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Characterization and Substrate Specificity of a Protein Carboxymethylase in the Pituitary Gland

(protein hormones/*S*-adenosyl-L-methionine/neuroendocrine/methanol-forming enzyme)

EMANUEL J. DILIBERTO, JR.* AND JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014; and * Research Associate in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **Protein carboxymethylase, an enzyme capable of methylating proteins and polypeptides, was purified from bovine pituitary. The anterior pituitary hormones, luteinizing hormone, follicle-stimulating hormone, adrenocorticotropic hormone, growth hormone, thyroid-stimulating hormone, and prolactin, were found to be substrates for this enzyme. The posterior pituitary hormones, oxytocin and vasopressin, did not serve as substrates. With luteinizing hormone as the substrate, protein carboxymethylase had a pH optimum near pH 5.5. A limiting K_m of 1.47 μM for *S*-adenosyl-L-methionine was obtained with luteinizing hormone as the methyl acceptor. Possible roles of this enzyme in the posterior and anterior pituitary are discussed.**

In a study of the distribution of phenylethanolamine *N*-methyltransferase in various tissues, Axelrod and Daly (1) observed the formation of a volatile product extractable in organic solvents when *S*-adenosyl-L-methionine was incubated with pituitary glands. Further investigation revealed that pituitary extracts contained an enzyme that forms methanol from *S*-adenosyl-L-methionine (1). Subsequently, several investigators isolated an enzyme(s) from various tissues that was capable of methylating protein in the presence of *S*-adenosyl-L-methionine and found that the methylated proteins are readily hydrolyzed at an alkaline pH to yield a volatile product (2–4). However, not until recently was it realized that the "methanol-forming" enzyme and the protein methylase(s) were the same enzyme (5). It was proposed that the protein methylase catalyzed the methylation of carboxyl groups to form methyl esters and that under mildly alkaline conditions the enzymically formed protein esters hydrolyzed to liberate methanol (5).

The "methanol-forming" enzyme has been identified in erythrocytes (6) and a variety of rat tissues (7). The enzyme was found to be most highly localized in the pituitary (1, 7), of which the posterior pituitary appears to have greatest activity (1); enzyme activity was also reported in the hypothalamus (7).

Several enzyme systems are known to be involved in the methylation of proteins: one that methylates the guanidino group of arginine residues in protein (8); another, located in the nuclei of various cell types, that methylates the ε-amino group of lysine residues in protein (9); and a third, the "methanol-forming" enzyme or the protein methylase, described here.

Abbreviations: SAM, *S*-adenosyl-L-[*methyl*-^{14}C]methionine; LH, luteinizing hormone (ovine).

The "methanol-forming" enzyme, which we will now call protein carboxymethylase, probably methylates the carboxyl groups of proteins by an ester linkage (3). Indirect evidence for this reaction was provided recently; conversion of free carboxyl groups of ribonuclease to an amide derivative decreased the methyl-acceptor activity by 85%, indicating that protein carboxymethylase probably methylates the free carboxyl groups (10). Several reports suggest that the enzyme catalyzes the formation of aspartyl and glutamyl methyl esters in the protein substrates using *S*-adenosyl-L-methionine as methyl donor (3, 4, 10).

This report describes the purification of a protein carboxymethylase from bovine pituitary and its ability to methylate all known anterior pituitary hormones.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine, 52.3 mCi/mmol, was purchased from New England Nuclear Corp. Luteinizing hormone (LH) (ovine), follicle-stimulating hormone (ovine), growth hormone (ovine), prolactin (ovine), and thyroid-stimulating hormone (bovine) were supplied by NIAMD. Lysine-vasopressin (synthetic), oxytocin (synthetic), adrenocorticotropic hormone (porcine), and bovine-serum albumin were obtained from Sigma Chemical Co. Poly(L-glutamic acid) and poly(L-aspartic acid) were purchased from ICN·K&K Laboratories, Inc. and Sephadex G-100 and DEAE–Sephadex A-50 from Pharmacia Fine Chemicals, Inc.

Enzymic Assay. Protein carboxymethylase activity was assayed by a modification of the method of Axelrod and Daly (1) or by the method of Kim and Paik (4). The former method was used to assay for enzymic activity based on methanol formation; the incubation mixture contained 50 μl of 0.5 M phosphate buffer (pH 6.0), 3.85 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (SAM), 52.3 mCi/mmol, purified enzyme (DEAE preparation), and the indicated amount of protein substrate in a final volume of 0.25 ml. The reaction was carried out at 37° for 15 min and terminated by the addition of 1 ml of 0.5 M borate buffer (pH 10.0) and 6 ml of 4:1 mixture of 3-methyl-1-butanol and toluene. After the sample was allowed to stand at ambient temperature for 15 min, it was agitated and centrifuged. Two 2-ml aliquots were transferred to two vials; to one, 10 ml of Bray's solution (11) was added and the sample was counted by liquid scintillation spectrometry (Beckman LS 250). The second aliquot was evaporated

TABLE 1. *Purification of protein carboxymethylase from bovine pituitary*

Enzyme preparation	Vol. (ml)	Protein conc. (mg/ml)	Specific activity (units/mg of protein)	Yield (%)	Purification	Ratio (endogenous/LH)
Whole homogenate	327	23	4.27	100	1.0	
Supernatant at 80,000 × *g* for 80 min	225	7.5	10.4	55	2.4	
Ammonium sulfate precipitate	40	13.5	23.9	42	5.6	0.82
Sephadex G-100 chromatography	80	0.256	574	37	134	0.14
DEAE–Sephadex A-50 chromatography	40	0.094	2,255	26	528	0.00

for 1 hr at 80° in a chromatography oven before radioactivity was determined. The radioactivity difference was taken as a measure of methanol formation. A blank in which enzyme was added after the incubation period was run simultaneously.

The second assay method involves precipitation of the methylated protein with trichloroacetic acid. Incubations were performed as described above and stopped by the addition of 0.5 ml of 30% trichloroacetic acid; after four 1-ml washings with 15% trichloroacetic acid, the precipitates were solubilized with 0.5 ml of NCS (Amersham/Searle Corp.) and counted. A blank, consisting of enzyme added after the incubation period, was also run.

All experiments were carried out in duplicate. Protein concentration was determined by the method of Lowry *et al.* (12) with bovine-serum albumin as standard. Enzyme specific activity was expressed as units per mg of protein where a unit equals 1 pmol of [^{14}C]methyl transferred or incorporated per min.

RESULTS

Purification of Protein Carboxymethylase from Bovine Pituitary. All procedures were carried out in the cold (0–4°). Bovine pituitaries (Pel-Freez Biological Inc.) (55 g) were homogenized in 275 ml of 0.25 M sucrose containing 3 mM $CaCl_2$ and 2.4 mM 2-mercaptoethanol in a Waring Blender for two 20-sec pulses with a 1-min interval. The homogenate was passed through three layers of sterile gauze and centrifuged (Spinco ultracentrifuge) at 80,000 × *g* for 80 min. To the supernatant (225 ml) was added ammonium sulfate to 50% saturation, and the precipitate was recovered by centrifugation. The precipitate was dissolved in 49 ml of the sucrose solution described above and was dialyzed against 5 mM phosphate buffer containing 5 mM EDTA and 2.4 mM 2-mercaptoethanol at pH 7.4 overnight with one buffer change.

TABLE 2. *Stoichiometric relationship of protein carboxymethylase activity assayed for methanol or for methylated protein formation*

Method	Specific activity (units/mg of protein)
Methanol formation	965
Methylated protein formation	986

Purified protein carboxymethylase was incubated with 3.85 nmol of SAM and 2 mg of ovalbumin at pH 6.0. The assay was carried out as described under *Methods* for methanol or for methylated protein formation.

The dialyzed preparation (40 ml) was applied to a Sephadex G-100 column (5.0 × 90.5 cm) that had been equilibrated with the 5 mM phosphate buffer (pH 7.4); the column was eluted with the phosphate buffer at a flow rate of 20 ml/hr. The most active fractions (80 ml) were pooled, concentrated by dialysis against 50% sucrose in 5 mM borate buffer with 5 mM EDTA and 2.4 mM 2-mercaptoethanol at pH 9.3, and redialyzed against 5 mM borate buffer with 5 mM EDTA and 2.4 mM 2-mercaptoethanol overnight. The dialyzed preparation was applied to a column (1.6 × 33 cm) of DEAE–Sephadex A-50 that had been equilibrated with the 5 mM borate buffer, pH 9.3. The column was eluted with 70 ml of borate buffer followed by a linear concentration gradient

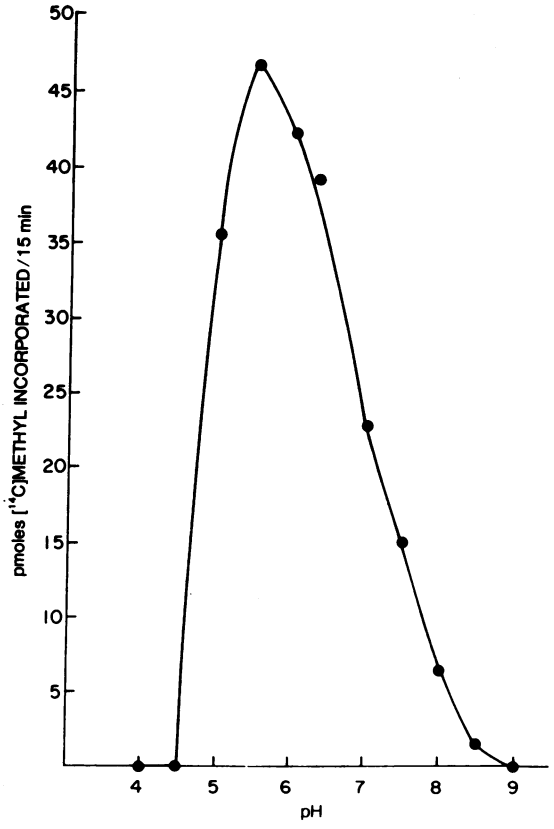

FIG. 1. Effect of pH on protein carboxymethylase activity. Sodium acetate buffer, 0.1 M, was used for the range of pH 4.0–5.5; 0.1 M sodium phosphate buffer for the range of pH 6.0–8.0; and 0.1 M Tris·HCl for pH 8.5–9.0. The assay was performed as described under *Methods* for trichloroacetic acid precipitation of methylated product using 1.6 μg of enzyme protein and 1 mg of LH as protein substrate.

to 0.4 M NaCl in borate buffer. The most active fractions were pooled and stored frozen at −20°. Table 1 lists the purification steps and purification achieved at each stage. The enzyme was purified 528-fold with a 26% yield. The ratio of carboxymethylase activity when assayed in the absence of added substrate (endogenous) to the activity when assayed in the presence of added substrate (LH) demonstrates that a 500-fold purification is required to separate the enzyme from the endogenous substrate(s) (last column, Table 1). The final step of the purification produced on enzyme preparation that, in the absence of added protein substrate, had no activity.

Characterization of Protein Carboxymethylase. Protein carboxymethylase was assayed for methanol formation or for methylated protein formation. Comparable results with the two methods were obtained when incubations were carried out with the purified enzyme at pH 6.0 (Table 2).

Protein carboxymethylase activity as a function of pH was examined with LH as the protein substrate (Fig. 1). The enzyme appears to have a narrow pH optimum near pH 5.5. The pH optimum probably reflects ionization characteristics of the enzyme and the protein substrate. Incubations were carried out at pH 6.0 where the enzyme is more stable (13).

A linear relationship between protein carboxymethylase activity and enzyme concentration was observed (Fig. 2). Incubations were carried out at pH 6.0 with LH as the protein substrate (methyl acceptor).

The Michaelis equation was plotted in the form usually ascribed to Lineweaver and Burk (1/v against 1/[S]) to calculate the kinetic parameters. Three plots were obtained from three fixed concentrations of LH and varying concentrations of SAM (Fig. 3). The intersecting or sequential pattern indicates that SAM and LH combine with the enzyme to form a ternary complex before product formation (14). The apparent K_m for SAM with LH (1 mg/0.225 ml of incubation mixture) as the methyl acceptor was 1.72 μM. A limiting

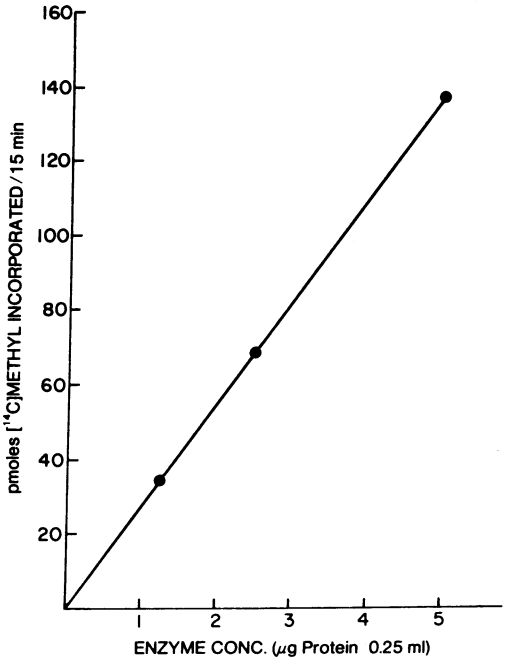

FIG. 2. Relationship between protein carboxymethylase activity and enzyme concentration. The assay was performed as described in the *legend* of Fig. 1 with 1 mg of LH as the protein substrate.

K_m of 1.47 μM for SAM with LH as the protein substrate was calculated from slope and intercept replots of Fig. 3; the V_m was 163 pmol/15 min per 4.7 μg of enzyme protein (2312 units/mg of protein). With prolactin (1 mg/0.225 ml of incubation mixture) as the protein substrate, the apparent K_m for SAM was 1.94 μM.

Substrate Specificity. The emphasis of the present report was to investigate the physiological role of the protein car-

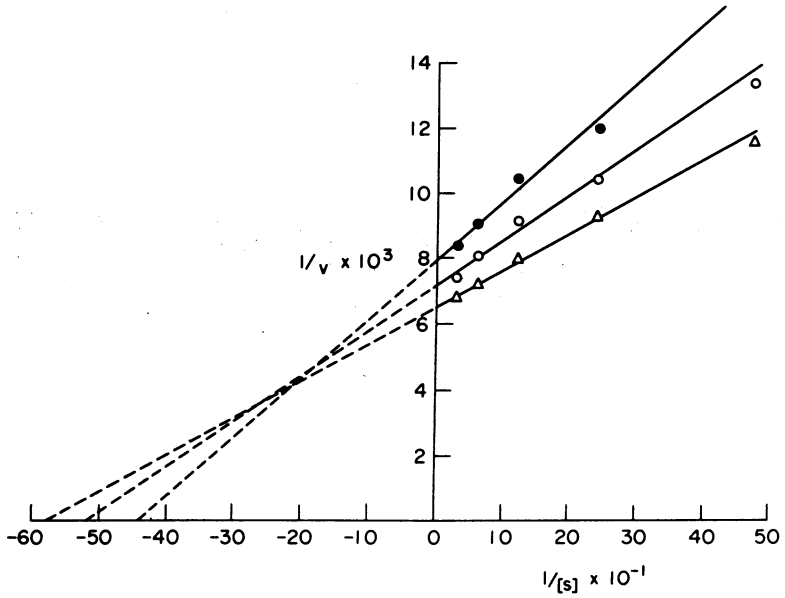

FIG. 3. Lineweaver and Burk plots of fixed concentrations of LH with varying concentrations of SAM. Protein carboxymethylase activity was measured as a function of SAM concentration with three fixed concentrations of LH: 0.028 mM (●——●), 0.056 mM (○——○), and 0.112 mM (△——△). The assay was performed as described under *Methods* for methanol formation using 4.7 μg of enzyme protein.

TABLE 3. *Substrate specificity of protein carboxymethylase*

Substrate protein	Specific activity (units/mg of protein)
Luteinizing hormone	2032
Follicle-stimulating hormone	1733
Adrenocorticotropic hormone	1702
Growth hormone	1644
Thyroid-stimulating hormone	1522
Prolactin	1457
Ovalbumin	967
Bovine-serum albumin	125
Vasopressin	120
Poly(aspartic acid)	108
Oxytocin	94
Poly(glutamic acid)	11

Protein carboxymethylase activity was assayed by the procedure described under *Methods* for methanol formation with 2 mg of substrate protein.

boxymethylase in the pituitary. In our search for possible endogenous substrates of protein carboxymethylase, the ability of protein pituitary hormones to serve as methyl acceptors was examined (Table 3). The most active substrate was the anterior pituitary hormone, LH; indeed, all anterior pituitary hormones were found to be effective methyl acceptors. Ovalbumin, a substrate for the spleen protein carboxymethylase (5), was less than half as active as LH. The posterior pituitary hormones, oxytocin and vasopressin, poly(L-aspartic acid), poly(L-glutamic acid), and bovine-serum albumin were essentially without effect as substrates.

DISCUSSION

The highest specific activity of protein carboxymethylase was found in the pituitary (1, 7). The localization of this enzyme and the substrate protein specificity reported here suggest a physiological function for protein carboxymethylase in the pituitary. All anterior pituitary hormones tested were effective substrates. The anterior pituitary hormones were found to be substantially more active than ovalbumin, which was previously reported to be the best substrate for the spleen protein carboxymethylase (5).

There is considerable evidence suggesting that protein carboxymethylase methylates the carboxyl groups of proteins (3, 10). Studies in our laboratory also suggest methylation of protein carboxyl groups (unpublished observations). Li and Fraenkel-Conrat (15) demonstrated that esterification of carboxyl groups of prolactin with methanol progressively lowered hormonal activity as more methyl groups were introduced into the molecule. It was concluded that carboxyl groups of prolactin are essential for biological activity (15). Since protein carboxymethylase has been shown to methylate prolactin and other anterior pituitary hormones on carboxyl groups, the function of this enzyme in the pituitary may be in the inactivation of anterior pituitary hormones. Inactivation by methylation might serve as a temporary storage form for the hormones since they are hydrolyzed by a small elevation of pH. The highest concentration of enzyme was found in the posterior pituitary (1), yet the posterior pituitary hormones were not effective as substrates. One possible explanation would be that the enzyme functions in the storage and/or the transport of the hormones by altering the binding proteins such as neurophysins.

Kinetic analysis demonstrated that SAM and the protein substrate, LH, combine with the enzyme to form a ternary complex prior to formation of the methylated protein. The limiting K_m of protein carboxymethylase for SAM with LH as the protein substrate was 1.47 μM, whereas, the apparent K_m values of catechol *O*-methyltransferase, hydroxyindole *O*-methyltransferase, and phenylethanolamine *N*-methyltransferase were reported to be 14, 14, and 10 μM, respectively (16). The fact that protein carboxymethylase has an apparent K_m value one order of magnitude less than the other methyltransferases suggests that the carboxymethylase may also function as a control mechanism for biogenic amine metabolism through its utilization of intracellular SAM.

Several characteristics of this enzyme, i.e., the pH optimum and the apparent K_m for SAM, and the characteristics of the methylated product appear to indicate that the pituitary protein carboxymethylase studied here is similar to the enzyme (protein methylase II) from calf thymus (4) and the protein methylase from calf spleen (3).

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