Arch. Pharm. Res. 10(3), 193-196 (1987)

Endogenous Proteinaceous Inhibitor for Protein Methylation Reactions

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(Received August 10, 1987)

Protein methylation occurs ubiquitously in nature and involves N-methylation of lysine, arginine, histidine, alanine, proline and glutamine, O-methylesterification of dicarboxylic acids, and S-methylation of cysteine and methionine[1-3]. In nature, methylated amino acids occur in highly specialized proteins such as histones, flagella proteins, myosin, actin, ribosomal proteins. hn-RNA-bound protein, HMG-1 and HMG-2 protein, fungal and plant cytochrome *c*, myelin basic protein, opsin, EF-Tu, EF-1α, porcine heart citrate synthase, calmodulin, ferredoxin, α-amylase, heat shock protein, scleroderma antigen, nucleolar protein C23 and IF-3*l*[4]

These methylations are carried out by several classes of highly protein-specific methyltransferases[3]. For example, protein methylase I (S-adenosyl-L-methionine: protein-arginine N-methyltransferase; EC 2.1.1.23), representing one such class, methylates the guanidino group of arginine residues; protein methylase II (S-adenosyl-L-methionine: protein-carboxyl O-methyltransferase; EC 2.1.1.24) methylates the carboxyl group of glutamyl or aspartyl residues; and protein methylase III (S-adenosyl-L-methionine: protein-lysine N-methyl-transferase; EC 2.1.1.43) methylates the ε-amino group of lysine residues[3].

The most remarkable characteristics of these methyltransferases is the high degree of specificity toward a particular amino acid residue in the substrate protein. However, there is increasing evidence of an additional level of specificity in the identity of the methyl-acceptor protein species. At least several examples of each of these methyltransferase classes have been characterized: Histone-, cytochrome *c*-, and calmodulin-specific protein methylase III's or histone- and myelin basic protein (MBP)-specific protein methylase I's[3]. Recently, a protein methyiase III has been partially purified from *Crithidia oncopelti* and this enzyme is highly specific for its own cytochrome *c*-557 but not for horse heart cytochrome *c*[5]. Furthermore, histone-specific protein methylase III has a counterpart enzyme ε-alkyllysinase (ε-Alkyl-L-lysine: oxido reductase; EC 1.5.3.4) that dealkylates methylated protein[6]. In addition to these enzymes, evidence indicates the presence of an enzyme that methylates histidine residues of muscle protein[7].

Earlier, we identified a factor in the rat liver which inhibited protein methylase II activity[8]. Not too long ago, Chiva and Mato[9] partially purified a cytosolic proteinaceous factor from rat liver, which inhibited phosphatidylethanolamine methyltransferase. Since none of these factors has been extensively purified and characterized, we felt it worthwhile to further purify the factor(s). A brief account of purification and characterization of this inhibitory factor which has been purified to apparent homogeneity is described herein.

RESULT

Purification of an inhibitor for protein methylase II

The inhibitor for protein methylase II was purified from rat liver particulate fractions to apparent homogeneity through gel filtration involving Sephadex G-25 column chromatography and HPLC employing Protein PAK 125 (separates proteins on the basis of hydrophobicity and molecular sieve) and a μ-Bondapak C_{18} column (separates proteins on the basis of hydrophobicity). Fig. 1 shows the elution profile of the inhibitor on HPLC μ-Bondapak column (final purification step). When ass ayed, all the protein methylase II inhibitory activity was found in a single major peak, indicated by an arrow in the figure. Twenty four micrograms inhibitor was obtained from 300 grams rat liver[10].

Purity of the inhibitor

When the inhibitor preparation which was purified using the HPLC μ-Bondapak C_{18} column (Fig. 1) was analyzed on two-dimensional paper electro-

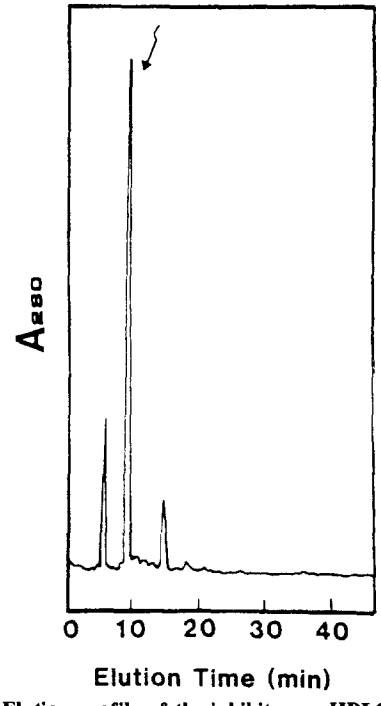

Fig. 1. Elution profile of the inhibitor on HPLC with a μ-Bondapak C_{18} column

Column was equilibrated with 0.1% trifluoroacetic acid in water. The peptides were eluted with a linear gradient of acetonitrile[10]

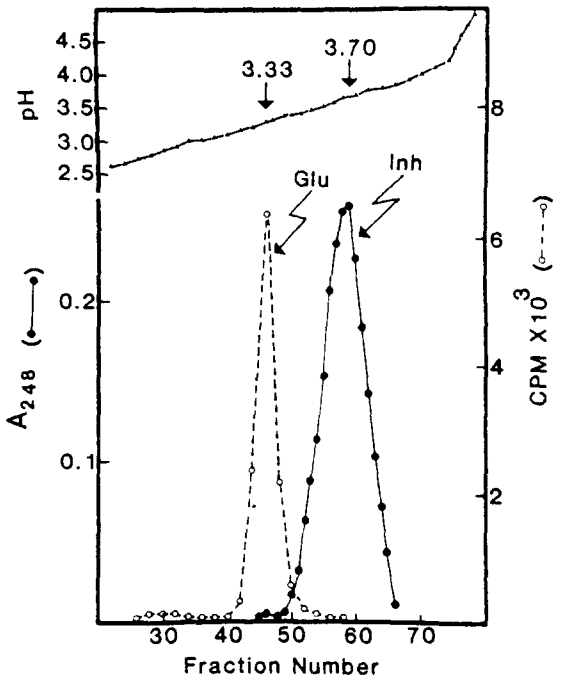

Fig. 2. Isoelectrofocusing of the HPLC-purified inhibitor.

Ampholine (pH range 2-4) was used. Inhibitor was monitored by measuring A_{246}. As an internal standard marker, L-(U-^{14}C)glutamic acid was run.

phoresis, only a single ninhydrin-positive spot was detected. In addition, the preparation yielded a single symmetrical peak with pI of 3.7 on an isoelectrofocusing column (Fig. 2). The results obtained by HPLC, two-dimensional paper electrophoresis and isoelectrofocusing demonstrate that the inhibitor has been effectively purified to homogeneity.

Spectral characteristics of the purified inhibitor

The compound has an absorbance peak at 248 nm in both acidic and neutral media (Fig. 3). In alkaline medium two absorbance peaks were observed at 206 nm and 248 nm. The peak at 206 nm, however, appears to be very unstable. When the compound was allowed to stand in the dark in alkaline medium for 24 hours, the 206 nm peak disappeared[10]. Fluorescence spectra of the purified inhibitor exhibited an excitation peak at 285 nm and an emission peak at 358 nm[10]. The heights of these two peaks depended greatly on the pH of the medium, being the highest in the alkaline pH.

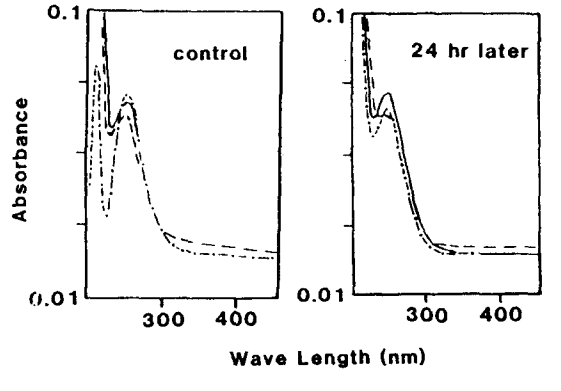

Fig. 3. Ultraviolet and visible light absorption spectra of the inhibitor solution.

(—)The spectra at pH 3.0, (----)pH 7.0, and (·····) pH 10.3. The pH of the solution was adjusted with either 0.1 M HCl or NaOH. The samples were kept in the dark for 24 hours at 4°C.

Table I. Amino acid composition of the inhibitor

Amino acids detected	Observed amount (pmol / sample)	Minimum number of residues calculated	Number of residues	Percent composition (mol / 100 mol)
Aspartic	27.8 ± 0.3	1.00	1	3.7
Glutamic	41.8 ± 0.5	1.50	2	7.4
Serine	96.8 ± 0.1	3.48	3	11.1
Glycine	296.5 ± 0.9	10.67	11	40.7
Alanine	67.2 ± 0.1	2.42	2	7.4
Tyrosine	43.3 ± 0.1	1.55	2	7.4
Methionine	77.0 ± 0.1	2.77	3	11.1
Isoleucine	57.8 ± 0.5	2.08	2	7.4
Leucine	28.7 ± 0.1	1.03	1	3.7
Total			27	

The molecular weight calculated from the total is 2,482 Da.

Structural nature of the inhibitor

Table I lists the amino acid analysis data. The minimum total number of amino acid residues is calculated to be 27, of which 11 residues (41%) are glycine. The inhibitor is devoid of any basic amino acid. Excluding the possible presence of a cofactor with fluorescence, the molecular weight of the inhibitor is calculated to be 2,482 Da. The inhibitor was found to be completely resistant to the action of DNase and RNase. Furthermore, inhibitory activity of the inhibitor was not affected either by treatment with trypsin or by heating it at 100 °C for 5 minutes.

Effect of inhibitor concentration on protein methylase II activity

Fig. 4 illustrates the effect of inhibitor concentration on protein methylase II activity. As shown in the figure, only 80% of the maximum inhibition was achieved with the highest concentration of the inhibitor.

Specificity of the inhibitor

Chiva and Mato[9] observed earlier that a proteinaceous inhibitor purified from rat liver cytosol inhibited phosphatidyl-ethanolamine methyltransferase, however, they did not attempt its effect on other S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases. As shown in Table II, both peaks I and II of Chiva and Mato[9] preparation also inhibited all of the other AdoMet-dependent methyltransferases examined so far,

Table II. Effect of purified inhibitor on various AdoMet-dependent methyltransferases[10]

AdoMet-dependent methyltransferases tested	Inhibitor for phosphatidylethanolamine methyltransfe. peak I (%)	peak II	Inhibitor for protein methylase II
Phosphatidylethanolamine	43	30	36
Phenylethanolamine	49	41	15
Catechol-O-methyltransferase	84	3	n.d.*
Thiol S-methyltransferase	14	23	n.d.
DNA	77	69	60
RNA	68	38	45
Protein methylase I	79	60	30
Protein methylase II	40	23	43
Protein methylase III	66	65	33

* Not determined.

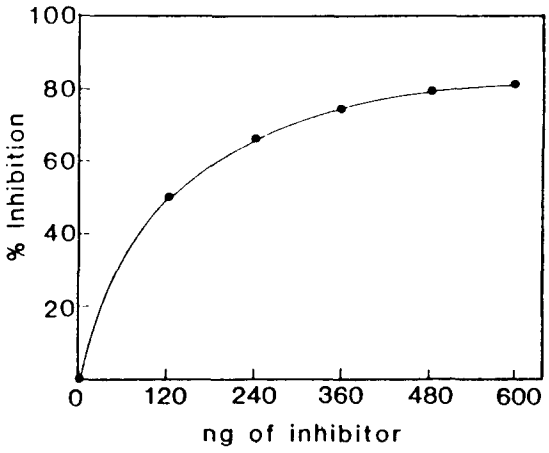

Fig. 4. Effect of the inhibitor concentration on protein methylase III
Total incubation mixture was 0.050 m*l*[10].

including protein methylase II. Likewise, the inhibitor we purified for protein methylase II also inhibited all of the AdoMet-dependent methyltransferases examined, including phosphatidylethanolamine methyltransferase. Therefore, the results in Table II together with the fact that both inhibitors were purified from rat liver strongly suggest that the membrane-bound inhibitor of protein methylase II is very similar, if not identical, to the cytosolic inhibitor described by Chiva and Mato. Furthermore, Table II emphasizes the fact that the proteinaceous inhibitor described here is not specific toward any specific single methyltransferase.

CONCLUSION

The wide distribution of the inhibitor among various subcellular fractions and rat organs[8] and its inhibitory activity on many AdoMet-dependent methyltransferases strongly suggest the importance of *in vivo* methylation. Since 120 micrograms inhibitor in 0.050 m*l* incubation mixture inhibits the protein methylase II activity by 50% (Fig. 4) and the molecular mass of the inhibitor is assumed to be 2,482 Da, the final concentration of the inhibitor needed to inhibit the enzyme activity by 50% is calculated to be 0.97 μM. This value is extremely close to the reported K_i values of S-adenosyl-L-homocysteine towards various methyltransferases (1-10 μM)[11]. Furthermore, since the present inhibitor is primarily membrane-bound, the *in situ* concentration of the inhibitor in the membrane might even be higher. To what extent the present inhibitor participates in modulation of biological methyltransferation remains to be explored.

ACKNOWLEDGEMENTS

This work was supported by the research grants DK09602 from the National Institute of Diabetes, Digestive and Kidney Diseases (WKP) and RG1765-A-1 from the National Multiple Sclerosis Society.

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