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Purification and Characterization of Protein Carboxyl Methyltransferase from *Torpedo ocellata* Electric Organ

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ABSTRACT: Posttranslational modification of proteins by the enzyme protein carboxyl methyltransferase (PCM) has been associated with a variety of cellular functions. A prerequisite for the understanding of cellular mechanisms associated with PCM is the characterization of purified PCMs from different tissues. We describe here the purification and characterization of PCM from the electric organ of *Torpedo ocellata*. The enzyme was purified to homogeneity by ion-exchange chromatography and ammonium sulfate precipitation, followed by chromatography on Sephadex G-100 and hydroxylapatite columns. When visualized by silver staining, the 700-fold-purified PCM exhibited a single band on sodium dodecyl sulfate–polyacrylamide gels, corresponding to a polypeptide of M_r 29 000. The molecular weight of the nondenatured enzyme (as determined by rechromatography on Sephadex G-100 column) was also 29 000, suggesting that the enzyme is a monomer. Two isoelectric forms of PCM (pI = 6.1 and pI = 6.4) were detected in the purified enzyme preparation. The enzyme methylates various exogenous and endogenous proteins, including the acetylcholine receptor. Of the four different polypeptides of the acetylcholine receptor, the γ and β polypeptides were selectively methylated by the purified PCM. Purified *Torpedo* PCM is highly sensitive to sulfhydryl reagents. The competitive inhibitor of PCM *S*-adenosyl-L-homocysteine (AdoHcy) protected the enzyme from inactivation by sulfhydryl reagents, suggesting the existence of a cysteine residue at the active site of the enzyme. The purified PCM has a low affinity toward DEAE-cellulose and toward AdoHcy–agarose. This property, as well as the relatively high molecular weight and the marked sensitivity to sulfhydryl reagents, distinguishes between the electric organ PCM and analogous enzymes of mammalian tissues.

The enzyme protein carboxyl methyltransferase (EC 2.1.1.24) (PCM)[1] catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to free carboxyl side chains of glutamyl or aspartyl residues of proteins. This enzymatic reaction results in the formation of protein methyl esters that are subsequently hydrolyzed, either spontaneously (Axelrod & Daly, 1965) or by a protein methylesterase (Gagnon et al., 1984), to generate the methyl acceptor protein and methanol. In mammals, PCM has an ubiquitous tissue distribution [for review, see Diliberto (1982) and Clarke (1985)], and the enzyme has been purified from various organs such as the pituitary gland (Diliberto & Axelrod, 1974), the testes (Cusan et al., 1981), the brain (Aswad & Deight, 1983;

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[1] Abbreviations: PCM, protein carboxyl methyltransferase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PCMB, *p*-(chloromercuri)benzoic acid; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Iqbal & Steenson, 1976), and the thymus (Kim & Paik, 1970) as well as from human erythrocytes (McFadden & Clarke, 1982; Kim et al., 1983). These PCMs exhibit broad substrate specificity and bring about a relatively low degree of protein methylation in vitro [for review, see Clarke (1985)]. They appear to be distinct from the bacterial methyl-accepting chemotaxis protein methyltransferases, which exhibit selective substrate specificity and result in the stoichiometric methylation of proteins (Kleene et al., 1977; Van Der Werf & Koshland, 1977). Also, bacterial PCM modifies the γ-glutamyl residues of proteins, whereas from mammalian PCMs the methylation products so far identified are the unusual β-methyl ester of D-aspartyl residues (McFadden & Clarke, 1982) and the α-methyl ester of isoaspartyl residues of diamidated proteins (Aswad, 1984).

In view of its ability to reversibly modify proteins, it was suggested that PCM might be a posttranslational modulator (Axelrod & Daly, 1965; Diliberto, 1982). In chemotactic bacteria, PCM-dependent methylations of γ-glutamyl residues on membrane chemoreceptors participate in the regulation of the chemotactic response (Springer et al., 1979; Koshland, 1979; Kleene et al., 1977). Putative functions of PCM in eucaryotic cells are associated with the secretion of hormones and neurotransmitters (Heisler et al., 1983; Borchardt et al., 1978; Diliberto et al., 1976; Gagnon et al., 1984), sperm motility (Gagnon et al., 1982), leucocyte chemotaxis (O'Dea et al., 1978), the processing of exportable proteins (Diliberto, 1982), modulation of calmodulin-dependent enzymes (Billingsley et al., 1985), repair of damaged proteins (Clarke, 1985), cell differentiation (Duerre & Fetters, 1985; Zukerman et al., 1982; Kloog et al., 1983; O'Dea et al., 1983), and modulation of photoreceptors (Swanson & Applebury, 1983) and of the acetylcholine receptor from *Torpedo* electric organ (Kloog et al., 1980; Flynn et al., 1982; Yee & McNamee, 1985; Nuske, 1986).

A prerequisite toward the understanding of cellular mechanisms associated with PCM is the characterization of purified PCMs from various tissues. This step is especially important in view of the variety of cellular functions that involve PCM-catalyzed reactions. Isolation and characterization of PCMs should thus provide valuable information on the possible existence of different types of eucaryotic PCMs.

In this work we purified PCM from the electric organ of *Torpedo ocellata*. The purified enzyme is capable of methylating the acetylcholine receptor by selectively methylating the β and γ receptor subunits and appears to possess certain properties distinct from those of mammalian PCMs.

Materials and Methods

Materials

S-Adenosyl-L-homocysteine (AdoHcy), AdoMet, gelatin, calmodulin, ovalbumin, trypsinogen, bovine serum albumin (BSA), iodacetamide, *p*-(chloromercuri)benzoic acid (PCMB), and *N*-ethylmaleimide (NEM) were from Sigma (St. Louis, MO). (Diethylaminoethyl)cellulose (Cellex-D) and hydroxylapatite (HTP) were from Bio-Rad (Richmond, CA). Sephadex G-100 and low molecular weight markers for gel electrophoresis (phosphoenilase *b*, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; α-lactolbumin, 14 200) were from Pharmacia (Uppsala, Sweden). AdoHcy–agarose was purchased from BRL, Bethesda, MD. Acrylamide, *N*,*N*′-methylenebis(acrylamide), sodium dodecyl sulfate (SDS), bromophenol blue, pyronine Y, and Coommassie brilliant blue were from Bio-Rad. Ampholines (pH 3–10, pH 5–8) were from LKB (Bromma, Sweden). All other chemicals were from E. Merck (Darmstadt, West Germany) and BDH (The British Drughouses Ltd., Poole, England).

Methods

Tissue Preparation. Electric organs were dissected out from live *Torpedo ocellata* and used either for purification of PCM or for the preparation of subcellular fractions. The preparation of the cytosolic fraction used for purification of PCM is described under Results. Synaptosomal fractions were prepared by the method of Michaelson and Sokolovsky (1976), and acetylcholine-enriched membranes were prepared by the method of Neubig et al. (1979).

Buffers and Chromatographic Procedures. Buffer A consisted of 40 mM sodium phosphate, pH 7.2, 5 mM ethylenediaminetetraacetic acid (EDTA), 14 mM 2-mercaptoethanol, 10 μM phenylmethanesulfonyl fluoride (PMSF), 5 units/mL aprotonine, and 5 μg/mL pepstatine A. Buffer B consisted of 5 mM phosphate buffer, pH 7.2, 1 mM EDTA, 14 mM 2-mercaptoethanol, and 100 μM PMSF. For AdoHcy–agarose affinity chromatography, 5 mM sodium phosphate buffer (pH 7.2) containing 5 mM EDTA and 14 mM 2-mercaptoethanol was used. In these experiments a partially purified PCM from human red blood cells was also used. This enzyme was purified according to the procedure of Gagnon (1979). Other chromatographic procedures are described under Results. The Sephadex G-100 columns were calibrated with dextran blue (void volume, V_0), *p*-nitrophenol (total volume, V_t), BSA (M_r 67 000), ovalbumin (M_r 43 000), and trypsinogen (M_r 24 000).

Enzyme Assay for PCM. PCM was assayed by the method of Diliberto et al. (1976). The reaction mixture contained (in a final volume of 50 μL) 100 μg of gelatin (or other protein substrate), 10 μL of enzyme preparation, 6.6 μM [*methyl*-^{3}H]AdoMet, and 0.01 M sodium acetate buffer, pH 6.5. Reactions were carried out in a microfuge tube at 37 °C for 10 min and terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA). Proteins were then precipitated by a 5-min spin in a Beckman microfuge and dissolved in 50 μL of 1 M sodium borate (pH 11) containing 3% methanol. The [^{3}H]methanol released by the alkaline solution was extracted into 1 mL of toluene–isoamyl alcohol (3:2 v/v), and 0.5 mL of the extract was counted in a liquid scintillation counter (LKB, 1128, Rackbeta), after the addition of 3 mL of Hydroluma (Lumac, Inc.). Blanks of assays carried out in the presence of 100 μM AdoHcy were substracted from the total counts.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis according to Laemmli (1970) was used to separate and identify the purified PCM. Prior to gel electrophoresis, the purified PCM samples were first dialyzed against 1 mM sodium phosphate buffer (pH 7.2), then lyophylized, and dissolved in 25–50 μL of water. Protein samples were diluted 1:2 (v/v) in 3× sample buffer (52.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol), boiled for 5 min, and then loaded onto 12.5% polyacrylamide slab gels. The samples were electrophoresed together with molecular weight standards at 40 mA/slab for 3–4 h and then stained with silver (Merril et al., 1981) from a Bio-Rad silver staining kit.

Acid/urea/SDS–polyacrylamide gel electrophoresis was used to separate carboxylmethylated proteins. This procedure is used in order to prevent degradation of the labile methyl groups (Kloog & Saavedra, 1983). The acidic gel system, consisting of 0.05 M sodium phosphate, pH 2.4, 8 M urea, 1% SDS, and 10% acrylamide, was used as described previously (Kloog & Saavedra, 1983). Aliquots of methylated

Table I: Purification of PCM from Electric Organ of *Torpedo ocellata*[a]

purification step	protein activity vol (mL)	mg	units	sp act. (units/mg)	yield (%)	degree of purification
80000*g* supernatant	252	756.0	20600	27.2	100	1.0
DEAE-cellulose filtrate	260	531.0	24000	45.3	116	1.7
$(NH_4)_2SO_4$ precipitate	30	121.5	7400	61.0	36	2.2
Sephadex G-100	136	3.4	7600	224.0	37	82.0
hydroxylapatite	14	0.128	2440	1906.0	12	698.0

[a] PCM activity was determined as described under Methods. A unit of activity is defined under Results. Data represent the results of a typical purification experiment. Similar results were obtained in 12 additional experiments.

proteins were diluted 1:1 in freshly prepared sample buffer (0.05 M sodium phosphate, pH 2.4, 3% SDS, 8 M urea, 20% glycerol, 0.05% pyronin Y, and 30 mM 2-mercaptoethanol), loaded onto slab gels, and electrophoresed at a constant current (70 mA/slab) until the pyronin Y tracking dye had migrated 12–15 cm. Marker proteins were electrophoresed in parallel lanes. Gels were stained with Coomassie brilliant blue and destained (Kloog & Saavedra, 1983). After 3–4 h the wet destained gels were cut into 2-mm sections and counted in 5 mL of 1:10 Luma Solve:Lipo Luma (Lumac Inc.). Base-labile volatile groups, measured in aliquots obtained from each sample loaded onto the gels, were taken as an index of the carboxylmethylated proteins loaded. The total number of counts found along the gel, after subtraction of the counts obtained in a parallel sample treated with base prior to electrophoresis, was used to calculate the recovery of protein carboxymethyl esters. Recoveries were usually between 85 and 100%.

Isoelectric focusing was performed by the method of O'Farrell (1975) in order to determine the isoelectric point (p*I*) of the purified PCM. Protein samples containing 0.05% (v/v) Triton X-100 and ampholines (1% pH 3.5–10.0 and 1% pH 5.0–8.0) were loaded onto acrylamide tube gels (0.4 × 13 cm) as described by O'Farrell and run at 300 V for 15 h followed by a run of 600 V for 2 h. The gels were then removed and sliced into 0.5-cm sections. The gel slices were immersed in 1 mL of degassed double-distilled water, and the pH gradient was determined. PCM activity in the gel slices was determined as follows: each gel section was incubated in 150 μL of buffer B for 4 h at 4 °C, and a 20-μL sample was then withdrawn for determination of enzyme activity. Preliminary exeriments indicated that the 4-h incubation time was sufficient to elute the PCM activity from the gel sections.

Proteins were determined by the method of Bradford (1976) with BSA as a standard.

Results

Purification of Torpedo PCM. *Torpedo* electric organ PCM was purified at 4 °C. The tissue (100 g) was homogenized in 2 volumes of buffer A with a Waring blender (4 min, full speed). PCM activity in the tissue homogenate was 276 units/g of organ (an enzyme unit is defined as 1 pmol of [^{3}H]methyl groups transferred per minute under the standard assay conditions described under Methods). The homogenate was centrifuged at 80000*g* for 45 min. The resulting supernatant, which was found to contain 75–80% of the tissue PCM activity, was collected and mixed with 250 mL (packed volume) of DEAE-cellulose, which was preequilibrated in buffer A. After being stirred for 30 min, the mixture was filtered over a Buchner funnel, and the filtrate, containing all of the 80000*g* supernatant activity (Table I), was collected. Solid ammonium sulfate was added to the filtrate to 70% saturation; 2 h later the protein precipitates were collected by centrifugation for 10 min at 31000*g*, dissolved in buffer B, and dialyzed

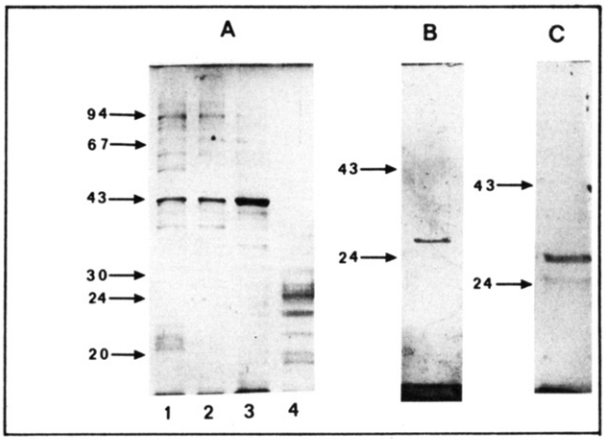

FIGURE 1: SDS–polyacrylamide gel electrophoresis of proteins in various purification steps. (A) Gel patterns of the 80000*g* supernatant proteins (1), DEAE-cellulose filtrate proteins (2), and Sephadex G-100 fractions corresponding to $0.12V_t$–$0.17V_t$ (3) and $0.17V_t$–$0.22V_t$ (4). Proteins (30 μg) were loaded onto each lane of the 12.5% SDS–polyacrylamide slab gel. The migration of molecular mass markers (kilodaltons) in a corun lane is indicated by arrows. Gels were silver stained. (B and C) Gel patterns of the purified *Torpedo* electric organ PCM. The hydroxylapatite-purified enzyme (15 μg) was run on 12.5% acrylamide gel. Gels of two separate preparations show a single M_r 29000 polypeptide (B) and an additional M_r 26000 polypeptide (C). Gels were silver stained. The migration of molecular weight standards is indicated by arrows.

for 12 h against 2 × 4 L of the same buffer. About 35% of the 80000*g* supernatant PCM activity was recovered in the ammonium sulfate precipitate, and a 2-fold purification was achieved (Table I).

The dialyzed ammonium sulfate precipitate was loaded onto a Sephadex G-100 column (4 × 97 cm) preequilibrated in buffer B, and 12-mL fractions were collected at a flow rate of 60 mL/h. Most of the protein was eluted at the void volume, whereas PCM activity was eluted at $0.12V_t$–$0.22V_t$. Sephadex G-100 column chromatography resulted in about 80-fold purification, with no detectable loss of enzyme activity (Table I). The active fractions, corresponding to $0.17V_t$–$0.22V_t$, were collected (130–140 mL) and loaded onto a hydroxylapatite column (3.7 × 19 cm), preequilibrated in buffer B. Proteins were eluted from the column by employing a linear sodium phosphate gradient (150 mL of 5 mM and 150 mL of 200 mM sodium phosphate, pH 7.2, each containing 1 mM EDTA and 14 mM 2-mercaptoethanol). The PCM activity came off the column as a sharp peak at 5.8–6.4 $m\Omega^{-1}$ (48–52 mM sodium phosphate). Specific activity of PCM in the pooled active fractions was 19000 units/mg of protein, and a 700-fold purification was achieved. The yield was 12% (Table I). This enzyme preparation was stable for at least 2 months when stored at −80 °C in the presence of 0.8 M sucrose.

SDS–polyacrylamide gel electrophoresis was used to characterize the proteins obtained in each of the purification steps. A comparison between the gel patterns of the super-

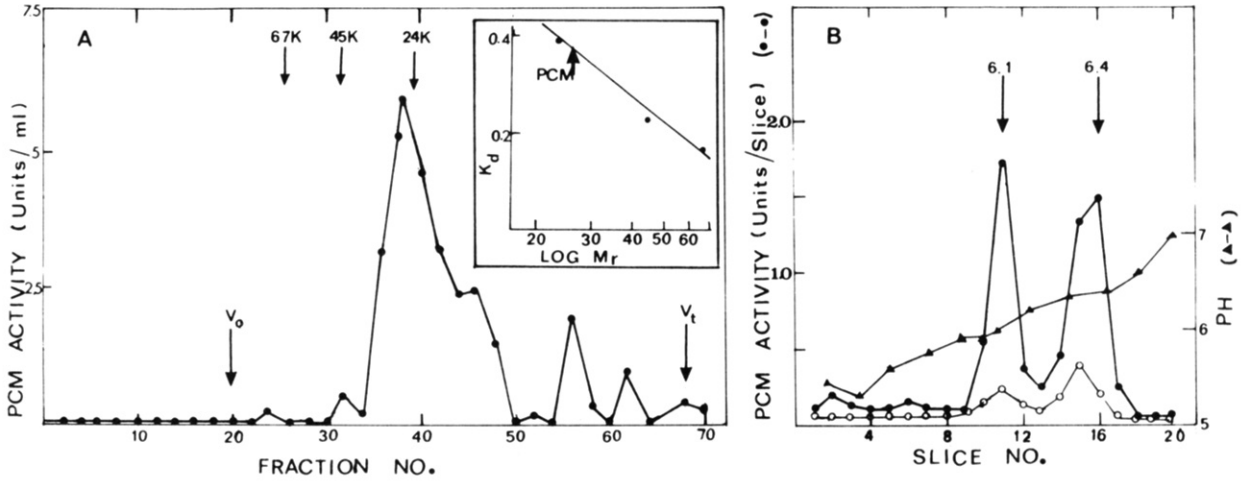

FIGURE 2: Determination of the molecular weight and p*I* of the nondenatured PCM from *Torpedo* electric organ. (A) Purified PCM (40 μg) was loaded onto a 1.5 × 45 cm Sephadex G-100 column and chromatographed with buffer B, and fractions (1.2 mL) were collected. PCM activity was determined in 10-μL samples. The column was calibrated with molecular weight markers (indicated by arrows). V_0 = void volume; V_t = total volume, (Insert) Plot of elution coefficient (K_d) as a function of the molecular weight of the marker proteins. $K_d = (V_t - V_0)/(V_e - V_0)$, where V_e is the elution volume of the protein. (B) Isoelectric focusing of purified PCM. Purified enzyme (2 μg) was focused on 0.5 × 13 cm tube gels. Gels were sliced and processed for determination of the pH gradient (▲) or the PCM activity in the absence (●) and in the presence (○) of 10^{-4} M AdoHcy, as described under Methods.

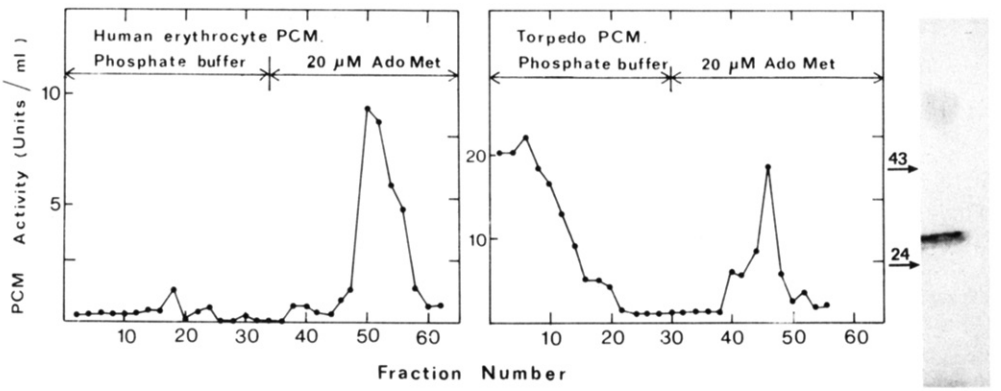

FIGURE 3: AdoHcy–agarose affinity chromatography of purified *Torpedo* electric organ PCM and of partially purified PCM from human erythrocytes. The affinity support was preequilibrated for 1 h at 4 °C with 12 μg of erythrocyte PCM (left) or with 9 μg of purified electric organ PCM (middle) in 5 mM phosphate buffer (pH 7.2) as described under Methods. The matrix was loaded onto a column (1 × 14 cm) and washed with the phosphate buffer, which was then replaced by phosphate buffer (pH 7.2) containing 20 μM AdoMet. Fractions (0.7 mL) were collected, and PCM activity was determined in 10-μL samples. (Left) Gel pattern of the activity peak fractions of electric organ PCM eluted with the AdoMet.

natant and those of the DEAE-cellulose filtrate (Figure 1) indicates that some proteins of apparent M_r 20000–25000 and of M_r 50000 were bound to the matrix. Gel patterns of proteins present in the pooled Sephadex G-100 fractions, corresponding to $0.12–0.17V_t$ and to $0.17V_t–0.22V_t$, are also shown in Figure 1. In the latter range of fractions, which contained about 80% of the PCM activity, most of the proteins were of M_r <40000 while in the former most of the proteins were of M_r >40000, with a polypeptide of M_r 43000 predominating (Figure 1). We preferred to use the fractions corresponding to $0.17V_t–0.22V_t$ for the subsequent stage, viz., hydroxylapatite column chromatography. The 700-fold-purified PCM prepreparation obtained by this latter procedure contained a major polypeptide with an apparent M_r of 29000 ± 1000 (Figure 1). In most of the preparations this was the only protein band detected (Figure 1), although in some of them a minor component with an apparent M_r of 26000 ± 1500 was also observed (see Figure 1).

Characterization of PCM. In order to estimate the molecular weight of the nondenatured PCM, the purified preparation was rechromatographed on a Sephadex G-100 column (1.5 × 45 cm) that was calibrated with BSA (M_r 67000), ovalbumin (M_r 43000), and trypsinogen (M_r 24000). The peak activity obtained corresponded to a protein of M_r 29000 ● 2000 (Figure 2A).

Isoelectric focusing in polyacrylamide tube gels was used to determine the p*I* of PCM. Two activity peaks were observed, at pH 6.1 and pH 6.4 (Figure 2B), suggesting that the purified preparation contains two isoelectric forms of PCM.

The K_m of *Torpedo* PCM for the methyl donor AdoMet was 1.5 ± 0.6 μM, as estimated by the standard assay and with various concentrations of AdoMet (0.8–100 μM). AdoHcy competitively inhibited the enzyme with an apparent K_i of 0.3 ± 0.1 μM, AdoHcy–agarose column chromatography demonstrated that the inhibitor binds the enzyme; the enzyme was eluted from the column by 20 μM AdoMet (Figure 3), and a single M_r 29000 band was detected in the SDS–polyacrylamide gel of the eluted activity peak (Figure 3).

It should be noted, however, that most of the enzyme did not bind to the AdoHcy–agarose column, in contrast to the PCM obtained from human erythrocytes which did bind to the affinity support (Figure 3). Increasing the pH of the buffer up to 8.5 or decreasing the ionic strength of the buffer solution failed to increase the binding of the *Torpedo* PCM to the column.

The effects of various metal ions on PCM activity were

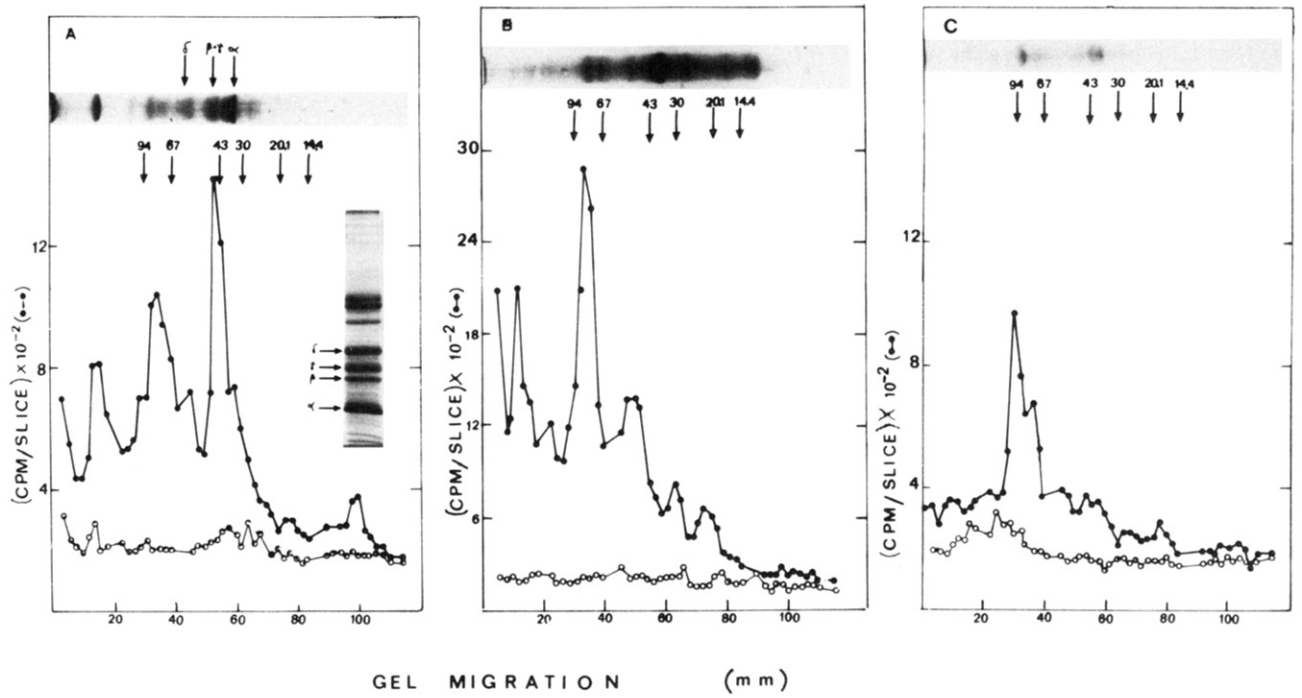

FIGURE 4: Acid/urea/SDS–polyacrylamide gel electrophoresis of methylated proteins from subcellular fractions of *Torpedo* electric organ. Proteins (50 μg in 0.1% Triton X-100) were methylated as described under Methods, with 0.5 μg of purified *Torpedo* electric organ PCM in the absence (●) and in the presence (○) of 100 μM AdoHcy. The methylated proteins were subjected to gel electrophoresis (7.5% SDS–polyacrylamide gel, pH 2.4) and processed for counting as described under Methods. Upper panels show the electrophoresed proteins stained by Coomassie brilliant blue; the arrows indicate the migration of the molecular weight standards. (A) Acetylcholine receptor enriched membranes. (Inset) The same preparation was run on a Laemmli (1970) 10% SDS–polyacrylamide gel in order to demonstrate the acetylcholine receptor subunits (α, β, γ, and δ). In the acidic gel system, the β and γ subunits are not well separated from each other. (B) Cytosolic proteins. (C) Synaptosomal proteins. In all cases there was 87–100% recovery of protein methyl esters from the gels.

studied under the standard assay conditions. Sodium, potassium, Ca^{2+}, and Mg^{2+} at concentrations of up to 100 mM did not affect PCM activity. Manganese (10 mM), Cd^{2+} (1 mM), and Cu^{2+} (0.1 mM) were strong inhibitors of the enzyme, suggesting the possibility that a sulfhydryl group is important for its activity. Indeed, all of the sulfhydryl reagents tested [Hg^{2+}, *N*-ethylmaleimide, iodoacetic acid, and *p*-(chloromercuri)benzoate] were found to be effective inhibitors of the PCM. Moreover, AdoHcy protected PCM from inactivation by *N*-methylmaleimide or by *p*-(chloromercuri)-benzoate (Table II).

Various endogenous proteins were capable of serving as methyl acceptors for the purified PCM (Figure 4), including cytosolic proteins and proteins of presynaptic origin (synaptosomes) and of postsynaptic origin (acetylcholine receptor enriched membranes). Of the cytosolic proteins the major methylated band corresponded to a polypeptide of $M_r \simeq 90\,000$, but other methylated polypeptides ($M_r > 100\,000$, $M_r \simeq 50\,000$, $M_r \simeq 31\,000$, and $M_r \simeq 22\,000$) were also detected (Figure 4B). In the synaptosomal fraction the major methylated band corresponded to a polypeptide of M_r 90 000, and a second methylated band appeared at $M_r \simeq 70\,000$. In the acetylcholine receptor enriched membranes, the major methylated band corresponded to the γ and β receptor subunits. The α and δ subunits were also methylated but to a lower extent (Figure 4A). Additional methylated polypeptides at $M_r \simeq 80\,000$ and $M_r > 100\,000$ were also detected in the receptor-enriched membranes. It is important to note the advantage of using acidic gels to separate the methylated polypeptides rather than the Laemmli gel system, since 80% of the methyl groups are recovered in the former case as compared with about 20% in the latter. However, the separation between the four different acetylcholine-receptor polypeptides obtained with the acidic gel system is not as good (see insert to Figure 4A). It is interesting to note that despite the low yields in the Laemmli gel system it was already possible to observe that the M_r 60 000 polypeptide was methylated to a greater extent than the other receptor subunits (Flynn et al., 1982).

Table II: Protection of *Torpedo* Electric Organ PCM Activity by AdoHcy from Inactivation by Sulfhydryl Reagents[a]

treatment	PCM activity (% of control)
no dialysis	
control	100
AdoHcy (10^{-4} M)	9
NEM (10^{-3} M)	40
PCMB (10^{-3} M)	30
+dialysis	
no additions	91
AdoHcy (10^{-4} M)	89
NEM (10^{-3} M)	38
PCMB (10^{-3} M)	26
10^{-4} M AdoHcy, then 10^{-3} M NEM	90
10^{-4} M AdoHcy, then 10^{-3} M PCMB	90

[a] Samples of purified PCM (4.5 μg) were preincubated at 4 °C in 50 mM sodium phosphate buffer, pH 7.4, for 30 min in the absence or in the presence of AdoHcy. The sulfhydryl reagents were then added for an additional 15 min. PCM activity was measured either immediately (no dialysis) or after dialysis for 12 h at 4 °C against the phosphate buffer. Data represent the mean of triplicate determinations.

In agreement with previous results (Kloog et al., 1980), we also found that the *Torpedo* PCM is capable of methylating various proteins such as ovalbumin, calmodulin, BSA, and neurophysin; i.e., the enzyme did not exhibit selective substrate secificity.

Discussion

Protein carboxyl methyltransferase was purified from the electric organ of *Torpedo* by means of DEAE-cellulose anion

exchange, ammonium sulfate precipitation, and Sephadex G-100 chromatography, followed by hydroxylapatite column chromatography. The purified enzyme appears to be nearly homogeneous as shown by SDS–polyacrylamide gel electrophoresis, which revealed either a single M_r 29 000 polypeptide or, occasionally, an additional minor component of M_r 26 000 (Figure 1). The purified enzyme, eluted by AdoMet from an AdoHcy–agarose column, also corresponds to a M_r 29 000 polypeptide (Figure 3). Rechromatography on a Sephadex G-100 column indicated that the apparent molecular weight of the nondenatured PCM is also 29 000, suggesting that the enzyme is a monomeric globular protein. Two isoelectric forms of PCM are present in the purified enzyme preparation. They could represent two polypeptides with a slightly different primary structure. A posttranslational modification of the PCM (e.g., phosphorylation/dephosphorylation) could also result in the appearance of two isoelectric forms of the enzyme.

The nature of the copurified M_r 26 000 polypeptide is not yet known. It could be a contaminant, or it could represent a degradative product of the M_r 29 00 polypeptide. Alternatively, it could represent a different form of PCM that might be present in low amounts in the electric organ of *Torpedo ocellata*. This last possibility is interesting, in view of the fact that an M_r 25 000 polypeptide was recently detected in the Sephadex G-100 elution pattern of *Torpedo* PCM, which was partially purified by means of a two-step (ammonium sulfate and Sephadex G-100) procedure (Lee & McNamee, 1985). Like these authors, we found the Sephadex G-100 sieving to be a useful step in the purification procedure, although the estimated molecular weight for PCM in our purest preparation, which contained only one polypeptide band, was 29 000. The differences in the estimated molecular weight values might be explained in terms of species differences (e.g., *T. californica* vs. *T. ocellata*). Another possibility is that the minor M_r 25 000 polypeptide detected in some of our preparations represents another active PCM.

The main problem we encountered during purification of the electric organ PCM was the marked loss of enzyme activity. We overcame this problem by adding antiproteases and using a relatively high concentration of 2-mercaptoethanol. We are currently investigating whether or not there is a correlation between the antiprotease activity and the appearance of the M_r 25 000 polypeptide. We are also attempting to determine whether the use of endogenous substrates, specifically those present in the fraction of M_r ~90 000 cytosolic proteins (Figure 4B) and in the fraction of acetylcholine receptor enriched membranes (Figure 4A), could result in a different PCM elution profile on the Sephadex G-100 column. (Although broad substrate specificity is a typical feature of all eucaryotic PCMs so far purified [for review, see Clarke (1985)], one cannot rule out the possibility that there are PCMs in eucaryotic cells that are substrate-specific and thus possess different physical and biochemical properties.) From the data now available we know that the *Torpedo* electric organ PCM has many features in common with PCMs of eucaryotic cells, such as cytosolic localization, broad substrate specificity, high affinity for AdoMet and for AdoHcy, a monomeric form of the active enzyme (Diliberto, 1982; Billingsley et al., 1985), and multiple isoelectric forms (Aswad & Deight, 1983; Cusan et al., 1984). We nevertheless encountered some notable differences between this PCM and mammalian PCMs as discussed below.

The *Torpedo* electric organ PCM is strongly inhibited by sulfhydryl reagents (Table II); AdoHcy can protect against this inactivation, suggesting that the enzyme has at least one cysteine residue that is important for its catalytic activity. In this respect the enzyme resembles PCMs from human (Billingsley & Lovenberg, 1985; Polastro et al., 1978) and horse erythrocytes and from rat brain (Billingsley & Lovenberg, 1985), all of which contain a cysteine residue. Polastro et al. (1978) have shown that the equine erythrocyte PCM is inhibited by sulfhydryl reagents, although they found that iodoacetamide and *N*-ethylmaleimide do not inhibit the enzyme unless previously denatured. In line with this report, we found that partially purified human erythrocyte PCM in a nondenatured state is hardly affected by iodoacetic acid or by *N*-ethylmaleimide. The *Torpedo* electric organ PCM is however inhibited by these reagents even in its nondenatured state, which could indicate that the active cysteine residue of this enzyme is more readily accessible than the cysteine residues of mammalian PCMs. The high sensitivity of the *Torpedo* PCM to Cu^{2+} ions would also be in line with this suggestion; PCMs of mammalian sources are not sensitive to this divalent metal ion [for review, see Diliberto (1982)].

Other properties of the electric organ PCM that differentiate between this enzyme and mammalian PCMs concern its affinity to DEAE-cellulose and to AdoHcy–agarose. The electric organ enzyme does not bind to the anion exchanger even at a pH of 8.3; in view of the p*I* values of the two isoelectric forms of the enzyme (6.1 and 6.4), it should have bound to the anion exchanger. It thus appears that the *Torpedo* PCM has low affinity toward the DEAE-cellulose, possibly due to the charged group(s) on this enzyme being inaccessible to the exchanger. In contrast, analogous enzymes from mammalian tissues do bind to DEAE columns (Polastro et al., 1978; Diliberto & Axelrod, 1974; Aswad & Deight, 1983). Similarly, mammalian PCMs bind efficiently to AdoHcy–agarose (Kim et al., 1978; Aswad & Deight, 1983; Walker et al., 1984), whereas the *Torpedo* PCM binds with only very low affinity, resulting in a poor yield (1–2%) in purification when this affinity support is used (Figure 3). Interestingly, the procaryotic PCM protein methyltransferase II from *Bacillus subtilis* (Burges-Cassler et al., 1982), like the *Torpedo* PCM, does not bind to DEAE or to AdoHcy affinity columns. There is a further resemblance between these two enzymes in their apparent molecular weight (30 000 for the bacterial PCM and 29 000 for the *Torpedo* PCM). The molecular weight values recorded for most of the mammalian PCMs are lower, e.g., 24 000–27 000 (Clarke, 1985; Billingsley & Lovenberg, 1985). It is important to note, however, that the bacterial methyl-accepting chemotaxis protein methyltransferases specifically methylate γ-glutamyl residues (Kleene et al., 1977; Van Der Werf & Koshland, 1977), whereas the site modified by the *Torpedo* PCM has not yet been identified. It is nevertheless interesting to note that in the acetylcholine receptor the preferred methylation sites appear to be localized on the β and γ receptor subunits. Thus in spite of the high degree of homology between the α, β, γ, and δ receptor subunits [for review, see Hucho (1986)], PCM selectively methylates sites on the β and γ polypeptides.

Some of the properties that distinguish between the electric organ PCM and analogous enzyme purified from mammalian tissues might be associated with a selective posttranslational modification of proteins of the electric organ. The purification of this enzyme from the electric organ of *Torpedo* should facilitate elucidation of its role in the functioning of this highly specialized tissue.

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Registry No. PCM, 9055-09-8; AdoMet, 29908-03-0; AdoHcy, 979-92-0.

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