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IDENTIFICATION OF GLUTATHIONE S-TRANSFERASE AS A SUBSTRATE AND GLUTATHIONE AS AN INHIBITOR OF IN VITRO CALMODULIN-STIMULATED PROTEIN METHYLATION IN RAT LIVER CYTOSOL

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SUMMARY: This report describes the isolation of the major calmodulin-stimulated methyl acceptor protein of adult rat liver cytosol. This M_r 29,000 methyl acceptor protein (MeAP29) has been purified to apparent homogeneity using ammonium sulfate precipitation and chromatography on DEAE-cellulose, phosphocellulose, hydroxylapatite and Sephadex G-75. Affinity chromatography on glutathione-Sepharose and assays of enzyme activity indicate that MeAP29 is a member of the glutathione S-transferase family. We further show that glutathione can act as an inhibitor of calmodulin-stimulated in vitro methylation of MeAP29 and that MeAP29 methylation is enhanced in non-dialyzed liver cytosol from rats with lowered glutathione levels.

INTRODUCTION: A previous report from this laboratory (1) showed that calmodulin, a major intracellular calcium-binding protein in eucaryotes, stimulates the in vitro methylation of three proteins (M_r 29,000, 32,000 and 45,000) in liver cytosol from adult rats. Calmodulin stimulated the methylation of the M_r 29,000 methyl acceptor protein (MeAP29) to a greater extent than for either MeAP32 or MeAP45. Dialysis also stimulated the methylation of these three proteins. The addition of calmodulin to methylation incubations containing dialyzed liver cytosol produced additive effects on the methylation of MeAP29, without significantly affecting the methylation of either MeAP32 or MeAP45. Calmodulin-stimulated MeAP29 methylation occurs in cytosol of liver and to a lesser extent in kidney and lung cytosol. This stimulation of protein methylation represented a previously unreported activity of calmodulin.

While the significance of protein methylation in eucaryotes is not yet known, a number of proteins have been shown to be post-translationally modified by methylation, including calmodulin (2), histones (3) and cytochrome c (4). This laboratory presented evidence (5) that calmodulin exists in both methylated and non-methylated states in some rat tissues. It has been shown that the N-methylation of calmodulin modulates its activation of NAD kinase (6). Other enzymes, such as cyclic nucleotide phosphodiesterase (7) and myosin light chain kinase (8), are activated to the same degree by either methylated or non-methylated calmodulins.

The observation that calmodulin could stimulate the methylation of specific proteins led to our interest in the possible significance of these events, especially in relation to calcium fluxes and subsequent calcium-calmodulin interactions within cells. To better understand protein methylation and the effects of calmodulin on this post-translational modification, we sought to isolate and characterize the calmodulin-dependent methyl acceptor protein MeAP29 and to

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determine the identity of the dialyzable inhibitor of this in vitro methylation. We report here the purification of MeAP29 to apparent homogeneity, as indicated by its appearance as a single band on 12.5% SDS-polyacrylamide gels. The retention of MeAP29 on glutathione-Sepharose affinity columns and its elution with buffer containing glutathione identified MeAP29 as a member of the glutathione S-transferase family; enzyme activity studies confirmed this classification. We also show that glutathione, a substrate of glutathione S-transferases, can act as an inhibitor of the calmodulin-stimulated methylation of MeAP29 and that methylation of MeAP29 is enhanced in non-dialyzed liver cytosol from rats with lowered hepatic glutathione levels.

MATERIALS AND METHODS

Materials: [^{3}H]-S-adenosyl-L-methionine was obtained from the NEN Division of DuPont Corporation. DEAE-cellulose (DE-52) was purchased from Whatman; Sephadex G-75 from Pharmacia Fine Chemicals; and, phosphocellulose (Cellex-P) and hydroxylapatite (HTP) from BioRad Laboratories. Ultrafiltration units and filters are products of Amicon. Holtzmann rats (Sprague-Dawley Corp., Madison, WI) were reared in our breeding colony. All other materials were obtained from Sigma Chemical Company.

Purification of MeAP29: The presence of MeAP29 in fractions was determined by the existence of an M_r 29,000 Coomassie Blue-stained protein band following electrophoresis on 12.5% SDS-polyacrylamide gels and the ability of this protein to be methylated in the presence of calmodulin, partially purified methyltransferase and [^{3}H]-S-adenosyl-L-methionine under in vitro methylation conditions.

Liver cytosolic fractions were obtained as described (1) from 200-300 gram male Holtzmann rats. These cytosolic fractions were brought to 75% ammonium sulfate saturation by the gradual addition of solid ammonium sulfate, followed by one hour of gentle stirring at 4°C. The suspension was then centrifuged at 100,000 x g for 30 minutes. The resulting supernatant was brought to 90% ammonium sulfate saturation, stirred for 1 hour, and then centrifuged as before. The 75-90% ammonium sulfate pellet was dissolved in Buffer A (20 mM Tris-HCl, pH 7.3) and then dialyzed overnight against this buffer.

The dialyzed ammonium sulfate fraction was clarified by a 30 minute centrifugation (100,000 x g) and then pumped (1 ml/min) onto a DEAE-cellulose column (21.5 cm x 3.5 cm), which had been previously equilibrated in Buffer A. The washthrough fraction from this column was immediately pumped (1 ml/min) onto a phosphocellulose column (20 cm x 2.5 cm), previously equilibrated in Buffer A. The washthrough fraction from the phosphocellulose column was dialyzed overnight against 50 mM sodium phosphate, pH 7.0 (Buffer B), reduced in volume by ultrafiltration (YM10 filter) and pumped (20 ml/h) onto a hydroxylapatite column (15 cm x 2.5 cm), previously equilibrated in Buffer B. The column was washed with Buffer B until the 280nm absorbance returned to baseline. Bound proteins were eluted with a linear, six column volume gradient to 300 mM sodium phosphate, pH 7.0. Fractions containing a methyl accepting 29,000 dalton protein were pooled and dialyzed overnight against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (Buffer C); MeAP29 eluted from hydroxylapatite during the latter half of the gradient. The dialyzed pool was reduced in volume by ultrafiltration to less than two ml and pumped (10 ml/h) onto a Sephadex G-75 column (90 cm x 2.5 cm), previously equilibrated with Buffer C. MeAP29 eluted from this sizing column with an apparent molecular weight of 50,000 daltons (data not shown), suggesting that it exists as a dimer in solution.

Partial purification of MeAP29 methyltransferase : The rat liver cytosol methyltransferase(s), which methylate(s) MeAP29, was isolated by a 35-55% ammonium sulfate precipitation followed by chromatography on DEAE-cellulose. The 35-55% ammonium sulfate pellet was dissolved in Buffer A, dialyzed overnight against this buffer, and then chromatographed on DEAE-cellulose. MeAP29 methyltransferase was eluted from DEAE-cellulose with a step-wise gradient of Buffer A containing 75 mM NaCl. Neither MeAP29 nor calmodulin was detectable in this enzyme preparation.

In vitro methylation : Methylation incubations, followed by SDS-PAGE and fluorography, were carried out essentially as described (1), and were scaled-up for some experiments. Glutathione was added to some incubations, as described in the figure legends.

Glutathione affinity chromatography : Glutathione was attached to epoxy-activated Sepharose as described (9). Glutathione S-transferases were purified using this column (10).
Glutathione S-transferase assay : Glutathione S-transferase activity was assayed by the method of Habig and Jakoby (11), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.
Buthionine sulfoximine treatment : Buthionine sulfoximine (BSO) was used to lower hepatic glutathione levels in rats by the method of Meister and Griffith (12). Animals were injected intraperitoneally with 4 mmoles BSO/kg body weight at time zero and again after 2 h, and sacrificed 2 h later. Control animals received injections of 0.9% sodium chloride.
Glutathione levels : Glutathione levels were assayed by the method of Tietze (13).

RESULTS AND DISCUSSION : MeAP29 was purified, to a single stained protein band on 12.5% SDS-polyacrylamide gels, through the successive use of ammonium sulfate precipitation and chromatography on DEAE-cellulose, phosphocellulose, hydroxylapatite and Sephadex G-75 (Fig. 1). Identification of fractions containing MeAP29 was determined by : 1) the presence of an M_r 29,000 protein on 12.5% SDS-polyacrylamide gels, and 2) its ability to accept methyl groups in methylation incubations containing calmodulin, partially purified methyltransferase and [^{3}H]-S-adenosyl-L-methionine. Amino acid sequence determination of the N-terminus of the purified MeAP29 suggested that it is a member of the glutathione S-transferase (GST) family (manuscript in preparation).

Others have shown that the members of the GST family can be purified by glutathione-Sepharose affinity chromatography (9,10). To further characterize MeAP29 as a member of the GST family, a scaled-up methylation assay was incubated under standard conditions using dialyzed liver cytosol and added calmodulin, followed by isolation of GSTs on GSH-Sepharose. Methylated MeAP29 bound to this column and was eluted with buffer containing GSH (Fig. 2). The GSH eluate from the GSH-Sepharose column contained both methylated MeAP29 (Fig. 2) and GST activity (TABLE I), indicating that MeAP29 is a glutathione S-transferase.

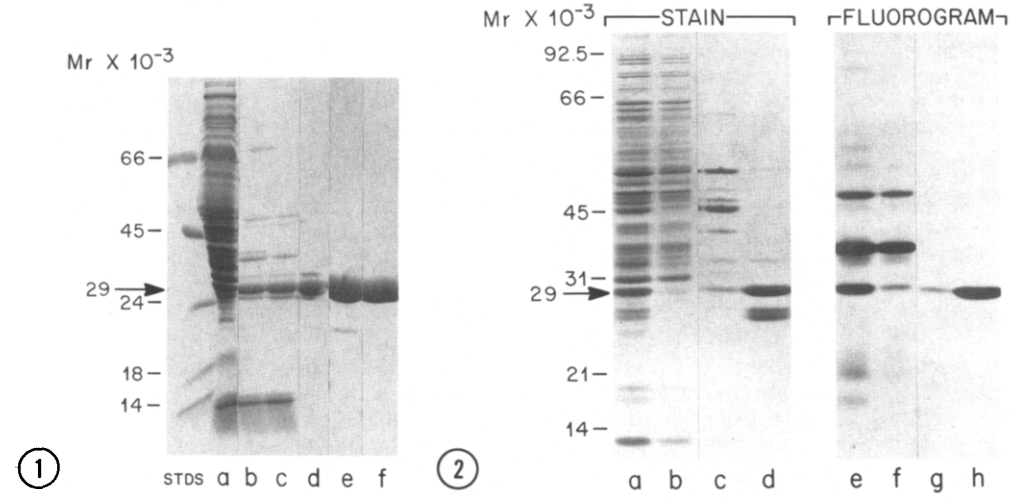

Figure 1. Purification of MeAP29 from adult rat liver cytosol. MeAP29 was isolated from liver of adult male rats, as described in Materials and Methods. Lane a, total liver cytosol; lane b, 75-90% ammonium sulfate pellet; lane c, DEAE-cellulose washthrough; lane d, phosphocellulose washthrough; lane e, hydroxylapatite pool and lane f, Sephadex G-75 pool.

Figure 2. Affinity purification of methylated MeAP29 on GSH-Sepharose. Left panel, Coomassie blue stain; right panel, fluorograph. Lanes a and e, total liver cytosol; lanes b and f, washthrough from GSH-Sepharose; lanes c and g, 50 mM Tris-HCl, pH 9.6 eluate from GSH-Sepharose; and lanes d and h, GSH eluate.

TABLE I

Affinity Purification of Liver Glutathione S-Transferase
Following Calmodulin-Stimulated Methylation

Fraction	mg Protein	Specific Activity (nmol/min·mg)	Total GST Activity
Dialyzed Cytosol	20.0	380	7,600
Post Methylation	12.7	534	6,800
Column Washthrough	6.6	28.3	187
pH 9.6 Eluate	0.32	438	142
Glutathione Eluate	0.51	9,160	4,670

Glutathione S-transferase activity was determined with CDNB as substrate.

Dialyzed rat liver cytosol was chromatographed on glutathione-Sepharose following in vitro methylation in the presence of calmodulin. Nonspecifically bound proteins were eluted from the column with 50 mM Tris-HCl, pH 9.6. Bound proteins were eluted with 50 mM Tris-HCl, pH 9.6 containing 5 mM glutathione.

This laboratory (1) had shown that calmodulin-dependent methylation of MeAP29 was subject to inhibition by a dialyzable inhibitor. Since GSH serves as a substrate in reactions catalyzed by GSTs, we decided to determine the effects of GSH on MeAP29 methylation in vitro. The methylation of MeAP29 was stimulated by dialysis or added calmodulin (Fig. 3). The combination of dialysis and added calmodulin resulted in additive effects on MeAP29 methylation. Inclusion of 0.1 mM GSH in the incubation slightly inhibited the methylation of MeAP29 in dialyzed cytosol compared to control; 1.0 mM GSH almost completely inhibited

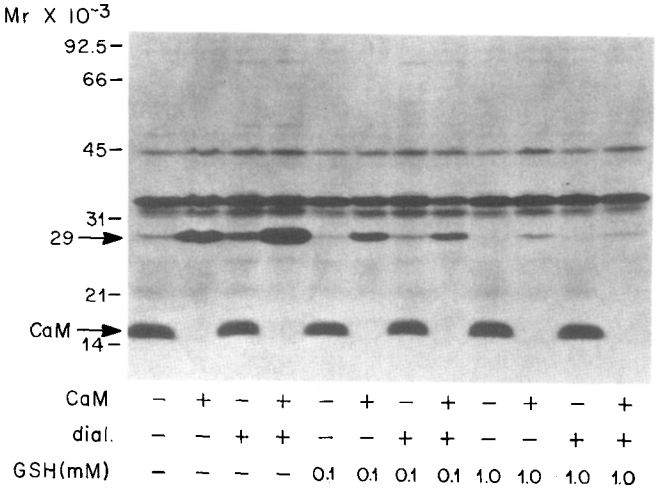

Figure 3. Effect of glutathione on in vitro methylation of MeAP29; fluorograph of SDS-polyacrylamide gel. GSH and/or calmodulin were added to methylation incubations, as indicated. MeAP29 source was either dialyzed or non-dialyzed liver cytosol, as shown. Methylation conditions as described in Materials and Methods.

MeAP29 calmodulin-stimulated methylation. Glutathione had no apparent effect on the in vitro methylation of any other protein.

Glutathione levels in normal adult rat liver cytosol are approximately 5 mM (14). Under a number of stresses, the level of GSH may drop substantially (for review, see 15). To determine the effect of lowered GSH levels on MeAP29 methylation, we injected rats with buthionine sulfoximine (BSO) (12). Rats injected with BSO showed only approximately 30% of the control levels of GSH (TABLE II). Fig. 4 shows the effect of lowered in vivo GSH levels on in vitro MeAP29 methylation (individual animals are shown; a typical control animal and four experimental animals). Lowered GSH levels resulted in enhanced methylation of MeAP29 in non-dialyzed liver cytosol. In the non-dialyzed cytosol plus added calmodulin lanes, there is a negative correlation between GSH levels and MeAP29 methylation (e.g., BSO-4 had the highest GSH level and under these in vitro conditions MeAP29 methylation was the lowest for this animal). Once again, no significant changes were observed in methylation of any of the other methyl acceptor proteins as a result of this drug treatment. The increased in vitro methylation of MeAP29 from BSO-injected animals was probably not due to an increase in GST levels, as there was actually a slight decrease in GST activity in the experimental animals compared to controls (TABLE III). These data suggest that GSH is the dialyzable inhibitor of calmodulin-stimulated in vitro MeAP29 methylation.

We classify MeAP29 as a member of the GST family from the following evidence:
1) retention of MeAP29 on GSH-Sepharose columns and its elution with buffer containing

TABLE II

Effect of Buthionine Sulfoximine Injection on Glutathione Levels in Rat Liver

Treatment		μmoles GSH/g tissue
Control	C-1	4.72
	C-2	6.25
	C-3	6.35
	C-4	7.10
		$\bar{X}$ = 6.10 ± 1.00
BSO-Injected	BSO-1	1.67
	BSO-2	1.12
	BSO-3	2.04
	BSO-4	2.88
		$\bar{X}$ = 1.93 ± 0.74 * (31.6% of control)

Male Holtzmann rats (200-300 g) were injected with 4 mmoles BSO/kg body weight at time zero and again after 2 hours, and sacrificed 2 hours later. Control rats received injections of 0.9% sodium chloride in a volume equal to those received by the experimental animals. Injections were made intraperitoneally. Data for individual animals is shown. * Using a two sample t test for independent samples $p < .0001$, indicating a significant difference between the control and BSO-injected data.

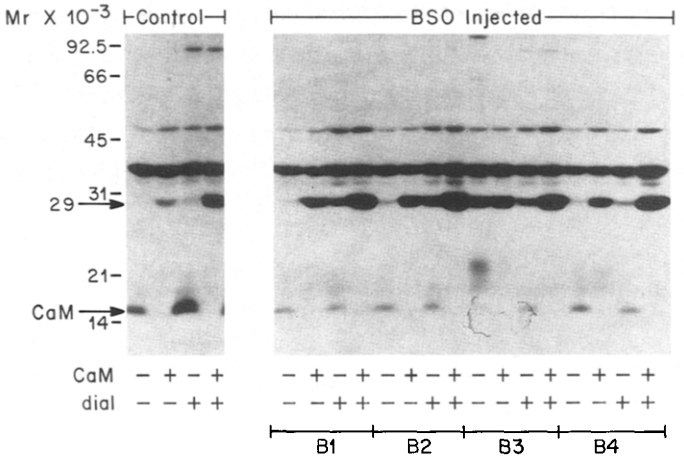

Figure 4. Effect of BSO-injection on in vitro MeAP29 methylation; fluorograph of SDS-polyacrylamide gel. Left panel, typical control; right panel, individual BSO-injected animals. Sample source was either dialyzed or non-dialyzed liver cytosol, as indicated. Alternate incubations contained 20 μg calmodulin.

glutathione; 2) comparable monomer molecular weights of the GSTs and MeAP29 (14, Fig. 2); 3) formation of dimers in solution (14) and 4) MeAP29 fractions purified through the Sephadex G-75 step contained GST activity (data not shown). Partial N-terminal amino acid sequence analysis supports this classification (manuscript in preparation).

Further studies point to GSH as the dialyzable inhibitor of MeAP29 calmodulin-dependent methylation. GSH added to dialyzed liver cytosol affected only the methylation of MeAP29 and not of other methyl acceptor proteins. Also, lowering GSH levels in liver enhanced the methyl

TABLE III

Glutathione S-Transferase Activity in Livers
of Control and Buthionine Sulfoximine Injected Rats

Treatment		Specific Activity (nmol/min•mg)
Control	C-1	359
	C-2	379
	C-3	330
	C-4	406
		$\bar{X}$ = 367 ± 31.7
BSO-Injected	BSO-1	318
	BSO-2	195
	BSO-3	277
	BSO-4	292
		$\bar{X}$ = 270 ± 53.1 * (73.6% of control)

Glutathione S-transferase activity was determined with CDNB as substrate. Data for individual animals is shown. Assays were run in duplicate for individual animals. * Using two sample t test for independent samples, control and BSO-injected results are not significantly different.

accepting activity of MeAP29 in non-dialyzed cytosol. Since glutathione levels in normal rat liver are high enough to inhibit MeAP29 methylation, this post-translational modification may only occur during times when glutathione levels are reduced and thus the methylation may constitute a stress response. Our current efforts to further characterize MeAP29 include studies of GST isoforms, the identification of methylated amino acid residue(s) and the determination of the physiological significance of this methylation.

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