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EVIDENCE OF THE CARBOXYMETHYLATION OF NASCENT PEPTIDE CHAINS ON RIBOSOMES

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SUMMARY

Protein carboxymethylase from bovine anterior pituitary is found to be capable of carboxymethylating proteins in an in vitro protein synthesizing system which includes S-adenosyl-L-methionine-[^{14}C methyl], wheat germ ribosomes and oviduct mRNA. Optimal carboxymethylation is inhibited by puromycin indicating the requirement for de novo protein synthesis. Ultracentrifugal profiles show that carboxymethylated proteins are associated with ribosomal absorption peaks. This is consistent with the carboxymethylation of proteins occurring on nascent peptide chains.

The initial discovery of the carboxymethylation of proteins in normal and tumor tissue by protein carboxymethylase, PCM (1, 2), an enzyme capable of methylating side chain carboxyl groups in proteins in the presence of S-adenosyl-L-methionine (SAM), was followed by the recognition that it coincided with the methanol forming enzyme described by Axelrod and Daley (1965), (Morin and Liss, 1973). The formation of carboxymethyl esters as a result of methylation is a transitory modification of protein molecules, which readily hydrolyzed under mild alkaline conditions to liberate methanol (1-3).

Protein carboxymethylase from whole bovine pituitaries has been shown to methylate several polypeptide hormones (5). A diverse number of secreted proteins and peptides have been found to be substrates of PCM from calf spleen (2, 5), bovine pituitaries and egg laying hen oviducts (our unpublished data). It has been suggested that PCM may play a role in adrenal medullary secretion (6). Recent evidence suggests it plays a role in the human monocyte chemotactic mechanism (7). In bacteria, the methylation and demethylation of a cytoplasmic membrane protein were found to be involved in the chemotactic responses (8). In this paper we wish to report the methylation of nascent peptide chains by

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bovine anterior pituitary PCM in an in vitro protein translation system using hen oviduct messenger ribonucleic acid as message, and discuss the possible role of PCM in protein secretion.

MATERIALS AND METHODS

Egg laying hen oviducts were purchased from Pel-Freez Biologicals Inc. and stored at -20°C. Bovine pituitaries were freshly obtained from a local slaughterhouse and wheat germ was from General Mills (Minneapolis, Minnesota). S-Adenosyl-L-methionine-[^{14}C methyl] (58.3 mCi/mMole) was a product of the New England Nuclear. Oligothymidilic acid-cellulose was from Collaborative Research, Inc. Creatine phosphate, creatine phosphokinase, ATP, GTP, spermine, and amino acids were products of Sigma Chemical Company.

Pituitary PCM was partially purified using a DEAE-A50 Sephadex column (1.5 x 30 cm) which had been equilibrated with 2 mM sodium bicarbonate buffer (pH 8.0) containing 1 mM DTT. The column was eluted with a linear NaCl gradient (0-0.3 M). Fractions containing the highest enzymatic activity were pooled and concentrated by ultrafiltration using Amicon PM 10 membrane.

Preparation of hen oviduct total RNA was as described by O'Malley et al. (9). Purification of Poly(A) containing oviduct mRNA using Oligo(dT)-Cellulose affinity chromatography was performed by the method of Aviv and Leder (10) with the omission of the 0.1 M KCl salt wash. Isolation of oviduct polysomes was by the method of Schimke et al. (11).

The wheat germ 30,000 xg supernatant (S-30) fraction was prepared by the procedure of Roberts and Paterson (12), but 12 grams of wheat germ were used instead of 6, and the pooled fraction of S-30 from Sephadex G-25 column was centrifuged at 30,000 xg and 2°C for 10 minutes. This reduced the protein concentration of the S-30 without reducing its translation activity.

The methylation of the nascent peptide chains was performed in an in vitro protein synthesizing system as follows. The system contained in a volume of 0.215 ml: 20 µl of wheat germ S-30, 20 mM of N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.0), 2 mM DTT, 1 mM ATP, 200 µM GTP, 8 µg creatine phosphokinase, 40 µM spermine, 8 mM creatine phosphate, 2.15 nmoles of radioactive SAM, 84 mM KCl, 3.5 mM magnesium acetate, 20 µM each of 20 amino acids, and when included, 20 µg of oviduct mRNA, 0.25 A_{260} units of oviduct polysomes, 27 µg of methylase protein, and 1 mM of puromycin. The incubation was for 1 hour at room temperature. After incubation, the mixture was diluted with 1 ml of a buffer containing 25 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$ and 140 mM sucrose. The mixture was then layered over 10.8 ml of a continuous sucrose gradient (1.5-0.5 M) and centrifuged at 2-4°C and 283,000 xg for 96 minutes in a SB-283 rotor (International Equipment). At the end of centrifugation, the tube was punctured from the bottom with a needle connected to a UV monitor and continuous A_{260} readings were recorded. Twelve 1 ml fractions were collected, and each fraction was then subjected to standard assay for carboxymethylated protein. The assay was based on the determination of radioactive methanol after hydrolysis of protein methyl esters under alkaline conditions as follows. To each 1 ml fraction, 0.2 ml of 2 N NaOH was added to convert the [^{14}C] methyl esters to [^{14}C] methanol. The sample was allowed to stand at room temperature for 15 minutes to form the hydrolysis product [^{14}C] methanol, which was then extracted by the addition of 6 ml of isopentyl alcohol. After centrifugation to break the emulsion, two separate 2 ml aliquots were removed. To one aliquot, the "wet" sample, 2 ml of ethanol was added followed by 10 ml of Liquifluor. The second aliquot was evaporated at 60°C under a water aspirator vacuum. To this "dry" sample, 2 ml of isopentyl alcohol, 2 ml of ethanol and 10 ml of Liquifluor were added. The radioactivity in each sample was determined in a liquid scintillation counter. [^{14}C] Methanol was calculated by the numerical difference in the "wet" and "dry" (13).

Table 1. PCM activity of cellular fractions from oviduct tissue of egg-laying hens.

Cell fraction assayed	[^{14}C]Methanol formed (pmoles)	
	Minus exogenous substrate	Plus ACTH
Whole homogenate	62	72
800 xg Supernatant	51	70
8,700 xg Supernatant	40	70
198,000 xg Supernatant	50	77
800 xg Pellet	13	33
8,700 xg Pellet	2	3
198,000 xg Pellet	4	12

Fresh oviduct tissue (3 grams) was homogenized in 3 volumes of cold buffer containing 50 mM triethanolamine HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose. PCM activity was determined in the whole homogenate and the various subcellular fractions. The various pellets were resuspended to their original pre-centrifugal volume using the same buffer. Assays contained 0.1 ml of either the supernatant fraction or the resuspended pellet, 0.1 ml of buffer (5 mM sodium phosphate, pH 7.4) or $_pACTH_{1-39}$ (0.5 mg/ml) and 0.42 nmoles of [^{14}C-methyl] SAM. Samples were incubated at 37°C for 1 hour. After incubation, 0.6 ml of 0.5 M sodium borate buffer (pH 10) was used to hydrolyze protein [^{14}C] methyl esters. [^{14}C] methyl groups incorporated into protein were calculated from the numerical difference in "wet" and "dry" samples as described in Materials and Methods.

RESULTS

In most tissues PCM is found primarily in the cytosol, but in ox brain a soluble and membrane form of the enzyme has been reported (14).

Table 1 shows that almost 90% of the PCM activity is found in the cytosol fraction of oviduct tissue from egg laying hens. Of the secreted proteins found in egg white, ovalbumin, conalbumin, ovomucoid, avidin, and lysozyme, only lysozyme and avidin cannot serve as a substrate in vitro (our unpublished data). Although carboxymethylated proteins have never been isolated from oviduct tissue, suggesting a transitory type of methylation in vivo, it was deter-

Table 2. Carboxymethylation of de novo synthesized proteins.

Experiment	Additions				[^{14}C]Methanol formed (pmoles)
	Wheat germ S-30	Oviduct mRNA	PCM	Puromycin	
1	+	+	+	-	106
2	+	-	+	-	26
3	+	+	-	-	40
4	+	+	+	+	41

Total protein carboxymethyl esters were measured as methanol after alkaline hydrolysis.

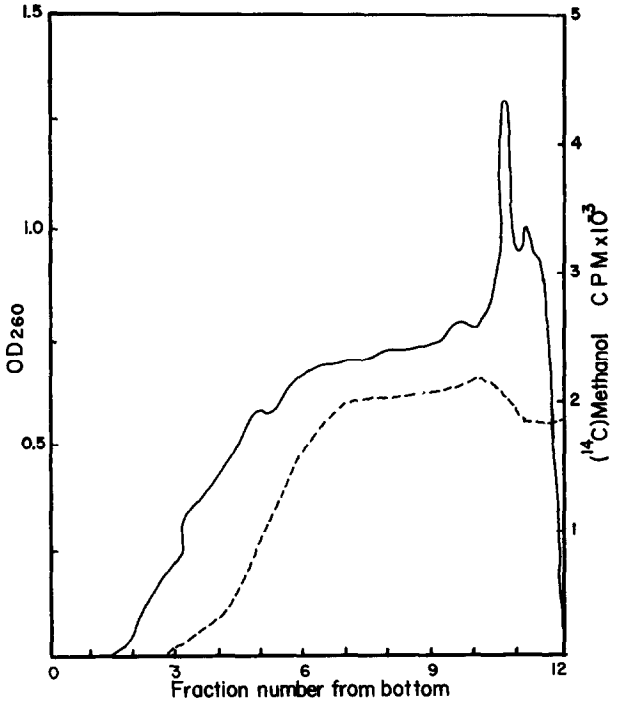

Fig. 1. Distribution of [^{14}C] carboxymethylated protein in the ribosomal and cytosol fractions determined after sucrose density gradient centrifugation as described in Materials and Methods. OD_{260}, solid line; CPM, broken line.

Table 3. Requirements for optimal carboxymethylation of proteins in an in vitro translation system.

Experiment	Additions					[^{14}C]Methanol formed (pmoles)
	Wheat germ S-105	Wheat germ ribosomes	Oviduct ribosomes	Oviduct mRNA	PCM	
1	+	+	+	+	+	126
2	+	-	+	+	+	149
3	+	+	-	+	+	176
4	+	-	-	+	+	67
5	+	+	-	-	+	57
6	+	-	-	-	+	51
7	+	-	-	-	-	39
8	-	-	-	-	+	6

The sum total amount of carboxymethylated proteins was determined. A wheat germ S-30 fraction was centrifuged at 105,000 xg for 2 hours to obtain a 105,000 xg supernatant (S-105) fraction and the ribosomal pellet. When necessary, wheat germ S-30 was reconstituted from the wheat germ S-105 and wheat germ ribosomal fractions to their original proportions.

mined whether PCM could methylate nascent polypeptide chains during translation in vitro.

The data in Table 2 reveal that optimal carboxymethylation of proteins by bovine anterior pituitary PCM in an in vitro protein translation system is dependent on wheat germ S-30 fraction and oviduct mRNA, and it is inhibited by puromycin suggesting de novo synthesis of proteins is necessary for optimal levels of carboxymethylation.

Further analysis indicated that optimal carboxymethylation of such proteins in the translation system used was dependent on the presence of wheat germ ribosomes and oviduct mRNA. The data in Table 3 show that the omission of wheat germ ribosomes from the system results in a drop of [^{14}C] methanol from 176 pmoles to 67 pmoles, while omission of oviduct mRNA

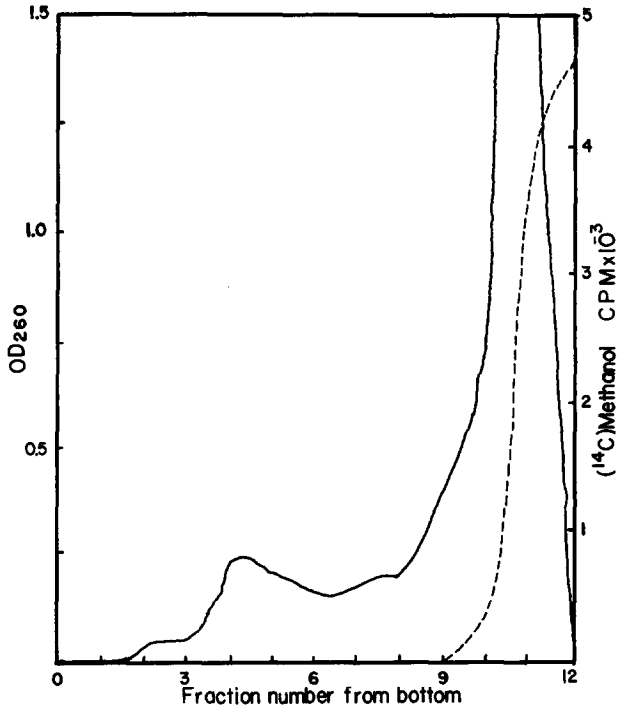

Fig. 2. Distribution of [^{14}C] carboxymethylated protein in the absence of added wheat germ ribosomes. Incubation and density gradient centrifugation otherwise the same as in Fig. 1.

results in a drop to 57 pmoles. When both wheat germ ribosomes and oviduct mRNA are omitted, the [^{14}C] methanol formed is only 51 pmoles. In order to determine whether the carboxymethylation of the proteins synthesized de novo can occur while they are nascent chains on ribosomes, experiments were carried out in an attempt to detect such ribosome associated carboxymethylated nascent chains. Figure 1 shows that carboxymethylated proteins are associated with ribosomal absorption peaks in a translation system including wheat germ ribosomes and oviduct mRNA. When wheat germ ribosomes (Fig. 2) or oviduct mRNA (Fig. 3) is not added, the association of carboxymethylated proteins with ribosomal peaks disappeared.

DISCUSSION

Of the secreted proteins found in egg white-ovalbumin, conalbumin, ovomucoid, avidin and lysozyme, those that can be carboxymethylated by PCM

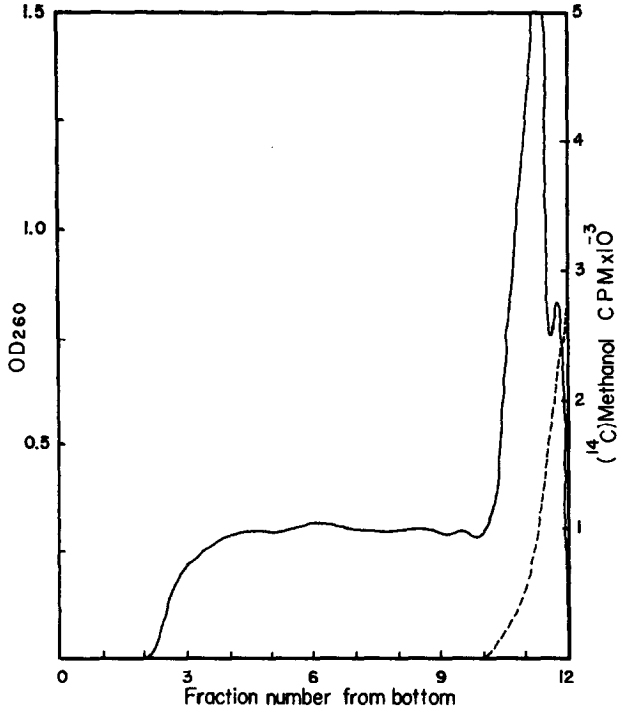

Fig. 3. Distribution of [^{14}C] carboxymethylated protein in the absence of added mRNA. Incubation and density gradient centrifugation otherwise the same as in Fig. 1.

in vitro are ovalbumin, conalbumin and ovomucoid. PCM is a cytosol enzyme in oviduct tissue, and if these proteins are carboxymethylated in vivo, this would be expected to occur while they are being synthesized on ribosomes, before they are sequestered within intracellular membraned vacuoles where they would not be accessible to the enzyme.

Evidence has been presented that indicates that carboxymethylation of proteins can occur while they are at the stage of nascent chains attached to ribosomes. Through charge neutralization this could facilitate the transport of and sequestration of secretory proteins within intracellular membraned organelles.

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