used (upper and lower primers respectively): 5′-GTG ACG AGG CCC AGA GCA AGA GAG-3′ and 5′-GGA GCC ACC GAT CCA CAC AGA GTA-3′ for $\beta$-actin; 5′-CAA GCG AAA GCA CAA GAA AGA CAT-3′ and 5′-ATA CCA AGG GAA AGA GGA GTG AGA-3′ for hsc-70; 5′-TGT CCA CGG CAC CCT CTT TCC TTG-3′ and 5′-CGA ATC CAC CCT CAT CAC CCA CAT-3′ for enolase-$\gamma$; 5′-AGC AGA AGA AGG AGT TGT CGG ATA-3′ and 5′-AAT CTC AGG GCT CCA CAT AGG CAC-3′ for aldolase C; 5′-CCA GAT GCC AGA CCA GGG AAT GAC-3′ and 5′-TGA CAG ACA TCC GCT CCT CAG TGC-3′ for TOAD64; 5′-CTC ACG GCA AAT TCA ACG GAC CAG+3′ and 5′-TTG GCA GGT TTC TCC AGG CGG CAC-3′ for GAPDH.

### Source material

PMOs were purified from bovine or rat brains or a neuroblastoma cell line, NB41A3. Bovine and rat brains were obtained immediately after slaughter and used to prepare either SVs by the procedure of Delorenzo and Freedman [13] or SPMs by the method of Cotman and Matthews [14].

PMO from either SPMs (i.e. plasma-membrane PMO) or SVs (vesicular PMO) were then prepared. The preparation method was identical for both types of membrane and involved in sequence: (a) protein extraction with 0.1 % Lubrol, (b) centrifugation at 100000 ***g*** for 4 h, (c) $(NH_4)_2SO_4$ precipitation of the supernatant and re-centrifugation, (d) chromatography on butyl-Sepharose, (e) chromatography on Superose S100 (Pharmacia); finally the active fractions were purified by PAGE under non-denaturing conditions, and PMO was revealed on native gel. Active bands were cut out and further submitted to SDS/PAGE for micro-sequencing.

PMOs were also purified and characterized from a neuroblastoma cell line, NB41A3. Conditions for cultivating NB41A3 cells and cell synchronization have been described previously [9,10]. Preparation of PMOs from NB41A3 cells was by a previously described procedure [9]. Briefly, the cells were surface labelled with iminobiotin, and the labelled plasma membranes were affinity-purified with avidin, anti-avidin antibodies and insoluble Protein A under high-stringency conditions, yielding very highly purified plasma membranes. The proteins were then extracted in 0.1 % Triton X-100 and submitted to PAGE under native conditions.

### Electrophoresis

Native PAGE was carried out as previously described [9]. After electrophoresis, the gel was immediately incubated with 0.275 mM Tetranitro Blue Tetrazolium (TNBT), 5 $\mu$M FAD and 0.65 mM NADH in 50 mM Tris/HCl buffer, pH 7.5, for 5–15 h at 4 °C, and the active bands were carefully excised. The active bands were then denatured at 100 °C in 1 % SDS/1 % 2-mercaptoethanol and submitted to SDS/PAGE. After electrophoresis, the proteins were revealed with Coomassie Blue or Amido Black for micro-sequencing or with silver stain (Pierce).

### Western blotting

Either commercial antibodies or those prepared in our laboratory were used for immunoprecipitation and immunodetection. Anti-hsc70 (monoclonal) was from Milan and recognizes both constitutive and inducible hsp70; anti-GAPDH (monoclonal) was from Anawa; anti-enolase-$\gamma$ (polyclonal) was from Milan and recognizes specifically the neuron-specific isoform of enolase; anti-(aldolase C/zebrinII) (monoclonal) was a gift from R. Hawkes (University of Calgary, Calgary, Alberta, Canada) and recognizes specifically the brain isoform; anti-TOAD was prepared against the synthetic microtubule-associated protein (MAP) peptide IPRITSDRLLIKGGK corresponding to the N-terminal segment of TOAD64. Polyclonal antibody 3C was raised against PMO III from bovine brain using the purified protein from excised native polyacrylamide gels as antigen.

Monoclonal antibody 9F6 was prepared against the purified major TNBT band from native PAGE. For the preparation of 9F6, biotinylated plasma membranes were prepared from a sheep neuroblastoma cell culture SCP. The purified plasma membranes were submitted to PAGE under native conditions and also displayed high PMO activity. The major band was excised and used as antigen for the preparation of monoclonal antibodies. Clone selection was performed by means of ELISA, screening positive clones against (in sequence): (a) purified sheep SPMs prepared from sheep brains; (b) biotinylated SCP plasma membranes; (c) TNBT-positive gel bands from NB41A3; (d) ADP–agarose (an affinity column for NADH dehydrogenases). After a final screening according to these criteria the antibody was found to be monoclonal. The final clone also detects the IPRITSDRLLIKGGK peptide (from TOAD64) on ELISA.

### Enzymic and protein determinations

PMO activity was determined as DCIP– or ferricyanide–NADH reductases by previously described procedures [9]. Inhibition studies were carried out under similar conditions, but before the assay the samples were incubated for 4 h at 4 °C with 3 mg of antibody.

Adenylate cyclase was measured as described by Moulet and Dreyer [15]. Other enzymic determinations were as described in [10]. Protein was usually determined by the micro-Pierce procedure, following the guidelines of the manufacturer.

For Western-blotting, extracts (usually raw but where specified prepurified by native PAGE) or purified protein preparations were submitted to SDS/PAGE. The gels were blotted on PVDF membrane for 1 h, and the membrane was then blocked with 1 % BSA, incubated with primary antibody (usually diluted 1:2000 for polyclonal antibodies and 1:500 for monoclonal antibodies) for 10 h at 4 °C, washed, incubated with the secondary

peroxidase-conjugated antibody and revealed by enhanced chemiluminescence (Amersham).

Micro-sequencing was performed on purified proteins submitted to SDS/PAGE and blotted on PVDF membranes.

### S1 nuclear-protection assay

This followed essentially the procedure of Sambrook et al. [16] with appropriate modifications. mRNA was isolated from NB41A3 cells. Before mRNA isolation, cells had been synchronized as described by Zurbriggen and Dreyer [10], then stressed for 1 h in the presence of agents as indicated. Purified mRNA was used for the preparation of cDNA fragments from $\beta$-actin (approx. 869 bp, to be used as a control) and from the five identified proteins [i.e. hsc70 (476 bp), aldolase C (602 bp), enolase-$\gamma$ (696 bp), GAPDH (607 bp) and TOAD64 (866 bp)] by standard reverse transcription and PCR using appropriate upper and lower primers (see above). PCR-amplified fragments were then cloned into pBS KS+ and transfected into competent bacteria. The fragments were checked for correct sequence amplification by restriction analysis and cDNA sequencing after purification of the fragments on CsCl. For S1 mapping, the cDNA was prepared, dephosphorylated with calf intestinal phosphatase and radiolabelled with [$\gamma$-$^{32}$P]ATP using $T_4$ polynucleotide kinase (50000–200000 c.p.m./probe). Hybridization was performed overnight at 50 °C for most probes except for aldolase C (40 °C) using 20–40 $\mu$g of mRNA from synchronized cells. S1 treatment was then performed with 150 units of S1 nuclease. A fragment of cDNA from $\beta$-actin (859 bp) was used as internal control in all cases. After incubation and washing, the probes were submitted to PAGE, and autoradioagraphy was performed for 20 h at room temperature. Evaluation was performed against the internal control.

**Table 1 Purification of PMO from bovine SVs, bovine SPMs and neuroblastoma NB41A3 cells**

Rat SVs were obtained from the cortices of 50 rat brains; bovine SVs were prepared from 100 g of bovine cortex; rat SPMs were prepared from 100 rat brains. PMO from NB41A3 cells was obtained from $5 \times 10^6$ cells. PMO was measured as NADH–DCIP reductase. Values in parentheses are percentages.

| | Total protein (mg) | Total activity (m-units) | Specific activity (m-units/mg) | Purification (fold) |
|---|---|---|---|---|
| Rat SVs | | | | |
| Vesicles | 12.5 (100) | 125 (100) | 10 | 1 |
| Solubilization | 6.25 (50) | 125 (100) | 20 | 1 |
| $NH_4(SO_4)_2$ | 0.5 (4) | 44 (35) | 88 | 9 |
| Gel filtration | 0.15 (1.2) | 41 (33) | 310 | 31 |
| Butyl-Sepharose | 0.03 (0.2) | 20 (16) | 710 | 71 |
| Bovine SVs | | | | |
| Vesicles | 21 (100) | 1155 (100) | 55 | 1 |
| Solubilization | 10 (50) | 1155 (100) | 110 | 2 |
| $NH_4(SO_4)_2$ | 0.7 (3.3) | 308 (27) | 440 | 8 |
| Gel filtration | 0.175 (0.8) | 231 (20) | 1320 | 24 |
| Butyl-Sepharose | 0.035 (0.16) | 106 (9) | 3036 | 55 |
| Rat SPMs | | | | |
| Vesicles | 31 (100) | 682 (100) | 22 | 1 |
| Solubilization | 15.5 (50) | 682 (100) | 44 | 2 |
| $NH_4(SO_4)_2$ | 1.24 (4) | 191 (28) | 154 | 7 |
| Gel filtration | 0.186 (0.6) | 171 (25) | 924 | 42 |
| Butyl-Sepharose | 0.074 (0.2) | 116 (17) | 1570 | 72 |
| NB41A3 neuroblastoma cells | | | | |
| Intact cells | 6.2 (94) | 53.9 (32) | 8.7 | 0.34 |
| Cell extract | 6.6 (100) | 168.3 (100) | 25.5 | 1 |
| Plasma membranes | 0.01 (0.0015) | 1.35 (0.008) | 135 | 5.3 |

## RESULTS

### Purification of PMOs from SPMs and SVs

PMO was purified from rat or bovine plasma membranes prepared from fresh brains (Table 1). The method of preparation of SPMs was developed by Cotman and Matthews [14] and Whittaker et al. [17] and gives membranes of high purity, with negligible contamination with dehydrogenase activity from other intracellular membranes, a strong requisite when the aim is to purify a dehydrogenase activity present in small amounts in the plasma membrane. Plasma membranes account for less than 1% of total intracellular membranes, so the SPM yield is very low. However, comparative electrophoretic analysis showed that PMO is also present in high amounts in recycling small vesicles (SVs), an intracellular component of synaptic terminals present in high concentration. Standard methods of Delorenzo and Freedman [13] modified by Benett et al.[18] have been designed for the preparation of SVs in good yield. For this reason, the methods of purification of PMO were generally developed using SVs (either bovine or rat) and later used for PMO preparation from SPMs. All biochemical properties (chromatographic behaviour, solubilization behaviour, electrophoretic mobility, inhibition pattern, etc.) were found to be identical for the two types of preparation.

PMO from SVs or SPMs were solubilized with different detergents, the most effective being Lubrol (at 1% in 5–10 mg/ml membrane protein in 10.9% saccharose). After incubation for 1 h at 4 °C followed by centrifugation at 150000 ***g*** for 1 h, the extract contained more than 50% of the total protein, and all the PMO activity was recovered in the supernatant. The extract was further fractionated by $(NH_4)_2SO_4$ precipitation. PMO activity was mainly recovered in pellets from the 50–70%-satd. fraction. A purification factor of about 8 was usually achieved at this step. Usually a first precipitation at 40% saturation was performed to remove most proteins and large quantities of detergent that floats on the supernatant under these conditions.

The active pellets from the 50–70%-satd. $(NH_4)_2SO_4$ precipitation step were diluted to about 30% $(NH_4)_2SO_4$ saturation and applied to a Superose 12 column equilibrated in 50 mM Tris/HCl buffer, pH 7.5, containing 0.1% Lubrol and 0.1 M NaCl (Figures 1A1 and 1A2). The chromatographic behaviour was reproducible and identical whatever the source of PMO (SPMs or SVs from either bovine or rat brain). At least threefold purification was achieved at this step.

Active fractions were pooled, reprecipitated at 70% $(NH_4)_2SO_4$ saturation and applied to a butyl-Sepharose column equilibrated in 100 mM Tris/HCl buffer, pH 7.5, that contained 0.1% Lubrol and 50%-satd. $(NH_4)_2SO_4$. After thorough washing, the enzyme was eluted at 30% $(NH_4)_2SO_4$ saturation (Figure 1B). Attempts to elute the enzyme in a stepwise or continuous gradient did not improve the yield or purity. Purification achieved at this chromatographic step was usually about 1.7-fold.

The active fractions were pooled, concentrated on Centriprep-10 (Amicon) and dialysed against 50 mM Tris/HCl, pH 7.5, until the $(NH_4)_2SO_4$ concentration was less than 0.2% saturation. The concentrated enzyme preparation was then submitted to PAGE under native conditions. Specific staining for PMO activity revealed two major intensively coloured bands. SV and SPM preparations from bovine membranes behave similarly to rat preparations in which two intensively coloured bands could also be detected (PMO II and PMO III; Figure 2A). The bands were excised, denatured at 100 °C for 3–10 min in 3% SDS/2 mM

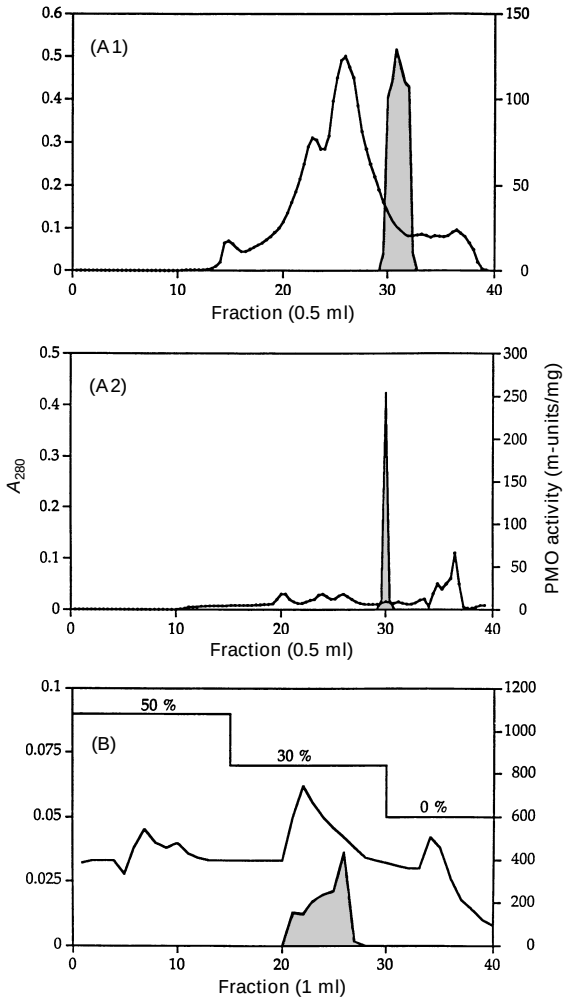


**Figure 1 Purification of PMO from bovine SVs and SPMs**

(**A1**) Purification of bovine vesicular PMO on Superose 12; (**A2**) purification of rat PMO on Superose 12 from SPMs [in both cases PMO was purified after membrane extraction in 1% Lubrol-PX and $(NH_4)_2SO_4$ precipitation at 60–70% saturation]; (**B**) purification of bovine PMO on butyl-Sepharose after Superose 12 chromatography and reprecipitation at 70% $(NH_4)_2SO_4$ saturation. ——, $A_{280}$; ⊡, PMO activity measured as DCIP reductase.

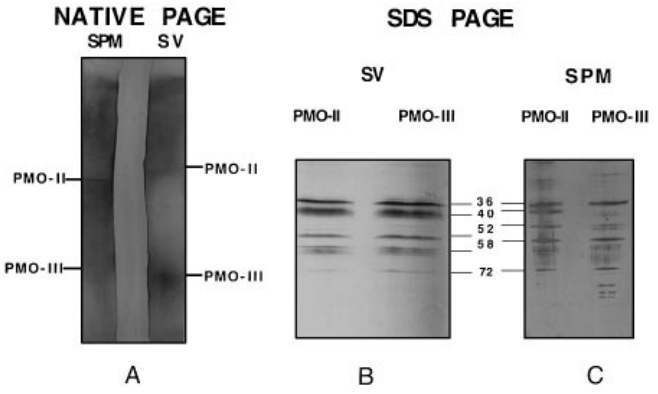


**Figure 2 PAGE of bovine PMO**

(**A**) Native PAGE of SV and SPM PMO detected by specific staining with TTNB (see the Materials and methods section). (**B**) SDS/PAGE of SV PMO II and PMO III. (**C**) SDS/PAGE of SPM PMO II and PMO III. For (**B**) and (**C**), the major bands from (**A**) were excised, denatured and submitted to SDS/PAGE.

**Table 2 Sequences of the component proteins of the PMO complex**

p52-I, p52-II and p73 are internal sequences from CNBr-cleaved peptides. The other sequences are N-terminal sequences.

| | Sequence | Identification | Identity (%) |
|---|---|---|---|
| p36 | VKVGVNGFGRIGRL | GAPDH | 100 |
| p40 | PHSYPALSAEQKKE | Aldolase C | 100 |
| p52-I | DVAASEFYRSGKYDLDFKSDDP | Enolase-$\gamma$ (neuron-specific) | 100 |
| -II | RIGAEVYHNL | Enolase-$\gamma$ | 100 |
| p58 | XPRITSDRLLIKGGK | TOAD64/Ulip/CMRP | 94 |
| p73 | VNHFIAEFXR | Hsc70 | 90 |

EDTA/1% 2-mercaptoethanol and submitted to SDS/PAGE (Figures 2B and 2C). SPM PMO II and PMO III displayed identical patterns of five bands on SDS/PAGE, with apparent molecular masses of 36, 40, 52, 58 and 75 kDa (Figure 2C). Preparations from SVs displayed the same pattern of two proteins after native electrophoresis (Figures 2A), which on SDS/PAGE resolved into five major proteins, with minor bands due to contamination; the major bands were identical with those obtained from SPMs (Figure 2B).

PMO was further purified in high amount by the same scheme from about 15 kg of bovine brain (50 brains), yielding a final amount of about 0.2 mg of purified protein after butyl-Sepharose chromatography, which was submitted to native PAGE and then SDS/PAGE. The five bands were submitted to micro-sequencing, by which the proteins could be unambiguously identified (Table 2). p36 displayed the N-terminal sequence VKVGVNVFRIGRL with 100% identity with bovine GAPDH. p40 displayed the sequence PHSYPALSAEQKKE with 100% identity with fructose bisphosphate aldolase C (brain-specific). p58 displayed the sequence XPRITSDRLLIKGGK which corresponds to TOAD64 with 94% identity over 15 amino acids, the first amino acid being unidentified.

p52 and p75 were blocked N-terminally. In order to identify these proteins we obtained a new preparation of the PMO complex from 100 bovine brains. The proteins were again prepared by the above protocol, and the purified p52 and p75 were submitted to CNBr treatment, liberating several peptides from purified p52. Two were micro-sequenced and had the sequences DVAASEFYRSGKYDLDFKSPDDP and RIGAEVYHNL, with 100% identity in both cases with enolase-$\gamma$ (after $Met^{243}$ and $Met^{182}$ respectively). For purified p75, CNBr treatment liberated five peptides; the major one was micro-sequenced and gave the N-terminal sequence VNHIFIAEFXR, which corresponds to bovine hsc70 (cleaved after $Met^{237}$) with 91% identity over 11 amino acids, one amino acid being unidentified.

### Purification of PMOs from NB41A3 cells

Plasma membranes from cultured cells can be efficiently affinity-labelled on the outer surface with iminobiotin and then purified to a very high degree with avidin, anti-avidin antibodies and Protein A–Sepharose [9]. The plasma membranes thus obtained are very pure but the yields are very low. Nevertheless plasma membranes from about $5 \times 10^8$ NB41A3 cells could be prepared

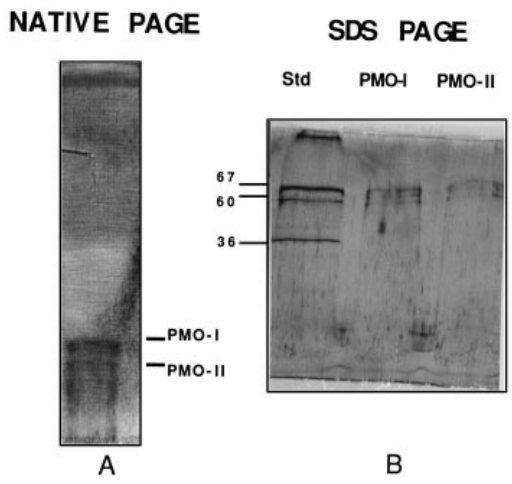


**Figure 3 Electrophoresis of PMOs from NB41A3 cells**

Conditions were similar to those in Figure 2, but preparations of iminobiotinylated, solubilized and affinity-purified plasma membranes from NB41A3 cells (about $10^6$) were used. In (**A**) the purified plasma-membrane preparation was submitted to native PAGE. In (**B**) the major bands from (**A**) were excised and submitted to SDS/PAGE.

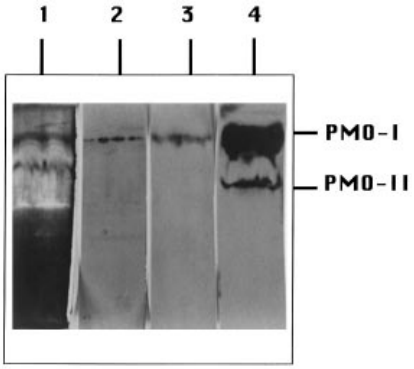


**Figure 4 Immunodetection of PMO components after native PAGE**

NB41A3-cell extracts were prepared from $10^6$ cells and submitted to PAGE under native conditions. After blotting on PVDF membrane, Western blot was performed with different antibodies: lane 1, specific activity stain with TTNB; lane 2, immunodetection with anti-TOAD; lane 3, immunodetection with anti-GAPDH; lane 4, immunodetection with anti-neuronal enolase-$\gamma$.

by this procedure (Table 1). After membrane solubilization the extract was submitted to PAGE under native conditions, which revealed two bands of PMO activity (see [13,14] and Figure 3A). Each band was excised and submitted to SDS/PAGE (Figure 3B). Both bands consisted of at least three proteins of about 67, 61 and 58 kDa. Immunodetection with 9F6 and anti-TOAD64 revealed the presence of TOAD64 in the 58 kDa band. Furthermore the 58 and 61 kDa bands were biotinylated proteins, since they could be identified with anti-biotin antibodies on blotted PVDF membranes after SDS/PAGE. Therefore these proteins do originate from the plasma membrane and display extracellular domains. The yield in such a preparation is low and therefore it is quite possible that the other proteins found in bovine plasma membranes as components of the PMO complex are present in NB41A3-cell preparations but below the level of detection under these conditions. p61 and p63 remain to be identified.

### Immunoprecipitation of PMOs

In an extract from NB41A3-cell plasma membranes two major PMO bands were detected by PAGE under native conditions (Figures 3A and 4). After blotting of native gels on PVDF membranes, immunodetection with specific antibodies against the different proteins revealed that PMO I contains TOAD64, GAPDH, enolase-$\gamma$ and hsp70. The last two proteins were also detected in PMO II (Figure 4).

The components of the PMO complex were immunoprecipitated with an antibody against one of the proteins. All immunoprecipitations were carried out in 0.75 M NaCl, i.e. under conditions of very high stringency to maximally prevent non-specific co-precipitation. Then the immunoprecipitates were washed thoroughly in 0.75 M NaCl. After SDS/PAGE and blotting on PVDF membranes, Western-blot analysis was performed using antibodies against the various components. The results of these studies are shown in Figure 5 and summarized in Table 3. In all immunoprecipitates the bands at 72, 58, 52, 40 and 36 kDa are clearly visible from the [$^{35}$S]Met autoradiography as major bands (Figure 5, top left gel, arrows). In all immunoprecipitates TOAD64 and neuron-specific enolase were detected. The monoclonal antibody raised against the extracellular domain of PMO was only detectable in its own immunoprecipitate and in the immunoprecipitate with monoclonal anti-enolase-$\gamma$ antibodies. Also anti-(aldolase C) antibody was only detectable in its own immunoprecipitate (Figure 5). Anti-hsp72 antibody was detected in all immunoprecipitates (not shown).

### Inhibition of PMO activity by antibodies

PMO activity in NB41A3-cell extracts was inhibited after preincubation with antibodies against the different components (Table 4). Incubation with a single antibody against only one identified protein of the complex caused partial inhibition (about 27–50 %), and combinations of antibodies against only two or three components yielded stronger but still incomplete inhibition (up to 80 %). However, the inhibition was almost complete ($> 95$ %) after incubation with antibodies against all five proteins, under which conditions the residual activity was barely higher than that of the control (without membrane proteins). The observed inhibition was specific and cannot be attributed to unspecific interference of the antibodies with the assay, as shown by controls performed with unrelated antibodies (not shown). All assays were repeated at least three times. In all cases the assays were reproducible, and standard deviation was less than 3 %.

### Induction of PMO activity after oxidative stress

The presence of five components in the PMO complex leads to the question of whether the proteins are induced after stress or whether they are constitutively expressed in unstimulated cells and aggregate in a stress situation. To answer this question, induction of the components of the PMO complex was investigated by an S1 nuclear-protection assay. Partial cDNA (about 476–869 bp in length) corresponding to the five identified proteins was prepared from mRNA of NB41A3 cells by reverse transcription–PCR. Cells were then stressed under various

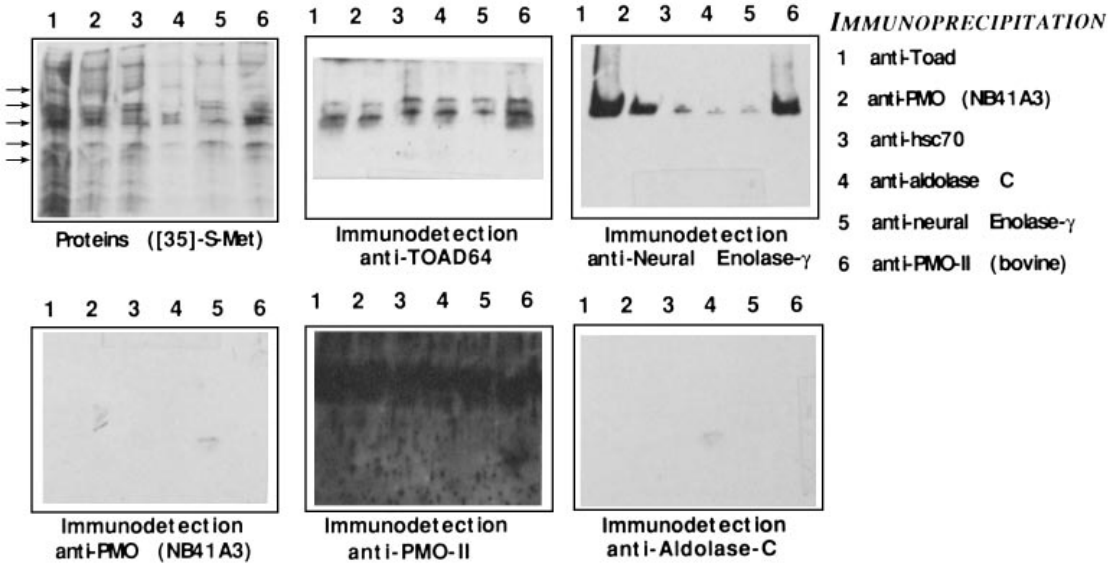


**Figure 5 Immunoprecipitation and immunodetection of PMO components**

Extracts from about $10^6$ NB41A3 cells, grown in the presence of [$^{35}$S]methionine, were immunoprecipitated with different antibodies, and the immunoprecipitates were Western-blotted for immunodetection. Top left gel, SDS/PAGE and PVDF blotting of immunoprecipitates (protein profiles from $^{32}$S autoradiography): lane 1, immunoprecipitation with anti-TOAD polyclonal antibody; lane 2, immunoprecipitation with anti-PMO (extracellular domain) monoclonal antibody; lane 3, immunoprecipitation with anti-hsc70 monoclonal antibody; lane 4, immunoprecipitation with anti-(aldolase C) monoclonal antibody; lane 5, immunoprecipitation with anti-enolase-$\gamma$ monoclonal antibody; lane 6, immunoprecipitation with anti-(PMO II) polyclonal antibody. The other five gels show immunodetection of the same blot with different antibodies as indicated.

**Table 3 Immunoprecipitation and immunodetection of components of the PMO complex**

Extracts from about $10^6$ NB41A3 cells were used for each immunoprecipitation. Western blots were performed on each immunoprecipitate after separation by SDS/PAGE of its components and blotting on PVDF membranes. ID, immunodetection; IP, immunoprecipitation.

| | IP anti-TOAD | IP 9F6 | IP anti-hsc70 | IP anti-GAPDH | IP anti-(aldolase C) | IP anti-enolase-$\gamma$ | IP 3C |
|---|---|---|---|---|---|---|---|
| ID anti-TOAD | + | + | + | + | + | + | + |
| ID 9F6 | + | + | + | | | + | |
| ID anti-hsc70 | + | | + | | | | |
| ID anti-GAPDH | + | + | + | + | | | |
| ID anti-(aldolase C) | | | | | + | | + |
| ID anti-enolase-$\gamma$ | + | + | + | + | | + | |

**Table 4 Inhibition of PMO activity with antibodies**

Cell extract (1 mg/ml) was incubated without substrate for 4 h at 4 °C with 3 mg of antibody as indicated, then DCIP reductase was measured spectrophotometrically. A control without antibody (no inhibitor) or without probe was run in parallel for comparison. All measurements were performed at least three times. The values are means ± S.D., 100% corresponding to the value of the control without inhibitor. Values in parentheses are percentage of control.

| Inhibitor | DCIP reductase activity (m-units/min per mg) |
|---|---|
| No inhibitor | 6.5 ± 0.2 (100) |
| Anti-PMO (monoclonal 9F6) | 4.4 ± 0.2 (68) |
| Anti-PMO (polyclonal) | 4.3 ± 0.2 (66) |
| Anti-Toad | 4.8 ± 0.2 (73) |
| Anti-hsc70 | 3.8 ± 0.1 (58) |
| Anti-GAPDH | 3.2 ± 0.1 (50) |
| Anti-(aldolase C) | 3.3 ± 0.2 (50) |
| Anti-enolase $\gamma$ | 4.0 ± 0.2 (61) |
| Anti-TOAD64 + Anti-hsc70 | 4.7 ± 0.1 (72) |
| Anti-(aldolase C) + Anti-enolase $\gamma$ | 1.4 ± 0.1 (22) |
| All antibodies combined | 0.4 ± 0.1 (6) |
| Control: all antibodies combined, no enzyme | 0.2 ± 0.1 (3) |

conditions and mRNA was isolated. The agents used to stress NB41A3 cells included *N*-acetylcysteine (up to 400 $\mu$M), DCIP (up to 400 $\mu$M) and $H_2O_2$ (up to 200 $\mu$M). Normally the agents were applied for between 20 and 60 min. As shown in Figure 6, a concentration-dependent increase in the components of the PMO complex was observed in stressed NB41A3 cells. The induction was not dramatic, varying from 100 to about 140%, but nevertheless was significant. Since the natural substrate of PMO is not known, one can assume that the conditions for induction are not yet optimized. Indeed induction is only observed when cells have previously been synchronized. In non-synchronized cells, induction is not observed because of the heterogeneity of the population.

## DISCUSSION

The enzyme activity isolated in this study is probably part of the PMO system previously described in neural cells [1–3,9–12]. In view of the important functions of PMO, a thorough characterization of its composition has long been desirable, but hampered by the difficulty of obtaining plasma membranes in high quantity (sufficient for protein purification and micro-

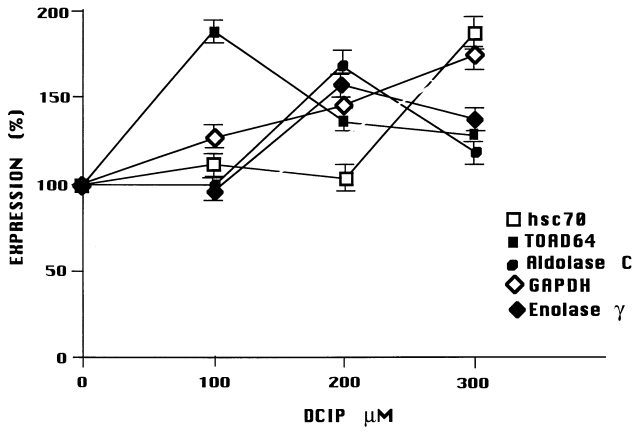


**Figure 6 Induction of PMO components by oxidative stress**

NB41A3 cells ($10^6$) were synchronized (see the Materials and methods section) and stressed with DCIP at the indicated concentrations. mRNA was isolated and used for the S1 nuclear-protection assay.

sequencing) and of good quality (uncontaminated by other intracellular membranes and oxidoreductases from intracellular sources). Plasma membrane represents a minute portion of total cellular membranes ($< 1\,\%$) and preparations of it are generally contaminated, mainly by oxidoreductase-rich microsomal membranes and mitochondria. Here we have used brain as a source material, as it is very rich in plasma membranes from synaptic terminals and axons and is also highly enriched in recycling SVs. Both types of membrane can be readily prepared in high yield and high purity, sufficient for our purposes, by following well-established standard protocols. We have therefore been able to purify PMOs from different brain sources and compare their properties.

PMOs were purified from either SPMs or SVs, and, in each case, they displayed identical composition and biochemical properties at each step of the purification. PMOs from bovine and rat brain cortices also have identical properties. Electrophoretic mobility differences among the two to six isoenzymes found on native PAGE may be traced to small changes in the stoichiometry of the various proteins in the complex, or to the presence of other as yet unidentified components that are more labile or present in smaller amounts. Isoforms may also originate from various degrees of post-transcriptional modification (phosphorylation) of some components. These points remain to be elucidated.

The complex is made up of at least the following five proteins identified by micro-sequencing: (1) NADH oxidoreductase homologous to GAPDH; (2) aldolase C; (3) neuron-specific enolase-$\gamma$; (4) a novel protein TOAD64; (5) a heat shock protein hsc70. The proteins remain tightly associated despite the drastic means used for purification (detergents, high ionic strength, chaotropic agents, electrophoresis, chromatographic separation according to different biophysical criteria, etc.).

GAPDH is a multifunctional glycolytic enzyme found in the cytoplasm, regulated by association with cellular structures, and closely associated with the plasma membrane [19]. A cAMP-binding regulated phosphoprotein [20] with protein kinase activity, auto-ADP-ribosylated by NO [21], it catalyses triad junction formation, regulates actin filament networks and bundles microtubules (together with aldolase) in an ATP-dependent process [22] and, like the SV nucleotide transporter [23], is associated with SVs.

GAPDH is stress-inducible, similar to the ATP-binding heat-inducible protein p37 [24], being inducible by stresses such as metals, chelators, heat [25] and *N*-methyl-D-aspartate [26]. Its expression is regulated by hypoxia [27], and it is markedly induced during age-induced cell death. Overexpression of the particulate membrane-associated GAPDH has a direct role in neuronal apoptosis [28]. GAPDH interacts specifically with the cytoplasmic domain (C-terminus) of Alzheimer's $\beta$-amyloid precursor protein [29]. In addition, GAPDH is identical with the nuclear uracil DNA glycosylase, can be translocated to the nucleus [30] and acts as a non-histone protein and a possible activator of transcription in neurons [31]. GAPDH is also related to $\pi$-cristallin, a stress protein [32], and NAD(P)H–quinone reductase/$\zeta$-cristallin, the expression of which is up-regulated by the antioxidant-response element [33].

It is well known that a wide variety of genes (over 200 in rodents) contain sequences that encode proteins with strong similarity to GAPDH [34,35]. The demonstration that distinct isoforms of GAPDH that possess widely disparate catalytic properties exist in mammals suggests that one potential explanation for the transcription of nominally 'non-functional' GAPDH messages is that the polypeptides encoded by those messages are utilized for a separate and distinct catalytic or regulatory function [7,34,35]. This agrees with the numerous observations on GAPDH.

Recently Glaser and Gross [7] demonstrated that a GAPDH isoform catalyses the rapid (millisecond) fusion of vesicles with the plasma membrane. The fusion is highly selective for plasmenylethanolamine, a major chemical constituent of SV membranes and plasma membranes that facilitates non-enzyme-catalysed membrane fusion by adopting an inverted hexagonal phase, thus decreasing the activation energy of the rate-limiting step of the fusion process. GAPDH was shown to be the protein that catalyses the fusion process in a highly plasmenylethanolamine-specific manner. It is probably this GAPDH isoform that is part of the PMO complex purified in the present study. Taking these data together, a function for GAPDH in the PMO complex can be rationalized.

The second component, aldolase C, preferentially associates co-operatively with GAPDH [36] and catalyses GAPDH inactivation in the presence of extracellular stressors [36], so its association within the complex may have a regulatory function. Oxidants (e.g. ferricyanide) also cause self-inactivation of aldolase [37]. Aldolase, like NADH dehydrogenases, binds NADH with high affinity ($K_D$ $6.0 \times 10^{-6}$), inducing strong conformational changes [38]. Aldolase levels are up-regulated in proliferating cells and during anoxia [39]. In addition, aldolase tightly interacts with the following cellular structures, the extent and nature of these interactions having great ontogenic significance: cytoskeletal elements [40]; SVs (via its association with SVAPP-120 [41]); c-adaptin (a clathrin assembly-promoting protein) [41]. Residues 32–52 of aldolase are highly similar to actin and to regions found in all actin-binding proteins [42]. Aldolase tightly and specifically associates with actin itself through residues $Lys^{107}$, $Arg^{148}$, $Lys^{229}$ and $Arg^{42}$ [43] and to tubulin, microfilaments and cytoskeletal proteins ($\alpha$-actinin, S-100) through the C-termini [43], acting to polymerize tubulin and cross-link microtubules [44]. These data suggest that the PMO complex is involved in the gliding of SVs along cytoskeletal proteins. Furthermore the aldolase C isoform is identical with zebrinII [45] and predominantly expressed in Purkinje cells of the cerebellar cortex. The zebrin$^+$ Purkinje cells are clustered together and arrayed to form a family of parasagittal zonation, sym-

metrical about the midline running rostrocaudally throughout the cerebellar cortex [46]. The spread of aldolase C/zebrinII expression follows clearly defined developmental stages and plays a critical role in developing cerebellum during functional maturation from disappearance of the embryonic molecular compartmentation to synaptic maturation of cerebellar circuitry, emergence of functional modules and receptive fields [46]. Compartmentation appears to be the epigenetic modification of local biochemistry to best fit local function in developing brain. These data nicely correlate with observations that PMOs are active at selective stages during the cell cycle [10] and play a role in growth and development [1–3]. Interestingly NADH diaphorase has also been described as a compartmentation marker complementary to aldolase C/zebrinII [46]. Notably, most diaphorases in brain tissue are unrelated to NO synthase, but derive from NAD(P)H-utilizing enzymes and could be related to PMOs, at least in part [11].

The third component, enolase-$\gamma$, is up-regulated during differentiation [47]. Transient expression is found at a period corresponding to the beginning of myelination, when cells have to form a large amount of membranous structure, coinciding with the formation of the protoplasmic processes [48]. Transcriptional control mechanisms regulating neuronal enolase gene expression are present at early developmental stages [49]. Enolase has the properties of a stress protein: $\alpha$-enolase is homologous to the multifunctional $\tau$-cristallin (which possesses enolase activity), encoded by a single gene and displays several activities and plays different roles during differentiation [50].

The other stress protein in the PMO complex, the heat shock cognate 70 kDa protein (hsc70), is the clathrin-uncoating ATPase, an essential protein in mammalian cells that belongs to the most abundant and conserved subset of stress proteins. It is located in the cytoplasm [51], but nuclear localization and accumulation near or at the plasma membrane in stressed cells and in synaptosomal membranes [52] has been observed. Hsc70 is identical with $\beta$-internexin, an MAP, and with a ubiquitous intermediate filament-associated protein [53]. Two other cytoskeleton-associated proteins (MAP69 and thermin A) are highly related to if not identical with hsc70 [54].

The fifth PMO component displays 100 % identity at its N-terminal sequence with several recently identified neuron-specific proteins, probably members of a same family: TOAD64, CRMP and ULIP, all of which are homologous ($> 80\,\%$) to the unc33 protein from *Caenorhabditis elegans*. The proteins display no similarity to NADH dehydrogenases, but high homology to allantoinase (reminiscent of $\delta$-cristallin, another stress protein with arginosuccinate lyase activity). TOAD64 (acronym standing for turned on after division), a neuron-specific protein [55], is among the earliest proteins expressed after cell differentiation, in advance of most other neuronal proteins, soon after the cell has committed itself to express a neuronal phenotype. This indicates a role for PMO in early development and growth, as observed Progenitor cells do not express TOAD64 and therefore it is a marker for postmitotic cells [55]. ULIP (acronym standing for unc33-like protein) was identified as a stathmin-binding phosphoprotein [56]. Stathmin (or oncoprotein-18) is a ubiquitous 19 kDa cytoplasmic phosphoprotein, highly expressed in neurons of the developing brain, and is a major substrate for MAP kinase during heat or chemical stress [57] and phosphorylated in response to neurotrophins [58]. Phosphorylation occurs in response to epidermal growth factor or *in vitro* by cAMP-dependent protein kinase and p34cdc2 [59]. CRMP (acronym standing for collapsin response mediator protein) [60] is 80 % homologous to unc33 in *C. elegans*, a protein without which nematode movements are unco-ordinated and axon outgrowth is aberrant, reminiscent of the disordered outgrowth of insect neurons with perturbations of semaphorin expression. Unc33 mutants induce nerve shivering and collapse. Collapsin itself is a member of the semaphorin family [61], a family of axon-guidance molecules that contribute to axonal pathfinding during neuronal development by inhibiting growth cone extension [62]. It induces the collapse and paralysis of neuronal growth cones. CRMP is a mediator protein required for blocking collapsin-induced growth cone collapse [60]. Several homologous proteins have been described that all contain semaphorin domains and act as repulsive molecules inducing axon guidance during growth.

A role for radicals in the regulation of neuronal outgrowth and remodelling or in growth cone collapse has been reported, so a function for PMO in this process is understandable. From all these considerations we believe that the PMO complex plays its major role as a regulator/sensor of the extracellular redox environment, a function that appears to be particularly important in axonal guidance during development. The presence of three chaperone-like proteins (enolase-$\gamma$, hsc70 and GAPDH) and a cytoskeletal-binding protein (aldolase) in addition to regulated phosphoproteins (GAPDH, TOAD64) as tightly bound components may be relevant to its function in cellular response to oxidative stress. Three proteins of the complex are multifunctional glycolytic enzymes, generating cytosolic ATP as required for e.g. vesicle recycling/gliding along the cytoskeleton; their $Ca^{2+}$- and substrate-dependent tight association ($K_D > 10^{-5}$ M) is well documented [39]. From its composition, the PMO complex is probably involved in cellular signalling through the MAP kinase pathway via either stathmin or collapsin/semaphorin. PMO-mediated activation of the MAP kinase pathway has been described. Also the presence of GAPDH, a plasmenylethanolamine-selective membrane-fusion protein [7], implies a function for PMO in vesicle fusion to the plasma membrane in response to extracellular oxidative stress and radicals.

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