EVIDENCE FOR A METHYLATED PROTEIN INTERMEDIATE IN PITUITARY METHANOL FORMATION

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Summary: Pituitary glands contain both the methanol-forming enzyme of Axelrod and Daly (1) and S-adenosylmethionine dependent protein carboxyl-methylase. It is proposed that the in vitro formation of methanol is the result of chemical cleavage of enzymatically formed protein methyl esters.

The enzymatic formation of methanol from S-adenosylmethionine was found originally by Axelrod and Daly in extracts of pituitary glands (1). The reaction could be perceived as a hydrolysis of S-adenosylmethionine or a methylation of water as follows:

S-adenosylmethionine + $H_2O \longrightarrow$ S-adenosylhomocysteine + methanol (1)

The methanol-forming enzyme has since been identified in red blood cells(2) and various rat tissues(3). Methanol formation from S-adenosylmethionine is normally assayed at pH 7.9-8.0 and after the addition of 0.5 M borate buffer of pH 10, it is extracted into isoamyl alcohol(1-3). We wish to present evidence for an alternative mechanism for the in vitro appearance of methanol in tissue extracts. This mechanism proposes the S-adenosylmethionine mediated formation of methyl esters of endogenous tissue protein by the enzyme, protein carboxyl-methylase(4-6). Protein carboxyl-methylase is capable of methylating side chain carboxyl groups of protein, but not of free amino acids; and the methyl esters are known to be very alkali labile with the formation of methanol (5).

Proposed Mechanism for Methanol Formation:

a. S-adenosylmethionine + Protein-COOH $\xrightarrow[\text{pH 6.2}]{\text{protein carboxyl-methylase}}$ S-adenosylhomocysteine + Protein-COOMe

b. Protein-COOMe $\xrightarrow{\text{pH 10}}$ Protein-COOH + MeOH

Summation: S-adenosylmethionine $\xrightarrow{\text{enzyme}}$ S-adenosylhomocysteine +MeOH

Such a mechanism would be consistent with the overall reaction described by Axelrod and Daly(1), but rather suggests that the methanol formed *in vitro* is by chemical cleavage of an enzymatically formed intermediate.

Materials: S-adenosylmethionine-$^{14}CH_3$ (53mCi/mmole) was a product of New England Nuclear, Inc. Ovalbumin was purchased from Mann and lyophilized hog pituitaries were obtained from Armour and Co.

Preparation of Pituitary Extracts: Lyophilized pituitary glands (600mg) were homogenized in 3 ml of 0.02 M sodium phosphate buffer, pH 6.2 with 3 mM mercaptoethanol. The sample was centrifuged at 40,000 x g for 1 hour, the supernatant recovered and dialyzed against the above buffer and finally, the supernatant solution was recovered after centrifugation of the dialyzed extract. The dialyzed extract served as source of enzyme.

Protein Carboxyl-Methylase Activity in Pituitary Extracts

Extracts of hog pituitary glands were found to contain protein carboxyl-methylase activity (Table I). Such extracts contain endogenous protein acceptor since addition of 5x-crystallized ovalbumin, an excellent substrate for spleen carboxyl-methylase (5) is without additive effect, and in fact may be slightly inhibitory. Methanol-forming activity is readily detected in such extracts prepared at pH 10.

The pH optimum for protein carboxyl-methylase activity is 6.2 in pituitary extracts, whereas assay of methanol-forming

Table I

Protein Carboxyl-Methylase and Methanol-Forming Activities of Pituitary Extracts

	Methylase Activity	Methanol Formation/2 ml isoamyl extract		
	cpm	cpm wet	cpm dry	cpm volatile
Complete system	4347	8520	262	8258
-OA	3571	7304	142	7162
0 time	198	263	135	128

Pituitary extract (2.6 mg protein) was incubated with 2.4 mg crystalline ovalbumin (OA) and 0.1 μCi of S-adenosylmethionine-$^{14}CH_3$, (SAM-$^{14}CH_3$) in 0.02 M sodium phosphate buffer, pH 6.2 containing 3 mM mercaptoethanol in a final volume of .21 ml for 1 hour at 37°. For methylase assay, 0.1 ml cold 15% TCA was added to incubation mixtures which were then washed 4x with 5%TCA. Precipitates were collected on Millipore filters and radioactivity measured on a gas flow counter. For methanol assay, 0.5 ml of 0.5 M borate buffer, pH 10, was added to the incubated samples which were then extracted with 6 ml of isoamyl alcohol. The samples were centrifuged. Separate 2 ml aliquots of isoamyl alcohol extract were counted: "wet" with 2 ml ethanol and 10 ml fluors and "dry" after evaporation of the 2 ml extract. To the dried sample, 2 ml isoamyl alcohol reagent, 2 ml ethanol and 10 ml fluors were added (1). Assay was in a Mark II liquid scintillation counter.

Table II

Incubation mixture	pH	Extraction Additive	Volatile cpm/2 ml isoamyl extract
Complete	6.2	0.5 ml H_2O	1086
		0.5 ml borate, pH 10	3210
-OA	6.2	0.5 ml H_2O	1222
		0.5 ml borate, pH 10	3501
0 time	6.2	0.5 ml H_2O	34
		0.5 ml borate, pH 10	40
Complete	7.9	0.5 ml H_2O	2473
		0.5 ml borate, pH 10	3027
-OA	7.9	0.5 ml H_2O	2351
		0.5 ml borate, pH 10	4246
0 time	7.9	0.5 ml H_2O	118
		0.5 ml borate, pH 10	42

Pituitary extracts (2.2 mg), SAM-$^{14}CH_3$(20,000 cpm) and OA (2.4 mg) were incubated in either 0.02 M sodium phosphate buffer, pH 6.2 or 0.5 M sodium phosphate, pH 7.9 in a final volume of of 0.2 ml for 60 min. at 37°. To the incubated mixtures either 0.5 ml of H_2O or 0.5 ml of 0.5 M borate buffer of pH 10 was added, followed by the addition of 6 ml isoamyl alcohol. The mixed samples were centrifuged and separate "wet" and "dry" 2 ml aliquots were counted as described in Table I.

Table III

Identification of ^{14}C-Labeled Protein

Treatment	Filter Disc; cpm
TCA washed	2569
0 time	422
Plus chloroform/methanol(2/1)	2514
Plus chloroform	2612
Plus ethanol(100%)	2545
Plus acetone	2641
Plus alcohol/ether(3/1)	2623
Plus isoamyl alcohol	2504
TCA washed after RNase pretreatment	2295

Dialyzed pituitary extract (10 mg) was incubated with SAM-$^{14}CH_3$ (200,000 cpm) in 0.02 M sodium phosphate, pH 6.2 containing 3mM mercaptoethanol for 60 min. at 37°, in a final volume of 2.0 ml. At zero time and after 60 min. incubation, 0.1 ml aliquots of the incubation mixture were separately applied to 23 mm Whatman No. 3 filter discs and air dried. The discs were washed by successive immersions in cold 10% TCA (30 min.), 5% TCA (twice, 15 min. each), and then air dried (7). Subsequent treatments of separate discs with organic solvents (10 min.) was followed after air drying by insertion of the discs in scintillation vials with 10 ml toluene-fluor mixture.

activity in pituitary extracts as described by Axelrod and Daly is done by incubation at pH 7.9 followed by a treatment with 0.5 M borate buffer of pH 10(1). It can be seen that the more alkaline the extraction and incubation conditions, the greater the yield of $^{14}CH_3OH$ (Table II). Such a result would be consistent with the chemical hydrolysis of enzymatically synthesized protein methyl esters (5).

The formation of endogenous protein carboxyl-methyl esters is indicated in Table III. The radioactivity is located in the protein fraction as seen by the fact that incubation of the pituitary extract with SAM-$^{14}CH_3$ yields trichloroacetic acid precipitable counts. That the labeled product is not a lipid is indicated by the fact that the radioactivity in the TCA precipitate could not be extracted with diverse lipid solvents. The radioactivity

Table IV

Extraction of Methanol from ^{14}C-Methylated Protein Intermediate after Borate Treatment

Protein Sample on Disc	CPM Disc Protein	CPM/8 ml Isoamyl Extract		
		wet	dry	volatile
TCA and ethanol washed	2545	2240	248	1992

Radioactive, methylated protein was collected on a filter disc after incubation and assay as described in Table III. The disc was washed with TCA, ethanol and air dried. The disc was cut into small strips and inserted into a conical, glass centrifuge tube. A 1 ml aliquot of 0.5 M borate buffer, pH 10 was added to the tube and the mixture was allowed to stand for 10 min. at room temperature. The mixture was then extracted with 8 ml isoamyl alcohol. The phases in the tube were separated by centrifugation. Aliquots of the isoamyl extract were counted "wet" and "dry" as described under Table I. The volatile counts represent the differance between the two samples. Evaporation of methanol was achieved by gentle aeration of the isoamyl extract for 30 min. at 60°.

in the TCA precipitate was not diminished by pretreatment of the incubated mixture with 200 µg of pancreatic RNase, excluding methylated RNA.

Formation of Methanol from ^{14}C-Labeled Protein.

Further evidence that exposure to 0.5 M borate, pH 10, results in the chemical cleavage of ^{14}C-methylated protein to yield methanol is seen in Table IV. The radioactive, labeled protein methyl ester is extractable into isoamyl alcohol only after the filter paper discs containing the labeled protein was treated with 0.5 M borate buffer, pH 10; a procedure which is part of the standard assay for methanol-forming enzyme (1). The radioactivity in the isoamyl extract is volatile since the radioactivity is lost when the isoamyl extract is gently aerated at 60°. Of the known protein methylases; lysyl-, arginyl-, and carboxyl-protein methylases, only carboxyl-methylase is known to yield methanol under mild hydrolytic conditions (4-6).

S-adenosylethionine could not serve as substrate for pituitary protein carboxyl-methylase, nor, as would be expected from the proposed pathway, could it serve in the formation of ethanol by pituitary extracts. Also, efforts to detect an esterase in pituitary extracts which could hydrolyze protein methyl esters to methanol have been as yet unsuccessful.

Discussion

It is proposed that methanol is formed, at least *in vitro*, primarily by the chemical hydrolysis of methyl esters of endogenous tissue proteins. The protein methyl ester intermediates are synthesized by the S-adenosylmethionine mediated activity of protein carboxyl-methylases.

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