Suenson, E., Lutzen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513-522.
Tran-Thang, C., Kruithof, E. K. O., & Bachmann, F. (1984) *J. Clin. Invest. 74*, 2009-2016.
Trexler, M., Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 7401-7406.
Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 2104-2110.
Wallen, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122-134.
Wu, H. L., Chang, B. I., Wu, D. H., Chang, L. C., Gong, C. C., Lou, K. L., & Shi, G. Y. (1990) *J. Biol. Chem. 265*, 19658-19664.
Zioncheck, T. F., Powell, L. M., Rice, G. C., Eaton, D. L., & Lawn, R. M. (1991) *J. Clin. Invest. 87*, 767-771.

# The Type II Isoform of Bovine Brain Protein L-Isoaspartyl Methyltransferase Has an Endoplasmic Reticulum Retention Signal (...RDEL) at Its C-Terminus†

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ABSTRACT: Bovine brain is known to contain two major isoforms of protein L-isoaspartyl methyltransferase (PIMT), an enzyme that facilitates repair of atypical L-isoaspartyl peptide bonds in proteins. Although the two isoforms can be separated by anion-exchange chromatography, they appear to have similar, if not identical, substrate specificities in vitro. The more basic type I isoform has been extensively characterized, and its complete sequence has been reported. The present study was undertaken in an attempt to understand the structural and functional uniqueness of the more acidic type II isoform. Electrospray mass spectrometry of the intact enzymes revealed that the type II isoform is approximately 43 amu heavier than the type I isoform. Cyanogen bromide cleavage followed by HPLC with on-line mass analysis revealed that the type II isoform contains a unique C-terminal fragment which is 43 amu heavier than the corresponding fragment from the type I isoform. Amino acid composition analysis and direct sequencing of this fragment indicate that the type II isoform ends in the sequence ...RDEL, while the type I is known to end in ...RWK. Since ...RDEL, like ...KDEL, serves as an effective endoplasmic reticulum retention signal, we propose that the type II isoform serves to repair damaged proteins within the endoplasmic reticulum or, perhaps, within some other specialized compartment of the cell. Comparison of the protein sequences of the two bovine brain isoforms to DNA sequences for rodent PIMT reported by others suggests that the type II isoform may be produced by splicing within the codon for $Arg_{224}$.

Protein L-isoaspartyl methyltransferase (PIMT,[1] E.C. 2.1.1.77), an enzyme that transfers the active methyl group from *S*-adenosyl-L-methionine (SAM) to the α-carboxyl of L-isoaspartyl residues in peptides and proteins, has been found in a wide variety of organisms, including several vertebrate and invertebrate animals, bacteria, a plant, and a fungus (O'Connor & Clarke, 1985; Johnson et al., 1991a; Fu et al., 1991). PIMT is unusual in that it has extremely broad substrate specificity, and natural polypeptides are typically methylated with a low stoichiometry by the purified enzyme (Clarke, 1985). The low stoichiometries of methylation are consistent with the view that isoaspartyl residues are found only in atypical subpopulations of polypeptides that have undergone spontaneous isomerization of aspartate residues and/or spontaneous deamidation of asparagine residues [reviewed in Johnson et al. (1990) and Ota and Clarke (1990)].

A normal, α-carboxyl-linked aspartate residue is one of the products that follows the spontaneous demethylation of the labile α-carboxyl methyl ester (Johnson et al., 1987a). The in vitro methylation of an isoaspartate-containing form of age-damaged calmodulin by purified bovine brain PIMT, coupled with spontaneous demethylation, has been shown to restore a substantial amount of its functional activity (Johnson et al., 1987b). It has been proposed that, in vivo, PIMT may play an important part in the repair of proteins that have been damaged by spontaneous isoaspartate formation (Aswad, 1984; Murray & Clarke, 1984).

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[1] Abbreviations: PIMT, protein L-isoaspartyl methyltransferase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; βME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CNBr, cyanogen bromide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid: TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.

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In mammals, it has been observed that PIMT exists in a number of isoforms, with isoelectric points ranging from 4.9 to 7.4, with varying tissue and species distributions. For example, rabbit brain exhibits four isoforms, of p*I* 5.7, 5.9, 6.2, and 6.5, while rabbit liver exhibits only two isoforms, of p*I* 5.5 and 5.8 (N. Freitag and D. Aswad, unpublished observations). Bovine brain shows two major isoforms, designated here as type I (p*I* 6.5), and type II (p*I* 5.6) (Aswad & Deight, 1983), while the calf thymus is reported to contain only a p*I* 4.95 isoform (Kim & Paik, 1978). Human erythrocytes possess two isoforms, of p*I* 6.6 and 5.5 (Ota et al., 1988), in roughly equal amounts (Gilbert et al., 1988). The PIMT isoforms found in rat testes change as the rat matures. Of the three isoforms observed (p*I* 6.1, 6.7, and 7.4), only the most basic one undergoes a marked increase in specific activity concomitant with the onset of spermatid production (Cusan et al., 1982). No clear differences have been observed in either the substrate specificity or the kinetic properties of the various PIMT isoforms examined (Aswad & Deight, 1983; O'Connor et al., 1984; Ota et al., 1988; Cusan et al., 1982).

The bovine brain type I and type II isoforms appear to have the same $M_r$ by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Aswad & Deight, 1983). The amino acid sequence of the type I isoform from bovine brain has been determined (Henzel et al., 1989), and its calculated molecular weight is 24476. Complete sequences have also been published for type I PIMT from human erythrocytes (Ingrosso et al., 1989) and rat brain cDNA (Sato et al., 1989), showing striking sequence identities of 97.3% and 96.0%, respectively, compared to the bovine type I sequence.

We, and others, have been interested in the structural basis for the different isoforms of PIMT in a given organism or cell type. Ingrosso et al. (1991) have reported that the more acidic type II PIMT isoform of human erythrocytes differs from the human erythrocyte type I isoform previously sequenced (Ingrosso et al., 1989), by the substitution of ...DD for ...WK at the C-terminus of the molecule. In the present study we have investigated the structural differences between the two major isoforms of bovine brain PIMT using enzymatic and chemical digestion, high-performance liquid chromatography, N-terminal sequencing, and mass spectrometry. We have determined that the last two amino acids of the bovine brain type I sequence, ...WK, are replaced by the sequence ...DEL in the type II enzyme. Electrospray mass spectrometry of the two isoforms and their cyanogen bromide fragments, combined with sequencing of 60% of the type II isoform, suggest that there are no other differences in sequence or posttranslational modifications. This substitution results in an ...RDEL sequence at the type II C-terminus. The ...RDEL sequence has been shown to result in the retention of certain proteins to the lumen of the endoplasmic reticulum (ER) (Andres et al., 1990; Nandan et al., 1990; Wang & Gudas; Clarke et al., 1991; Haugejorden et al., 1991). This suggests that the type II isoform may be specialized for the repair of proteins in the ER or other compartments of the cell.

## Experimental Procedures

*Purification of PIMT.* Purification procedures, except HPLC, were carried out at 4 °C. A crude cytosolic fraction was prepared from frozen bovine cerebral cortex and subjected to batch filtration through Whatman DE-23 anion-exchange resin, ammonium sulfate precipitation, and dialysis as previously described (Aswad & Deight, 1983). The dialysate was chromatographed on a 5- × 45-cm column of Q-Sepharose Fast Flow anion-exchange resin (Pharmacia) as previously described (Henzel et al., 1989). The type II isoform of PIMT was eluted from the column with a 4-L linear gradient of 0–0.4 M sodium chloride in the equilibration buffer. Most of the type I isoform eluted in the 1.5-L isocratic wash before the gradient. The type I and type II pools were concentrated separately by precipitation with ammonium sulfate (70% saturation). Ammonium sulfate pellets produced by centrifugation at 13000*g* for 15 min were dissolved in a minimal volume of 10 mM Tris-HCl, pH 8.25, 0.5 mM EDTA, 15 mM βME, and 100 μM PMSF and dialyzed against 20 mM potassium phosphate, pH 7.2, 2 mM EDTA, 15 mM βME, and 100 μM PMSF until the conductance of the dialysate measured less than 1 mmho. Affinity chromatography of the dialyzed pools using SAH coupled to Sepharose 4B, dialysis, and concentration to approximately 0.5 mg/mL by ultrafiltration were performed as described previously for type I PIMT (Henzel et al., 1989). Enzyme activity was monitored throughout by reaction with [*methyl*-$^3$H]SAM using γ-globulin as substrate, according to Aswad and Deight (1983). One unit of activity corresponds to the transfer of 1 pmol of methyl groups to the substrate/min.

Final purification of the affinity-purified PIMT by reversed-phase HPLC was carried out on a 2.1- × 100-mm Aquapore RP-300 C8 column with a 3.2- × 15-mm C4 guard column (Applied Biosystems). Solvent A was 0.1% (w/v) TFA in water. Solvent B was 0.1% (w/v) TFA in a 4:1 (v/v) acetonitrile/water mixture. A linear gradient from 0 to 100% B over 40 min was performed at 0.5 mL/min. Absorbance was monitored at 280 nm.

*Enzymatic and Chemical Digestion of PIMT.* (*A*) *Cyanogen Bromide (CNBr) Digestion.* Ten nanomoles of PIMT was digested in 700 μL of 70% (v/v) formic acid containing 1% (w/v) CNBr. Reaction tubes were purged with nitrogen, sealed, and left in the dark at room temperature for 9 h. Digests were then frozen at −70 °C until used.

(*B*) *Lys-C Digestion.* Three units of endoproteinase Lys-C (Boehringer Mannheim) was dissolved in 100 μL of 0.1 M Tris-HCl, pH 8.2, containing 10% (v/v) glycerol. This was kept at −70 °C until used. A total of 24 μg of HPLC-purified PIMT that had been evaporated to dryness in a Speed Vac (Savant) was dissolved in 100 μL of 0.2 M Tris base and 5 M urea. The pH was adjusted to 8.5 with 30 μL of 0.1 M hydrochloric acid. A total of 5 μL of the above Lys-C solution was added, and the mixture was incubated at room temperature for 24 h and then frozen at −70 °C until used.

(*C*) *Asp-N Digestion.* Endoproteinase Asp-N (Boehringer Mannheim) was dissolved in water to a concentration of 0.04 mg/mL. A total of 0.6–3 nmol of PIMT that had been evaporated to dryness was dissolved in 50 mM sodium phosphate, pH 8, and 0.01% (w/v) sodium dodecyl sulfate, and an appropriate volume of the Asp-N solution was added to give final concentrations of 7 μg/mL protease and 277 μg/mL PIMT. Digestions proceeded for 17 h at 37 °C and were stopped by freezing at −20 °C.

(*D*) *Chymotrypsin Digestion.* Five hundred picomoles of the CNBr C-terminus of type II PIMT (purified as described below) was dried and redissolved at a concentration of 100 μg/mL in 10 mM triethanolamine, pH 8.5, and chymotrypsin (Sigma) was added to a final concentration of 2 μg/mL. The peptide was digested for 12 h at room temperature and then frozen at −70 °C until sequencing was done.

*Reversed-Phase HPLC of Digests of PIMT.* In order to purify the C-terminal CNBr fragment, a CNBr digest of 5 nmol of type II PIMT was diluted 1:10 in HPLC solvent A [0.1% (w/v) TFA in water] and injected onto a 4.6- × 100-mm Aquapore RP-300 C8 column with a 4.6- × 30-mm C8 guard

column (Applied Biosystems) at 16.9% solvent B [0.1% (w/v) TFA in a 4:1 (v/v) mixture of acetonitrile and water]. At a flow rate of 1.5 mL/min, a linear gradient to 31.9% B over 40 min caused the nonformylated C-terminal peptide to elute between 27 and 28 min. Absorbance was monitored at 214 and 280 nm. The 27–28-min peak was collected and concentrated by evaporation.

Lys-C digests of approximately 800 pmol of either type I or type II PIMT were injected onto a 2.1- × 100-mm Aquapore RP-300 C8 column with a 3.2- × 15-mm C4 guard column (Applied Biosystems). Solvents used were the same as those described in the previous paragraph. A linear gradient from 0 to 68.7% B over 55 min was employed after the system was held at 0% B for 10 min, using a flow rate of 0.5 mL/min. Absorbance was monitored at 214 and 280 nm. Peaks were collected and concentrated by evaporation for sequencing and composition analysis.

Asp-N digests of approximately 500 pmol of either type I or type II PIMT were injected onto a 4.6- × 100-mm Aquapore RP-300 C8 column with a 4.6- × 30-mm C8 guard column (Applied Biosystems). Solvent A was 0.1% (w/v) TFA in water, and solvent B was 0.1% (w/v) TFA in acetonitrile. A linear gradient from 0 to 45% B over 90 min was employed after a 5-min hold at 0% B, using a flow rate of 0.5 mL per min. Absorbance was monitored at 214 and 280 nm. Peaks were collected and concentrated by evaporation for sequencing and composition analysis.

*Mass Spectrometry.* Electrospray mass spectrometry was conducted essentially as described by Ling et al. (1991). Briefly, intact PIMT, at 0.5 mg/mL in 30% (v/v) acetonitrile and 0.05% (v/v) TFA in water, was infused at 5 μL/min into a SCIEX API III triple-quadrupole Ionspray mass spectrometer using a Harvard Apparatus infusion pump. Quadrupole 1 was scanned from 300 to 2000 Da using a 0.5-Da step size and a 1.2-ms dwell time, resulting in a total scan time of 4.26 s. The spectra were collected in the multiple-count averaging mode, with 30–40 individual scans per spectrum. Parameters were the same for mass spectrometry of the CNBr digests, except that all scans were saved separately in a continuous scanning mode.

*HPLC of CNBr Digest for Mass Spectrometry.* A CNBr digest of approximately 10 nmol of PIMT was injected onto a 4.5- × 150-mm, 5-μm C18 Nucleosil reversed-phase column maintained at 40 °C. Solvent A was 0.05% (w/v) TFA in water, and solvent B was 0.05% (w/v) TFA in acetonitrile. A linear gradient from 0 to 70% B over 60 min was performed at 0.5 mL/min. The output stream was split 30:1 with a Valco tee, resulting in a flow rate into the mass spectrometer of 17 μL/min. UV-absorbing peaks (214 nm) were also collected by hand for further analysis.

*Peptide Sequencing.* Automated N-terminal sequencing by Edman degradation was carried out on an Applied Biosystems Model 477A pulsed liquid-phase sequencer equipped with an on-line 120A PTH-amino acid analyzer. Routinely, 5–200 pmol of peptide material was sequenced for 10–30 amino acid cycles.

*Acid Hydrolysis and Amino Acid Composition Analysis.* Approximately 200 pmol of the C-terminal CNBr fragment of type II PIMT was dried under vacuum. Empty hydrolysis tubes were subjected to identical treatment and used to monitor background contamination. After being purged with nitrogen, the hydrolysis tubes were heated at 105 °C for 24 h with 6 N hydrochloric acid vapor in a Savant AP100 hydrolyzer. Hydrolysates were derivatized with *o*-phthaldialdehyde, and amino acids were quantified by reversed-phase HPLC according to Jones (1986) with reference to amino acid standard mixture H (Pierce). A 4.6- × 100-mm C18 column with a 4.6- × 15-mm C18 guard module (Rainin Microsorb 80-OPA-C3) was used to separate the derivatized amino acids. Solvent A was 0.1 M sodium acetate, pH 7.2, 4.5% (v/v) methanol, and 0.5% (v/v) tetrahydrofuran. Solvent B was methanol. Derivatized amino acids were eluted at 1 mL/min using the following gradient: 0–8% B over 5 min, 8–15% B over 1 min, 15–30% B over 15 min, 30–45% B over 4 min, and 45–80% B over 20 min. Peaks were detected using a Gilson 121 fluorometer, with excitation at 305–395 nm and emission at 430–470 nm.

*Rat Brain Subcellular Fractionation and Isoelectric Focusing.* Six male Sprague-Dawley rats, each weighing ~380 g, were anesthetized with Nembutal and decapitated. Whole brains were rapidly removed and placed in ice-cold homogenization buffer (0.3 M sucrose, 20 mM potassium-HEPES, pH 7.4, 5 mM magnesium chloride, 10 mM sodium chloride, and 5 μg/mL aprotinin). All subsequent procedures were performed at 4 °C. Brainstems were removed and the brains (11.7 g total) were minced and homogenized, in several steps, in 15 volumes of homogenization buffer using 5 strokes of a Wheaton 55-mL Teflon/glass homogenizer. The subcellular fractionation scheme of Diliberto and Axelrod (1976) was followed. The homogenate was centrifuged at 1000*g* for 10 min. The resulting supernatant was centrifuged at 17400*g* for 30 min to pellet mitochondria and synaptosomes. The resulting supernatant was centrifuged at 100000*g* for 1 h to pellet microsomes. The resulting high-speed supernatant was designated as the "cytosolic" fraction. The microsomes were lysed by resuspending with an Ultra Turrax SDT homogenizer in 5.5 mL of deionized water. After 1 h at 4 °C, 5.5 mL of 0.3 M potassium chloride was added and the washed membranes were removed by centrifugation for 1 h at 100000*g*. The supernatant (soluble "microsomal" fraction) and the cytosolic fraction were dialyzed overnight against 4 changes of 4 L of 10 mL Tris-HCl, pH 8.25, 0.5 mM EDTA, 15 mM βME, and 100 μM PMSF. Glycerol was added to 10% (v/v), and the dialysates were stored at −20 °C until they were concentrated to 140–340 units of PIMT activity/mL using YM10 membranes in an Amicon ultrafiltration system. Protein concentrations were determined by the method of Lowry et al. (1951), after proteins were precipitated with 10% (w/v) trichloroacetic acid.

Nondenaturing isoelectric focusing gels contained 5% (v/v) glycerol, 4% (w/v) acrylamide, 0.21% (w/v) bisacrylamide, 0.1% (w/v) Triton X-100, 1% (w/v) each of pH 5–7 and pH 6–8 carrier ampholytes, 0.07% (w/v) riboflavin, 0.1% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. Gels were cast in 6- × 140-mm tubes and allowed to polymerize for 8 h at room temperature, 300 cm away from a 20-watt fluorescent light. Electrophoresis and gel elution were carried out at 4 °C. Gels were overlaid with 140 μL of overlay solution [5% (v/v) glycerol and 0.5% (w/v) each of pH 5–7 and pH 6–8 carrier ampholytes] and prefocused for 15 min at 200 V, 30 min at 300 V, and then for 30 min at 400 V, with 20 mM sodium hydroxide in the upper tank (negative terminal) and 10 mM phosphoric acid in the lower tank (positive terminal). Gel tops were rinsed with deionized water and loaded with samples (140 μL) and overlay solution (140 μL). Samples contained 20–70 units of PIMT in 10% (v/v) glycerol and 0.5% (w/v) each of pH 5–7 and pH 6–8 carrier ampholytes. The gels were focused for 12 h at 400 V and then for 1 h at 800 V. All gels were run in duplicate. Bovine brain type I and type II PIMT were run in parallel as a control. To elute

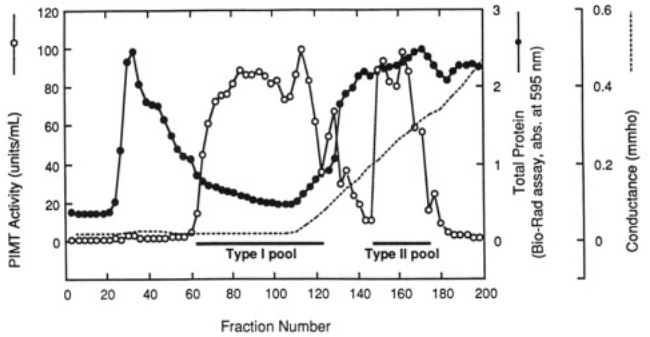


FIGURE 1: Separation of type I and type II PIMT by anion-exchange chromatography. A total of 480 mL of DE-23 filtrate containing 4.6 g of protein and 120000 units of PIMT activity was chromatographed on a 5- × 45-cm Q-Sepharose Fast Flow column as described in Experimental Procedures. Fractions (25 mL) were collected throughout. Horizontal bars indicate the fractions combined for subsequent concentration and further purification. A 20-μL portion of every third fraction was assayed for PIMT activity (open circles) as described by Aswad and Deight (1983). For protein determinations (closed circles), 100 μL of every third fraction was mixed with 2 mL of a 5:1 dilution of Bio-Rad protein assay concentrate, and absorbance at 595 nm was monitored. The sodium chloride gradient (dotted line) was monitored by diluting 100 μL of every fifth fraction in 5 mL of deionized water and measuring conductance.

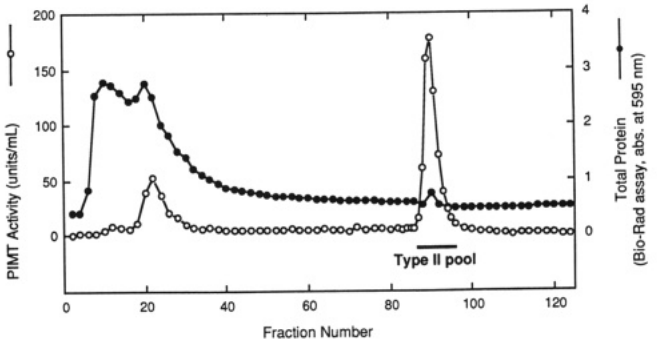


FIGURE 2: Affinity chromatography of type II PIMT. After dialysis, 50 mL from the Q-Sepharose pool of the type II isoform, containing 470 mg of protein and 23 600 units of PIMT activity, was loaded onto a 2.5- × 10-cm column of SAH-coupled Sepharose 4B and chromatographed as described for the type I isoform in Henzel et al. (1989). Fractions (10 mL) were collected throughout. SAM (20 μM) was included in the elution buffer beginning at fraction 82, and fractions 87–95 were combined for concentration and HPLC purification, as described in Experimental Procedures. PIMT activity and protein concentration were monitored as described in the legend to Figure 1.

proteins from the gels, 1-cm slices of the gels were soaked for 2 days in 1.3× gel weight of 10 mM potassium phosphate, pH 7.2, 2 mM EDTA, 15 mM βME, 25 μM PMSF, and 5% (v/v) glycerol. Slices from blank gels used for pH determinations were soaked in 1 mL of boiled, deionized water. The gels spanned a pH range from 4.3 to 7.5. The isoelectric points of the type I and type II isoforms from rat brain were 7.2 and 6.1, respectively, while the bovine brain type I and type II isoforms had isoelectric points of 6.6 and 5.7, respectively.

Eluents from the gel slices were assayed for PIMT activity using synthetic isoaspartyl δ-sleep-inducing peptide (Bachem Bioscience, Inc.) as substrate. Reactions contained 60 μL of gel eluent, 10 μL of substrate (50 μM final), 20 μL of 5× buffer (final concentrations: 20 mM sodium citrate, 62 mM sodium phosphate, and 2 mM EDTA, pH 6.1), and 10 μL of [*methyl*-$^3$H]SAM (5 μM final, ~2000 dpm/pmol). After a 2-min preincubation at 30 °C, the SAM solution was added and the incubation was continued for 6 min. Reactions were stopped by adding 100 μL of 0.4 M sodium borate, pH 10.2, 4% (w/v) sodium dodecyl sulfate, and 4% methanol. Carboxyl methyl esters were quantified using a methanol diffusion assay (Johnson et al., 1991b).

## RESULTS

*Purification of the Type II Isoform of PIMT.* The type I and type II isoforms of bovine brain PIMT were separated by anion-exchange chromatography on Q-Sepharose Fast Flow (Figure 1), as described in the type I purification scheme of Henzel et al. (1989). Subsequent affinity chromatography on SAH-Sepharose (Figure 2) resulted in a pure type I pool, judged by Coomassie staining after SDS–PAGE (Figure 3, lane 4). However, several contaminants remained in the type II pool after affinity chromatography (Figure 3, lane 2). It was therefore necessary to purify further the type II pool by reversed-phase HPLC (Figure 4). As a control, identical treatment of the type I pool was carried out. The affinity-purified type I PIMT yielded a single peak on reversed-phase HPLC (Figure 4, bottom trace), whereas the affinity-purified type II PIMT yielded a number of peaks (Figure 4, top trace). The largest of these was taken to be pure type II PIMT, since it had the same retention time as type I PIMT and appeared as a single band of the appropriate $M_r$ by SDS–PAGE (Figure 3, lane 3). All subsequent analyses were made using HPLC-purified PIMT.

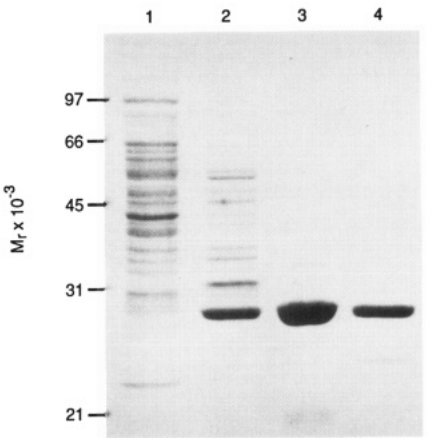


FIGURE 3: SDS–PAGE of PIMT. Lane 1: type II, Q-Sepharose pool (Figure 1), 5 μg of protein. Lane 2: type II, after affinity chromatography (Figure 2), 2 μg of protein. Lane 3: type II, after reversed-phase HPLC purification (Figure 4), 3.5 μg of protein. Lane 4: type I, after affinity chromatography, 1.7 μg of protein. The gel was prepared according to Laemmli and Favre (1973) with 4.8% (w/v) acrylamide in the stacking gel and 10% (w/v) acrylamide in the separating gel.

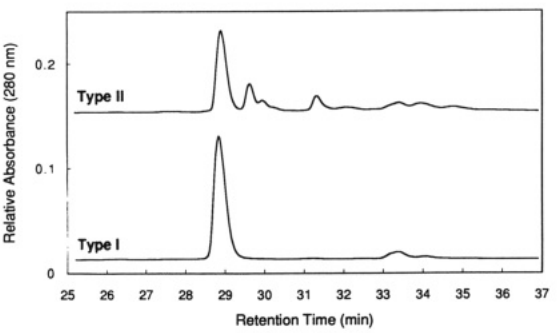


FIGURE 4: Reversed-phase HPLC purification of PIMT. For each trace, 24 μg of affinity-purified and concentrated protein was injected after being mixed with 0.1 volume of 10% (w/v) TFA and was chromatographed as described in Experimental Procedures. Both isoforms elute at 28.9 min. The small peak at 33.3 min was also present in a blank run where no sample was injected.

*Comparison of Type I and Type II Isoforms by Liquid Chromatography/Mass Spectrometry.* The mass of the type

Table I: Masses of CNBr Fragments of PIMT

| peptide no. | residues | predicted mass | mass[a] type I | mass[a] type II |
|---|---|---|---|---|
| 1 | 1–31 | 3460.94[b] | 3460.53 ± 0.05 | 3460.39 ± 0.17 |
| 2 | 32–46 | 1721.91 | | |
| 3 | 47–63 | 1738.87 | 1738.42 ± 0.13 | 1738.35 ± 0.20 |
| 4 | 64–98 | 3635.11 | | |
| 2, 4[c] | 32–46, 64–98 | 5355.02 | 5354.18 ± 0.07 | 5354.03 ± 0.13 |
| 5 | 99–128 | 3213.59 | 3213.03 ± 0.18 | 3213.31 ± 0.18 |
| 6 | 129–144 | 1638.89 | 1638.82 ± 0.08 | 1638.40 ± 0.21 |
| 7 | 145–190 | 4517.17 | 4516.27 | 4517.40 |
| 8 | 191–204 | 1605.76 | 1605.39 ± 0.00 | 1605.61 ± 0.48 |
| 9 | 205–208 | 439.56 | 439.20 | 439.31 |
| 10 | 209–226 (WK) | 2233.60 | 2232.77 ± 0.25 | |
| 10 | 209–227 (DEL) | 2276.57 | | 2276.02 ± 0.03 |

[a] Formylation is a side reaction of CNBr cleavage in formic acid. Several peptides were observed in both the formylated and unformylated forms. In these cases, the mass given is the mean of the two masses and the error is half the difference, after the mass of a formyl group (28.01 amu) is subtracted from the observed mass of the formylated peptide. Before correction, the mean difference between the 14 such pairs observed was 28.021 ± 0.395. After 28.01 was subtracted from the formylated masses, the mean of the absolute differences between the pairs was 0.30 ± 0.24. This provides a useful gauge of the reliability of the mass determinations of these peptides. [b] Includes the mass of the N-terminal acetyl group. [c] Refers to disulfide-linked pair.

I isoform, as determined by electrospray mass spectrometry, was 24 478.6 ± 1.8. The type II isoform, with a mass of 24 521.7 ± 1.5, was 43.0 ± 2.3 amu heavier. In an attempt to localize the cause(s) of this mass difference, the two PIMT isoforms were cleaved with cyanogen bromide (CNBr), and the resulting peptides were separated by reversed-phase HPLC with continuous on-line analysis via electrospray mass spectrometry. The type I chromatogram revealed peaks with masses corresponding to 8 of the 10 expected CNBr cleavage products (Figure 5; Table I). No peaks were observed with the masses expected for fragments 2 and 4 (residues 32–46 and 64–98, respectively) (Table I). These are presumed to be joined by a disulfide bond because a doublet with a mass corresponding to the sum of their masses (without the two hydrogen atoms lost upon oxidation of the Cys residues) was observed at 38 min (Figure 5; Table I). Fragment 1 (residues 1–31) from both isoforms had a mass consistent with acetylation at the N-terminus of the protein.

The only apparent differences between the UV traces of the type I and type II CNBr digests were in the 28–31-min region, which has been expanded in Figure 5B. Two of the type I peaks, at 28.7 and 29.3 min (designated as peaks 10 and $10_f$), had masses of 2233.01 and 2260.53 amu, respectively. The first of these had a mass expected for the C-terminal CNBr fragment (residues 209–226). The second peak had a mass corresponding to a formic acid adduct of this peptide, about 28 amu greater. (Formylated versions of most of the CNBr fragments of both isoforms were observed; see Table I and Figure 5.) In the type II trace, these are replaced by two new peaks at 29.7 and 30.3 min (designated as peaks 10′ and $10_f'$), with masses of 2275.99 and 2304.06 amu, respectively. The mass difference between peak 10 of the type I trace and peak 10′ of the type II trace was 42.98 amu. The mass difference between peak $10_f$ of the type I trace and peak $10_f'$ of the type II trace was 43.53 amu. Considering the variability in these mass determinations (see Table I, footnote *a*), these differences of 42.98 and 43.53 amu match the difference found for the intact PIMT isoforms of 43.0 ± 2.3 amu. This suggests that this C-terminal peptide is likely to contain the only difference between the two isoforms.

*Purification and Characterization of the C-Terminal CNBr Fragment of PIMT II.* A CNBr digest of 10 nmol of HPLC-purified type II PIMT was separated by reversed-phase HPLC, as described in Experimental Procedures, in order to purify the C-terminal peptide (not shown). A portion of the HPLC-purified peptide was subjected to acid hydrolysis and amino acid composition analysis. The resulting composition (Table II) is consistent with the C-terminal CNBr fragment expected for type I PIMT, except for the absence of one Lys residue, and the presence of an extra Asx, an extra Glx, and an extra Leu residue. Cys, Pro, and Trp were not determined. A mass difference of 43 amu would be consistent with the substitution of an Asp, a Glu, and a Leu residue for a Trp and a Lys residue. The yields of Val, Ile, and Tyr were less than expected, presumably due to incomplete hydrolysis, a common problem with hydrophobic regions of polypeptides (Jones, 1986).

Table II: Amino Acid Composition of Type II C-Terminal CNBr Fragment

| | observed[a] | expected ...RWK | expected ...RDEL[b] |
|---|---|---|---|
| Asx | 1.9 ± 0.05 | 1 | 2 |
| Glx | 3.0 ± 0.13 | 2 | 3 |
| Ser | 1.0 ± 0.06 | 1 | 1 |
| His | | 0 | 0 |
| Gly | 0.9 ± 0.09 | 1 | 1 |
| Thr | 1.0 ± 0.05 | 1 | 1 |
| Arg | 1.0 ± 0.04 | 1 | 1 |
| Ala | | 0 | 0 |
| Tyr | 0.6 ± 0.06[c] | 1 | 1 |
| Met | | 0 | 0 |
| Val | 1.1 ± 0.06[c] | 2 | 2 |
| Phe | | 0 | 0 |
| Ile | 0.2 ± 0.01[c] | 1 | 1 |
| Leu | 2.2 ± 0.13 | 1 | 2 |
| Lys | 2.2 ± 0.11 | 3 | 2 |

[a] Recovered picomoles from a given hydrolysate was divided by a constant that minimizes the total error between the observed values and the type I composition. Values are means of quadruplicate determinations (± standard deviations). Trp, Pro, and Cys were not determined. [b] Based on the composition ultimately revealed by sequencing (Tables III and IV). [c] Reduced yield presumably due to incomplete hydrolysis in a hydrophobic region of the peptide (Jones, 1986).

Table III: Sequencing of Type II C-Terminal CNBr Fragment[a]

| cycle | pmol of PTH-amino acids recovered R | D | E | L |
|---|---|---|---|---|
| 16 | 1.5 | 1.7 | 2.4 | 0.8 |
| 17 | **10.6** | 1.6 | 2.0 | 1.4 |
| 18 | 8.4 | **5.4** | 1.6 | 0.9 |
| 19 | 6.4 | 5.4 | **3.0** | 1.1 |
| 20 | 4.7 | 3.9 | 3.1 | **1.9** |

[a] Automated N-terminal sequencing by Edman degradation was performed on approximately 45 pmol of peptide. The first 16 cycles gave unambiguous matches to the type I sequence, from $Gly_{209}$ to $Ser_{223}$.

The C-terminal CNBr fragment of the type II isoform was sequenced by Edman degradation, revealing a possible substitution of ...DEL in the type II sequence for the teminal ...WK of the type I sequence (Table III). This sequence is consistent with both the mass spectrometry data and the composition data mentioned above. However, because of the ambiguity of the sequencing results due to low yields, we decided to carry out a second sequence analysis. To this end, the type II C-terminal CNBr fragment was further digested using chymotrypsin, to produce shorter, more easily sequenced fragments. When the whole digest was sequenced, three peptides were detected, consistent with cleavage at $Tyr_{212}$ and at $Trp_{222}$. As suggested by our first findings, one of these peptides had the sequence SRDEL (Table IV).

*Analysis of Proteolytic Fragments of PIMT.* Although the mass data suggest that the two PIMT isoforms are the same up to $Arg_{224}$, the possibility remained for alternate ordering

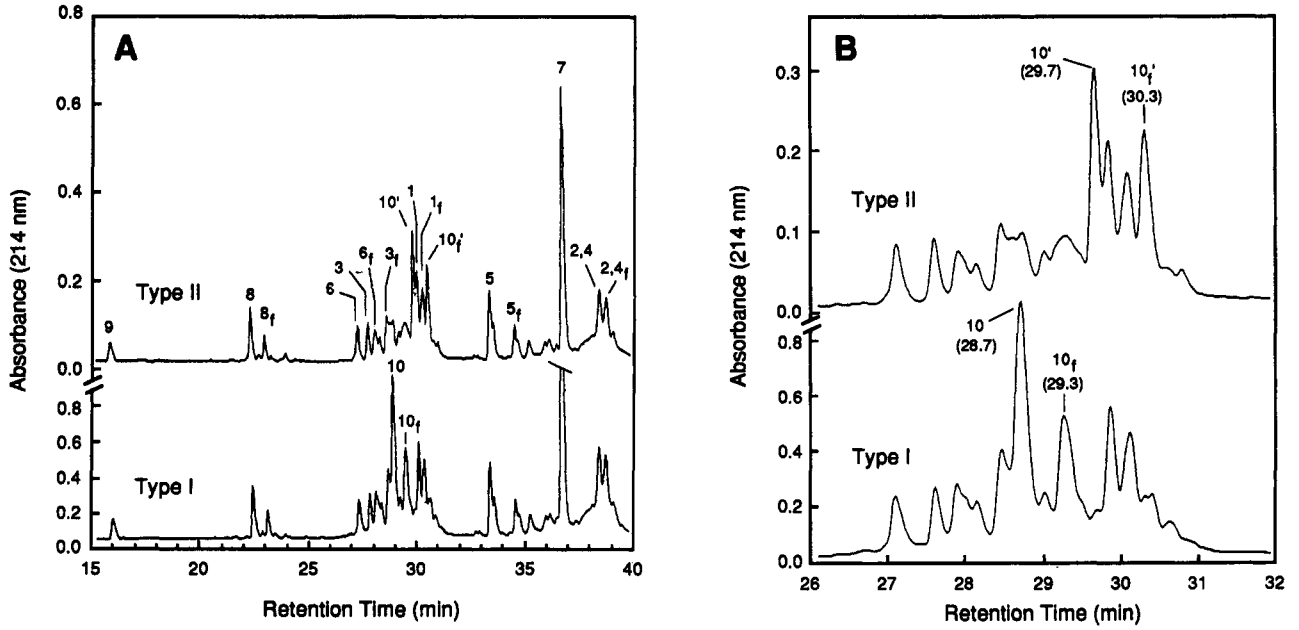


FIGURE 5: Reversed-phase HPLC of CNBr digests of PIMT. CNBr digests of approximately 10 nmol of PIMT were chromatographed as described in Experimental Procedures. (A) UV profiles (214 nm) of type I (bottom trace) and type II (top trace) PIMT CNBr digests, including all peaks identified by mass spectrometry (Table I). Peaks have been numbered according to their position in the sequence of type I PIMT. Labels with f subscripts designate formylated versions of the indicated peptides. (B) Expanded plot of the 26–32-min region from Figure 5A. Numbers in parentheses refer to retention times of the peaks that differ between the two isoforms, as referred to in the Results section.

Table IV: Sequencing of Chymotrypsin Digest of Type II C-Terminal CNBr Fragment

| | residue (yield, in pmol) | | |
|---|---|---|---|
| cycle[a] | 1 | 2 | 3 |
| 1 | G (50) | S (25) | V (15) |
| 2 | V (65) | R (5) | P (17) |
| 3 | I (50) | D (40) | L (6) |
| 4 | Y (45) | E (35) | T (2) |
| 5 | V (46) | L (10) | |
| 6 | P (50) | | |
| 7 | L (35) | | |

[a] Sequencing was not carried out past cycle 7.

```
                                                                    50
Type I  Ac-AWKSGGASHS ELIHNLRKNG IIKTDKVFEV MLATDRSHYA KCNPYMDSPQ
Type II Ac-   -------                                           ----
                                                                   100
        SIGFQATISA PHMHAYALEL LFDQLNEGAK ALDVGSGSGI LTACFARMVG
        ---------  - --         --------  --
                                                                   150
        PSGKVIGIDH IKELVDDSIN NVRKDDPMLL SSGRVQLVVG DGRMGYAAEA
            ------ ---------- ----------  - --    -   ------
                                                                   200
        PYDAIHVGAA APVVPQALID QLKPGGRLIL PVGPAGGNQM LEQYDKLQDG
          -------- ----   - - ---------- ---------- ----    --

        SVKMKPLMGV IYVPLTDKEK QWSRWK
        ---------- ---------- ----DEL 227
```

FIGURE 6: Bovine brain PIMT sequence. The sequence of the type I isoform is shown (from Henzel et al., 1989). Dashes indicate residues in the type II isoform that match the type I sequence, as determined by peptide sequencing in the present study. Blanks indicate regions not sequenced, or residues for which sequencing data were unclear. The DEL at residues 225–227 (in place of WK at residues 225–226 in the type I sequence) was the only mismatch detected in the type II sequence. In both isoforms, the amino terminus is acetylated, and Cys residues at positions 42 and 94 are disulfide-bonded.

of some residues and for substitutions that would not affect the mass, e.g., Ile⇔Leu or Gln⇔Lys. Some of these differences would be expected to change the behavior of protein fragments during reversed-phase HPLC; however, no such differences were detected during HPLC of CNBr digests (Figure 5). To further address the possibility that such changes exist, the two isoforms were also digested by endoproteinase Lys-C or by endoproteinase Asp-N and subjected to reversed-phase HPLC (not shown). The only HPLC peak that was different between the chromatograms of Lys-C digests of the two isoforms had the amino acid composition and UV absorbance ratio (280/214 nm) expected for the C-terminal peptide resulting from cleavage at $Lys_{220}$ (not shown). N-Terminal sequencing of this peptide was unsuccessful, presumably because of pyroglutamate formation at $Gln_{221}$.

Upon reversed-phase HPLC of endoproteinase Asp-N digests, a peptide ending at $Arg_{224}$ was obtained and sequenced. This peak was not present in the chromatogram of the Asp-N digest of the type I isoform (not shown). There were several other differences in the HPLC profiles of the Asp-N digests of the two isoforms, but sequencing revealed that these were caused by a greater cleavage of the type II isoform at glutamate residues present in both sequences. Endoproteinase Asp-N digestion of the type II isoform produced a fragment that had the amino acid composition of residues 1–10 of type I PIMT but yielded no sequence. This is consistent with the blocked amino terminus of an N-acetylated peptide. The same peak was observed in the type I chromatogram. Various fragments from the Lys-C and Asp-N digests of the type II isoform were sequenced, comprising 60% of the total sequence. Except for the WK⇔DEL substitution observed at their C-termini, the sequencing revealed no differences between the isoforms (Figure 6).

## Discussion

The results presented here strongly suggest that the two isoforms of PIMT from bovine brain differ only at their C-termini. Peptide sequencing revealed that the C-terminal amino acids ...WK present in the type I isoform are replaced by ...DEL in the type II isoform. The mass difference predicted for this change, an additional 43 amu, was observed for the whole proteins, as well as for their C-terminal CNBr fragments. The masses of all of the other CNBr fragments were the same for both isoforms. Reversed-phase HPLC in conjunction with mass spectrometry also confirmed the presence of a disulfide bond in both isoforms, between the only Cys residues in the sequence, $Cys_{42}$ and $Cys_{94}$. Sequencing

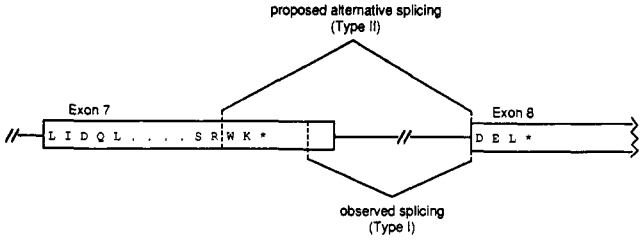


FIGURE 7: Splicing scheme for the production of mRNAs coding for type I and type II PIMT. Asterisks represent stop codons. The splicing observed in the 3′ untranslated region results in a sequence coding for the ...RWK ending (type I), like the unspliced version. The proposed alternative splicing within the $Arg_{224}$ codon would result in a sequence coding for the ...RDEL ending (type II).

of peptides from type II PIMT comprising 60% of the total sequence revealed no other differences between the isoforms.

The sequences of a number of tryptic fragments of type II PIMT from bovine erythrocytes have been previously reported by Clarke and co-workers (Gilbert et al., 1988). The C-terminal peptide was not sequenced, but of the 154 residues sequenced, all but one match the type I sequence from bovine brain. The mismatch was a Lys⇔Trp substitution at position 173. We found a Lys residue at both bovine brain sequences at this position. In a recent study of human erythrocyte PIMT, Clarke and co-workers concluded that the C-terminal ...WK of the type I isoform was replaced by ...DD in the type II isoform. No other mismatches were found in the 202 residues that were sequenced (Ingrosso et al., 1991).

On the basis of their findings, Ingrosso et al. (1991) suggested that the observed difference between the human erythrocyte PIMT isoforms might arise through differential splicing of the same pre-mRNA. Our data are consistent with a similar origin for the two isoforms of bovine brain PIMT (Figure 7). In cDNA for the type I isoform of PIMT from rat brain (Sato et al., 1989) and mouse testis (Romanik et al., 1991, 1992)[2] and in exon 7 of the mouse PIMT gene (Romanik et al., 1991, 1992), there is a candidate 5′-splice site (CAG:GTGGAA) that could lead to splicing within the codon for $Arg_{224}$, the last amino acid shared by the type I and type II isoforms from bovine brain (the present study) and from human erythrocytes (Ingrosso et al., 1991). This type of 5′-splice site, which matches a nine-nucleotide consensus sequence in the first six positions, is used in the processing of a number of vertebrate pre-mRNAs (Mount, 1982; Jacob & Gallinaro, 1989).

Two cDNAs for the type I isoform of PIMT have been cloned from mouse testis: one that contains the unspliced nucleotide sequence of exon 7 and one that results from pre-mRNA splicing within exon 7 to introduce a different 3′-untranslated region (Romanik et al., 1991, 1992). The sequence that is introduced by this splicing would complete the Arg codon and supply codons for Asp-Glu-Leu-(Stop) if it were added at the candidate 5′-splice site within the codon for $Arg_{224}$ instead of in the untranslated region (Figure 7). This alternative splicing would result in the C-terminal ...RDEL found in the present study for bovine brain type II PIMT. The cDNA for type I PIMT from rat brain corresponds to the spliced version of the mouse gene, and it also contains codons for Asp-Glu-Leu-(Stop) at the same location, although the nucleotides used in the third position of the codons for Asp and Glu are different from those in the mouse cDNA (Sato et al., 1989).

[2] Genbank locus MUSPCMAA, Accession Number M60320, submitted by L. C. Killoy, 1991.

The two isoforms of PIMT have similar substrate specificities (Aswad & Deight, 1983; O'Connor et al., 1984; Ota et al., 1988; Cusan et al., 1982), and both isoforms facilitate the conversion of isoaspartyl peptides into aspartyl peptides in vitro (Johnson and Aswad, unpublished). This raises questions about how the two isoforms could be serving different roles in vivo. The discovery that $Arg_{224}$ is followed by DEL in the type II isoform may shed light on the functional difference between the two isoforms. It is well established that the C-terminal sequence ...KDEL is responsible for preventing the secretion of certain proteins residing in the lumen of the endoplasmic reticulum (ER) (Pelham, 1988, 1989, 1990; Andres et al., 1990; Haugejorden et al., 1991; Munro & Pelham, 1987; Rose & Doms, 1988; Vaux et al., 1990). Andres et al. (1990) have shown that when codons for the sequences KDEL, DKEL, KNEL, and RDEL are appended to cDNA coding for the normally secreted neuropeptide Y, the gene products are retained by transfected secretory cells. Another mutation study demonstrated that a number of substitutions for the ...KDEL sequence of protein disulfide isomerase and the ...KEEL sequence of ERp72 continued to serve as ER retention signals (Haugejorden et al., 1991). For both protein disulfide isomerase and ERp72, ...RDEL mutants showed slightly better retention of the corresponding protein than did the wild-type cells. At least one natural form of protein disulfide isomerase (or a closely related protein) found in the sarcoplasmic reticulum of rabbit skeletal muscle has ...RDEL as its C-terminus (Fliegel et al., 1990). Another protein, known variously as colligin, gp46, and J6, has the C-terminal sequence ...RDEL and is localized to the lumen of the ER in rat and mouse cells (Clarke et al., 1991; Wang & Gudas, 1990; Nandan et al., 1990). We conducted a search of protein databases for sequences ending in ...KDEL and ...RDEL.[3] Of the eukaryotic proteins found whose subcellular localization has been studied, all were predominantly or entirely localized to the ER. Thus, the ...RDEL sequence of type II PIMT from bovine brain might serve to localize the enzyme to the ER. Alternative splicing that alters the C-terminus of certain proteins has been shown to result in different subcellular localization of different isoforms (Smith et al., 1989).

Previous reports of membrane-associated versions of PIMT (Iqbal & Steenson, 1976; Sellinger et al., 1987) and of the carboxyl methylation of secretory proteins in intact cells (Kloog & Saavedra, 1983; Nguyen et al., 1987) are also consistent with the entry of some form of PIMT into the ER. We have conducted a preliminary experiment to address the possible retention of the type II isoform by the ER, using cytosolic and microsomal fractions prepared from fresh rat brain (see Experimental Procedures). The soluble proteins in these fractions were separated on nondenaturing isoelectric focusing gels. The gels were sliced and the proteins were eluted and assayed for PIMT activity. The ratio of the activities of the isoforms was found to be about 1:1 both in the microsomal fraction and in the cytosolic fraction, thus revealing no enrichment of type II PIMT relative to type I PIMT in microsomes. The specific activity of total soluble PIMT was found to be similar in both fractions (cytosol, 26 units/mg; microsomal, 20 units/mg); however, it is not known what proportion of the PIMT found in the microsomal fraction originated from the lumen of the ER and what proportion was from some other vesicular com-

[3] Database searches were conducted using the facilities of the National Center for Biotechnology Information and the GENINFO Experimental BLAST Network Service (Altschul et al., 1990). The GenPept, NBRF PIR, and Swiss-Prot databases were searched (updated as of February 6, 1991).

partment(s) and/or from cytosolic contamination of this fraction.

The type I isoform of bovine brain PIMT has been shown to be N-acetylated at its amino terminus (Henzel et al., 1989), and the present results suggest that this is also the case for the type II isoform. In most tissues, N-acetylation occurs primarily in the cytoplasm and is catalyzed by a ribosomal acetyltransferase (Driessen et al., 1985). The presence of the N-acetyl group at the amino terminus of the mature type II isoform of PIMT therefore suggests that there was no cleavage of a typical, amino-terminal leader peptide that signals co-translational translocation of proteins into the ER (Walter & Lingappa, 1986). Various indices of hydrophobicity also failed to reveal a good candidate for an internal, noncleavable signal sequence such as that which is involved in the efficient post-translational ER translocation of ovalbumin (Tabe et al., 1984), a secretory protein that retains its amino-terminal N-acetyl group (Palmiter et al., 1978). However, weakly hydrophobic, internal signal sequences with hydrophobicities similar to portions of the PIMT sequence have recently been identified in plasminogen activator inhibitor type 2 (von Heijne et al., 1991). These sequences are inefficient signals and thus result in the presence of the protein in both cytoplasmic and secretory compartments (von Heijne et al., 1991), which may bear on our preliminary findings on the subcellular localization of the isoforms of rat brain PIMT.

Further work is needed to determine conclusively if PIMT is translocated to some extent into the ER. If it is, the C-terminal ...RDEL of the type II isoform should retain the enzyme in the ER, where it might catalyze the repair of iso-aspartyl proteins damaged while awaiting the formation of their correct tertiary and quaternary structures (Rothman, 1989). It is interesting in this regard that the ER-retained ...KDEL proteins, protein disulfide isomerase and BiP, may participate in the repair of misfolded proteins (Rothman, 1989). Because the type I isoform of PIMT lacks an ER retention signal, it presumably would be free to enter later compartments of the secretory pathway if it were translocated into the ER, and it could then repair proteins damaged during storage in these compartments. If PIMT is not translocated into the ER, the possibility remains open that the C-terminal ...RDEL of the type II isoform might be involved in targeting the enzyme to some other cellular comparment.

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## References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403–410.

Andres, D. A., Dickerson, I. M., & Dixon, J. E. (1990) *J. Biol. Chem. 265*, 5952–5955.

Aswad, D. W. (1984) *J. Biol. Chem. 259*, 10714–10721.

Aswad, D. W., & Deight, E. A. (1983) *J. Neurochem. 40*, 1718–1726.

Clarke, E. P., Cates, G. A., Ball, E. B., & Sanwal, B. D. (1991) *J. Biol. Chem. 266*, 17230–17235.

Clarke, S. (1985) *Annu. Rev. Biochem. 54*, 479–506.

Cusan, L., Andersen, D., Tuen, E., & Hansson, V. (1982) *Arch. Androl. 8*, 285–292.

Diliberto, E. J., & Axelrod, J. (1976) *J. Neurochem. 26*, 1159–1165.

Driessen, H. P. C., de Jong, W. W., Tesser, G. I., & Bloemendal, H. (1985) *CRC Crit. Rev. Biochem. 18*, 281–325.

Fliegel, L., Newton, E., Burns, K., & Michalak, M. (1990) *J. Biol. Chem. 265*, 15496–15502.

Fu, J. C., Ding, L., & Clarke, S. (1991) *J. Biol. Chem. 266*, 14562–14572.

Gilbert, J. M., Fowler, A., Bleibaum, J., & Clarke, S. (1988) *Biochemistry 27*, 5227–5233.

Haugejorden, S. M., Srinivasan, M., & Green, M. (1991) *J. Biol. Chem. 266*, 6015–6018.

Henzel, W. J., Stults, J. T., Hsu, C-A., & Aswad, D. W. (1989) *J. Biol. Chem. 264*, 15905–15911.

Ingrosso, D., Fowler, A. V., Bleibaum, J., & Clarke, S. (1989) *J. Biol. Chem. 264*, 20131–20139.

Ingrosso, D., Kagan, R. M., & Clarke, S. (1991) *Biochem. Biophys. Res. Commun. 175*, 351–358.

Iqbal, M., & Steenson, T. (1976) *J. Neurochem. 27*, 605–608.

Jacob, M., & Gallinaro, H. (1989) *Nucleic Acids Res. 17*, 2159–2180.

Johnson, B. A., & Aswad, D. W. (1990) in *Protein Methylation* (Paik, W. K., & Kim, S., Eds.) pp 195–210, CRC Press, Boca Raton, FL.

Johnson, B. A., Murray, D. E., Clarke, S., Glass, D. B., & Aswad, D. W. (1987a) *J. Biol. Chem. 262*, 5622–5629.

Johnson, B. A., Langmack, E. L., & Aswad, D. W. (1987b) *J. Biol. Chem. 262*, 12283–12287.

Johnson, B. A., Shirokawa, J. M., Hancock, W. S., Spellman, M. W., Basa, L. J., & Aswad, D. W. (1989) *J. Biol. Chem. 264*, 14262–14271.

Johnson, B. A., Ngo, S. Q., & Aswad, D. W. (1991a) *Biochem. Int. 24*, 841–847.

Johnson, B. A., Shirokawa, J. M., Geddes, J. W., Choi, B. H., Kim, R. C., & Aswad, D. W. (1991b) *Neurobiol. Aging. 12*, 19–24.

Jones, B. N. (1986) in *Methods of Protein Microcharacterization* (Shively, J. E., Ed.) pp 121–149, Humana Press, Clifton, NJ.

Kim, S., & Paik, W. K. (1978) *Methods Cell. Biol. 19*, 79–88.

Kloog, Y., & Saavedra, J. M. (1983) *J. Biol. Chem. 258*, 7129–7133.

Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575–599.

Ling, V., Guzzetta, A. W., Canova-Davis, E., Stults, J. T., Covey, T. R., Shushan, B. I., & Hancock, W. S. (1991) *Anal. Chem. 63*, 2909–2915.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McFadden, P. N., & Clarke, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2460–2464.

Mount, S. M. (1982) *Nucleic Acids Res. 10*, 459–472.

Munro, S., & Pelham, H. R. B. (1987) *Cell 48*, 899–907.

Murray, E. D., & Clarke, S. (1984) *J. Biol. Chem. 259*, 10722–10732.

Nandan, D., Cates, G. A., Ball, E. H., & Sanwal, B. D. (1990) *Arch. Biochem. Biophys. 278*, 291–296.

Nguyen, M. H., Harbour, D., & Gagnon, C. (1987) *J. Neurochem. 49*, 38–44.

O'Connor, C. M., Aswad, D. W., & Clarke, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7757–7761.

O'Connor, C. M., & Clarke, S. (1985) *Biochem. Biophys. Res. Commun. 132*, 1144–1150.

Ota, I. M., & Clarke, S. (1989) *Biochemistry 28*, 4020–4027.

Ota, I. M., & Clarke, S. (1990) in *Protein Methylation* (Paik, W. K., & Kim, S., Eds.) pp 179–194, CRC Press, Boca Raton, FL.

Ota, I. M., Ding, L., & Clarke, S. (1987) *J. Biol. Chem. 262*, 8522–8531.
Ota, I. M., Gilbert, J. M., & Clarke, S. (1988) *Biochem. Biophys. Res. Commun. 151*, 1136–1143.
Palmiter, R. D., Gagnon, J., & Walsh, K. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 94–98.
Pelham, H. R. B. (1988) *EMBO J. 7*, 913–918.
Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol. 5*, 1–23.
Pelham, H. R. B. (1990) *Trends Biochem. Sci. 15*, 483–486.
Romanik, E. A., Killoy, L. C., Ladino, C. A., D'Adrenne, S. C., & O'Connor, C. (1991) *J. Cell Biol. 115*, 152A.
Romanik, E. A., Ladino, C. A., Killoy, L. C., D'Adrenne, S. C., & O'Connor, C. (1992) *Gene* (in press).
Rose, J. K., & Doms, R. W. (1988) *Annu. Rev. Cell Biol. 4*, 257–288.
Rothman, J. E. (1989) *Cell 59*, 591–601.
Sato, M., Yoshida, T., & Tuboi, S. (1989) *Biochem. Biophys. Res. Commun. 161*, 342–347.
Sellinger, O. Z., Kramer, C. M., Fischer-Bovenkerek, C., & Adams, C. M. (1987) *Neurochem. Int. 10*, 155–166.
Smith, C. W. J., Patton, J. G., & Nadal-Ginard, B. (1989) *Annu. Rev. Genet. 23*, 527–577.
Tabe, L., Krieg, P., Strachan, R., Jackson, D., Wallis, E., & Colman, A. (1984) *J. Mol. Biol. 180*, 645–666.
Vaux, D., Tooze, J., & Fuller, S. (1990) *Nature 345*, 495–502.
von Heijne, G., Liljeström, P., Mikus, P., Andersson, H., & Ny, T. (1991) *J. Biol. Chem. 266*, 15240–15243.
Walter, P., & Lingappa, V. R. (1986) *Annu. Rev. Cell Biol. 2*, 499–516.
Wang, S.-Y., & Gudas, L. J. (1990) *J. Biol. Chem. 265*, 15818–15822.

# Identification in Bovine Liver Plasma Membranes of a Gq-Activatable Phosphoinositide Phospholipase C

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ABSTRACT: Phosphoinositide phospholipase C (PLC) activity extracted from bovine liver plasma membranes with sodium cholate was stimulated by GTPγS-activated $G\alpha_q/G\alpha_{11}$, whereas the enzyme from liver cytosol was not. The membrane-associated PLC was subjected to chromatography on heparin–Sepharose, Q Sepharose, and S300HR, enabling the isolation of the G-protein stimulated activity and its resolution from PLC-γ and PLC-δ. Following gel filtration, two proteins of 150 and 140 kDa were found to correspond to the activatable enzyme. These proteins were identified immunologically as members of the PLC-β family and were completely resolved by chromatography on TSK Phenyl 5PW. The 150-kDa enzyme was markedly responsive to GTPγS-activated α-subunits of $G\alpha_q/G\alpha_{11}$ or to purified $G_q/G_{11}$ in the presence of GTPγS. The response of this PLC was of much greater magnitude than that of the 140-kDa enzyme. The partially purified 150-kDa enzyme showed specificity for PtdIns(4,5)$P_2$ and PtdIns4P as compared to PtdIns and had an absolute dependence upon $Ca^{2+}$. These characteristics were similar to those of the brain PLC-$\beta_1$. The immunological and biochemical properties of the 150-kDa membrane-associated enzyme are consistent with its being the PLC-β isozyme that is involved in receptor–G-protein-mediated generation of inositol 1,4,5-triphosphate in liver.

It is well documented that calcium mobilizing agonists induce the rapid hydrolysis of phosphatidylinositol (4,5)-diphosphate (PtdIns4,5$P_2$) by a specific phospholipase C (PLC)[1] (Berridge & Irvine, 1984). Breakdown of this phospholipid results in the formation of two second messengers: inositol 1,4,5-triphosphate and diacylglycerol (Majerus et al., 1986), which amplify the initial signal and ultimately regulate cellular events.

Over the past 6 years, many different PLCs have been purified and characterized, and in most tissues, the enzyme has been shown to be heterogeneous [see Meldrum et al. (1991) for a review]. In 1987, three cytosolic PLCs were purified from bovine brain and were found to be discrete gene products, namely PLC-β1, PLC-γ1, and PLC-δ1 (Ryu et al., 1987; Katan & Parker, 1987). They have since been cloned and sequenced (Suh et al., 1988; Stahl et al., 1988; Katan et al., 1988) and have become representatives of three principal classes of phospholipase C isozymes: PLC-β (150 000–154 000), PLC-γ (145 000–148 000) and PLC-δ (85 000–88 000) according to the nomenclature proposed by Rhee et al. (1989). More recently, a cytosolic phospholipase of 85 kDa that is immunologically distinct from PLC-δ has been desig-

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[1] Abbreviations: PLC, phosphoinositide-specific phospholipase C; $G_q(G\alpha_q)/G_{11}(G\alpha_{11})$, a class of pertussis toxin insensitive G-proteins (and their α-subunits) defined by Strathmann and Simon (1990); PtdIns4P, phosphatidylinositol 4-phosphate; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; APMFS, (4-amidinophenyl)methanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; FPLC, fast protein liquid chromatography.

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