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SHORT COMMUNICATIONS

Protein carboxylmethylase isozymes in rat brain subcellular organelle

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Protein carboxylmethylase (PCM*; *S*-adenosylmethionine-:protein carboxylmethyltransferase, EC 2.1.1.24, protein methylase II) catalyzes the methylation of free carboxyl groups of proteins [1, 2] and has been linked to the chemotaxic response of bacteria [3–6]. It may also modulate functions such as excitation–secretion coupling [7, 8], the chemotaxic response of leucocytes [9, 10], and calmodulin regulation [11, 12]. These studies have been the subject of recent reviews [13–15] and books [16] to which the reader is referred for more detailed discussions.

In most tissues, PCM has been found in the cytosolic portion of cell homogenates [16]. In brain, however, considerable activity is found in synaptosomes [17]. Iqbal and Steenson [18] isolated both soluble and detergent-solubilized enzymes in partially-purified forms.

In addition to soluble and bound forms of PCM, brain has relatively high concentrations of methyl acceptor proteins (MAP) for these enzymes [17]. With the exception of calmodulin [11, 12] none of the endogenous MAPs has been identified or isolated, however, and little or no progress has been made in defining the functional significance of either the enzymes or the acceptors. In the following report, the results of a study on membrane-bound carboxylmethylases in brain synaptosomes are presented. The formation of methylated proteins by brain synaptosomal preparations was investigated recently by Eiden *et al.* [19].

Materials and methods

[^{3}H]AdoMet (*S*-adenosyl-L-[^{3}H-*methyl*]methionine, 68–80 Ci/mmole) and Aquasol were obtained from the New England Nuclear Corp. (Boston, MA). AdoMet and Triton X-100 were bought from the Sigma Chemical Co. (St. Louis, MO). Radioactivity was measured in a liquid scintillation spectrometer.

Preparation of synaptosomes. Synaptosomes were prepared from whole brain by the procedure of Gray and Whittaker [20]. Rats (Sprague–Dawley, 150–200 g) were killed by decapitation. The brains were removed, cooled on ice, weighed and used to isolate the synaptosome-enriched P2-fraction. This was resuspended in sucrose (0.32 M), layered over a sucrose gradient (0.8 to 1.2 M) and centrifuged for 2 hr at 100,000 *g*. The synaptosomal fraction (Fraction B) at the 0.8 to 1.2 M interface was diluted with water (1:2) and centrifuged (125,000 *g*). The precipitate was gently resuspended in Krebs–Ringer buffer [21] containing sodium azide (0.02%) using a glass tissue homogenizer. Fraction A (myelin) and Fraction C (mitochondria) from the sucrose gradient were also retained. In some experiments, Fractions B and C were subjected to further purification by methods described by Gurd *et al.* [22]. This procedure includes several washing steps for mitochondria and synaptosomes are separated by flotation on a Ficoll gradient.

Lysed synaptosomal fractions. Synaptosomal preparations were centrifuged at 4,000 *g* for 10 min, and supernatant fraction was retained. The pellet was resuspended in cold water, allowed to stand for 15 min, and then centrifuged at 125,000 *g* for 15 min. This step was repeated, and the aqueous supernatant (soluble) fractions were combined. The pellet was resuspended in Krebs–Ringer buffer and made to 0.2% with Triton X-100 followed by heating at 37° for 30 min. The suspension was then centrifuged at 125,000 *g* for 30 min. The supernatant fraction (STx) was retained. The precipitate had little or no activity and was discarded.

PCM assay. Protein carboxylmethylase was assayed by a modification of the procedure described by Diliberto and Axelrod [17]. Lysed synaptosomal preparations (30–180 μg protein) were added to the following at pH 6.3 in a total volume of 200 μl: sodium phosphate buffer (50 mM), EDTA (4.5 mM) and 25 μl [^{3}H]AdoMet (17.8 pmoles, 1.25 μCi). In a second series, the medium was supplemented with gelatin (2.5%). The mixtures were heated at 37° for 30 min in glass-stoppered vessels; then 0.5 ml of 0.5 M sodium borate (pH 10.0) was added and the heating was continued for 1 min. The vessels were placed in ice and extracted with a mixture of isoamyl alcohol and toluene (2:3). After vigorously mixing (vortex) for 15 sec, and centrifuging, an aliquot (2 ml) was retained for counting while another was placed in an oven and heated at 90° for 1 hr to drive off the volatile methanol. Aquasol (5 ml) was added to all samples prior to determining the radioactivity content of each. The amount of [^{3}H]methanol formed was the difference in radioactivity between the heated and non-heated samples. All assays were done in duplicate and were corrected for controls that contained no synaptosomal protein. The assay procedure gave linear results with protein concentrations ranging up to 210 μg and with time up to at least 30 min. Units were arbitrarily defined as one pmole methyl groups incorporated per hr. Protein concentrations were monitored by the procedure of Lowry *et al.* [23], using bovine albumin as standard.

Electrophoresis. Poly-acrylamide gel electrophoresis was done using a modification of the procedure of Davis [24]. Acrylamide gels (5%, 12.5 cm long) were prepared and pre-run for 1 hr. Protein samples (30–100 μg) containing 1.2 M sucrose (50%) and a few drops of bromophenol blue (0.5%) were layered on duplicate gels and electrophoresis was performed at 2 mA per gel for about 4 hr. Tris-borate (0.09 M) at pH 8.4 containing Triton X-100 (0.2%) was the running buffer. At the end of the run, one gel was fixed with sulfosalicylic acid (20%) overnight in preparation for staining with Coomassie blue (0.25% in methanol), while the other was immediately sliced into segments (1.5 to 2.0 mm). Each segment was homogenized in 0.5 ml assay buffer with gelatin added (2.5%), and aliquots were assayed for PCM activity. The activity was plotted against segment number to reveal the relative positions of the active peaks. These were then compared to the stained gels to identify the appropriate enzyme band.

Results and discussion

Figure 1 shows the results obtained when water-soluble and STx fractions from synaptosomes were subjected to gel electrophoresis. The two preparations had remarkably similar protein and activity patterns which, in fact, appear to be identical and superimposable. Each sample had two major and one minor active peaks. Some differences in the

* Abbreviations: PCM, protein carboxylmethylase; MAP, methyl acceptor protein; AdoMet, *S*-adenosyl-L-methionine; and STx, Triton X-100 solubilized fraction.

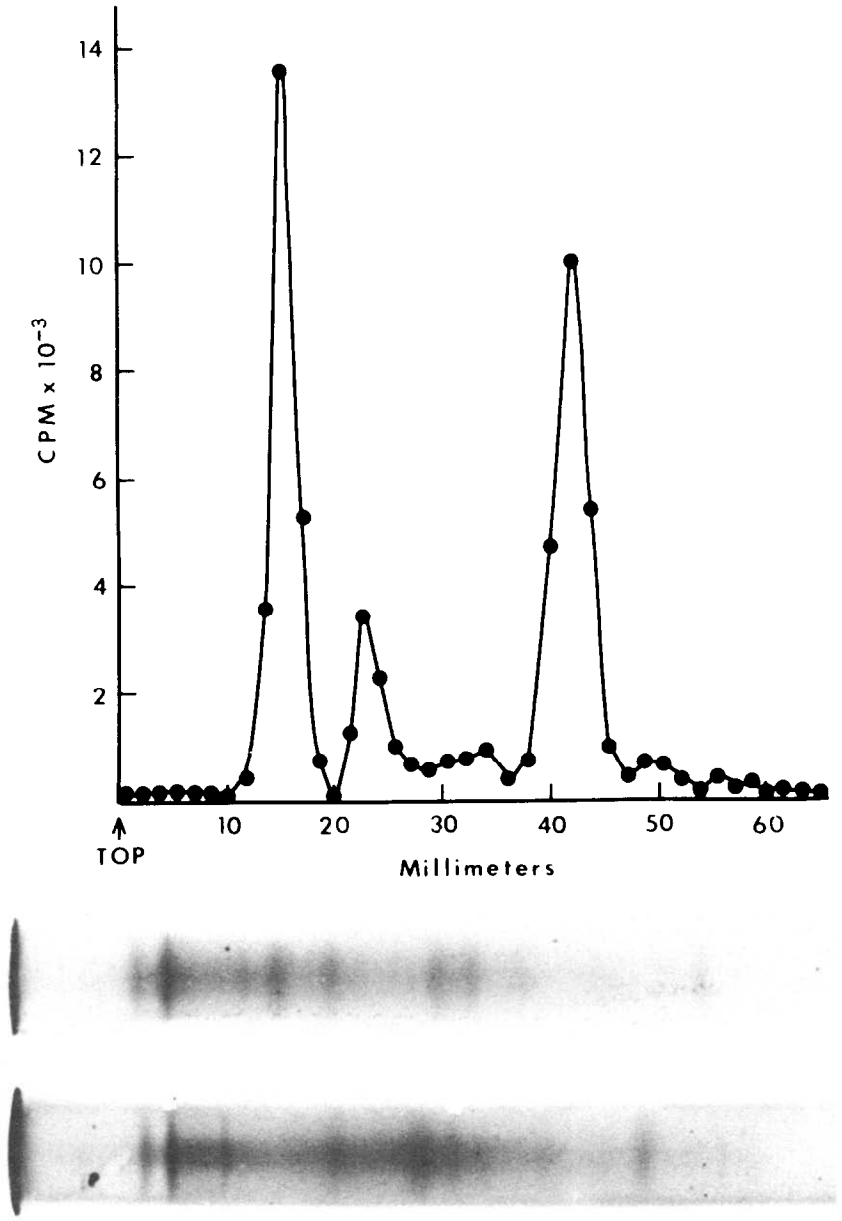

Fig. 1. Gel electrophoretic analysis of fractions from rat brain synaptosomes. The electrophoretic patterns are for STx (upper) and water-soluble (lower) fractions. The contained 31 and 23 μg of protein respectively. Consecutive slices assayed with gelatin gave identicaly activity (●——●) patterns for each preparation as shown. Other experimental details are described in the text.

properties were noted, however. Soluble preparations were well-resolved in the absence of detergent and were stable; STx lost activity within a few days when the detergent was removed, and it was not resolved by electrophoresis in the absence of detergent.

Data for cytosolic PCM analogous to those in Fig. 1 were quite recently reported by Aswad and Deight [25]. They isolated two major isozymes from bovine brain that were chromatographically distinct but which had the same molecular weight. These were electrically resolved, however, into three entities with pI values of 6.5, 5.7, and 5.6.

The appearance of similar, if not completely identical, patterns (Fig. 1) for soluble and membrane-bound enzymes was not entirely unexpected. In their studies of brain PCMs, Iqbal and Steenson [18] found that the two forms had almost identical properties with respect to molecular weight, pH optima, and K_m for AdoMet. The major differences, as noted for my own preparations, were that the soluble activity was stable, while the membrane-bound enzyme irreversibly lost activity when detergent was removed. Unlike the data in Fig. 1, Iqbal and Steenson [18] found only one active component in each preparation.

In whole bovine brain, the ratio of soluble to bound enzyme is about 60:40 respectively [17, 18]. The relative content, however, varies with the area of the brain. In the cerebellum, 76% of the activity is in the bound form,

whereas the pituitary has only 29% in this form [18]. The data in Table 1 show that the amount of membrane-bound activity also varies in subcellular brain organelles. With either endogenous MAP or exogenous acceptor, mitochondria and synaptosomes have higher specific activities than myelin. In some experiments, the activities of more highly purified synaptosomes and mitochondria were compared. These were analogous to the data in Table 1 except that with either endogenous or exogenous substrate the mitochondrial fraction had a higher specific activity than did synaptosomes. In the presence of Triton X-100, this difference was not observed (data not presented). Interestingly, mitochondria from two other tissues, which incidentally yielded no synaptosomal fraction, had little or no activity.

Table 1. Carboxylmethylase activity of particulate fractions from different tissues*

Tissue fraction	Activity (units/mg protein)	
	Substrate	
	Endogenous	Gelatin
Brain		
A	1.21 ± 0.25	4.80 ± 1.16
B	4.65 ± 0.23	15.9 ± 0.7
C	3.17 ± 0.65	19.9 ± 1.8
Heart		
C	0.48 ± 0.05	3.02 ± 0.23
Liver		
C	0.47 ± 0.09	0.49 ± 0.11

* Designations for fractions were derived from Gray and Whittaker [20] as follows: A, myelin; B, synaptosomes; and C, mitochondria. The data are mean values ± S.E. (N = 5). Liver mitochondria were prepared by the procedure of Schneider [26].

Evidence presented here suggests that, once solubilized, the bound forms of brain PCM are identical to the cytosolic forms, when isolated by electrophoresis under non-denaturing conditions. It is difficult at this time to reconcile this observation with the fact that the two forms differ in solubility and stability or to explain why some molecules are bound while others are not. Answers to these questions could reside in some subtle, as yet unrevealed differences in the membrane-bound isozymes. Experiments to isolate sufficient quantities of these for characterization are underway but are incomplete at this time.

Electrophoretic analysis of cytosolic and detergent-solubilized preparations of PCM under non-denaturing conditions showed that they have essentially the same protein pattern. Furthermore, each have two major and one minor enzymatically active peaks. In other words, once solubilized, protein carboxylmethylases in the two preparations appear to be the same. In contrast to peripheral tissues, brain mitochondria also have high levels of activity. Taken all together, the results presented here suggest the possibility that the soluble and bound enzymes could be synthesized by the same mechanisms, but that some functional or metabolic process peculiar to brain requires that they exist in different post-translation states, i.e. some are embedded in the membranes, some are not. This could explain why mitochondria of brain have a large component of bound enzyme, whereas liver and heart organelles are relatively deficient. It should be remembered also that synaptosomes are only resealed nerve cell endings and have their own complement of mitochondria [20].

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