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REVERSIBLE INACTIVATION OF SOLUBLE LIVER GUANYLATE CYCLASE BY DISULFIDES

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SUMMARY

Guanylate cyclase activity was reduced in soluble fractions from rat liver slices incubated with diamide, N-ethylmaleimide, or 5',5'-dithiobis-2-nitrobenzoate; addition of GSH or dithiothreitol to assays restored activity. Inactivation of purified rat liver guanylate cyclase by p-hydroxymercuribenzoate at 0°C was completely reversed by dithiothreitol. p-Hydroxymercuribenzoate and GSSG caused reversible inactivation of calf liver guanylate cyclase. Dithiobisnitrobenzoate and cystamine were more potent than GSSG; all were more effective at 30°C than at 0°C. It appears that guanylate cyclase activity in intact cells could be modulated by reversible modification of critical sulfhydryl groups, e.g., by thiol:disulfide exchange involving glutathione.

INTRODUCTION

Processes involving oxidation-reduction and/or free radicals are considered to be important in the modulation of guanylate cyclase activity (1,2). Protein sulfhydryl groups have also been implicated in stabilizing purified preparations of guanylate cyclase (3) as well as in modifying responsiveness of the enzyme to nitric oxide, nitroprusside and other related compounds (3-6). In other studies, a thiol-reducing agent, dithiothreitol (DTT), prevented the spontaneous increase in guanylate cyclase activity observed during aerobic incubation of crude tissue extracts or soluble preparations (7-9) and prevented or reversed activation of soluble spleen guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides (10). We have now shown, as reported here, that highly purified preparations of soluble guanylate

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Abbreviations: DTT, dithiothreitol; PHMB, p-hydroxymercuribenzoate; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; NEM, N-ethylmaleimide.

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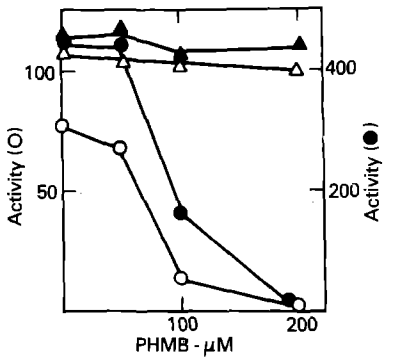
cyclase from liver are rapidly inactivated by GSSG, several other disulfide compounds, or PHMB and can be reactivated with DTT or GSH. In addition, we have found that soluble guanylate cyclase activity is decreased by incubation of rat liver slices with diamide, DTNB, or NEM and is restored by the addition of DTT or GSH to assays. These observations support the hypothesis that reversible modification of critical sulfhydryl groups in guanylate cyclase may be a mechanism for altering its activity in intact cells.

MATERIALS AND METHODS

Purification of Guanylate Cyclase. Rat liver was homogenized (Waring blender) in two volumes of cold 0.25 M sucrose/2 mM DTT/1 mM EDTA/0.4 mM phenylmethylsulfonyl fluoride/10 mM Tris(Cl^-), pH 7.4. The supernatant (10,000 x g, 30 min) was filtered (cheesecloth) and mixed with 0.65 volumes of DE52 (Cl^- form). The supernatant was removed and the DE52 was washed with homogenization buffer containing 75 mM NaCl. Guanylate cyclase activity was eluted with buffer containing 300 mM NaCl, precipitated with $(NH_4)_2SO_4$ (70% saturation), then dissolved in and dialyzed against buffer containing 50 mM NaCl. The solution was clarified by centrifugation (12,000 x g, 30 min) and guanylate cyclase was purified by chromatography on DEAE-Bio-Gel, phenyl-Sepharose, Ultrogel AcA 34, and GTP-Agarose as described (11,12). As previously observed (11), addition of activator fraction from rat liver markedly increased cyclase activity and, in its presence, activity was proportional to enzyme concentration.

Purification of Activator Fraction. Activator fraction from rat liver prepared as described earlier (11) was further purified as follows. NaOH was added to a final concentration of 0.5 N. After standing overnight at room temperature, the solution was concentrated using an Amicon hollow filter cartridge (PM 10), neutralized, and centrifuged (100,000 x g, 1 h). The supernatant was dialyzed against 10 mM Tris(Cl^-), pH 7.4/0.25 M sucrose/1 mM EDTA, then applied to a column of DEAE-Bio-Gel (Cl^- form) equilibrated with the same buffer. Activator, which before alkali treatment was excluded from DEAE-Bio-Gel (11), was bound to the column and eluted with a gradient of NaCl, 50 to 300 mM. Active fractions (eluted with ~ 200 mM NaCl) were pooled. An equal volume of 2 N HCl was added and, after 3 h at room temperature, the precipitate was sedimented by centrifugation. It was washed once with 1 N HCl, then suspended in 10 mM Tris(Cl^-), pH 7.8/0.25 M sucrose/1 mM EDTA. After adjusting the pH to 7.8 with NaOH, the solution was centrifuged at 100,000 x g for 1.5 h. The clear, slightly yellow supernatant was used as the activator fraction. Maximal effects in the standard assay were produced with 15 to 35 µg based on protein determination (Coomassie Brilliant Blue G-250 dye binding assay from BioRad Laboratories) with bovine serum albumin as a standard.

Incubation and Assay of Guanylate Cyclase. Just before each experiment, DTT present in guanylate cyclase preparations was removed (or its concentration decreased to 100 µM) on columns (V_t = 10.0 ml) of Sephadex G-25 equilibrated with 0.25 M sucrose containing 10% glycerol and 10 mM Tris-HCl, pH 7.4. Samples of enzyme were incubated at 0°C or 30°C in the same buffer with additions as indicated and, in some cases, further incubated at 0°C or 30°C with DTT before guanylate cyclase assays were initiated. Assays in a total volume of 0.1 ml containing 5 mM $MnCl_2$, 1 mM [α-^{32}P]GTP and 50 mM Tris(Cl^-) buffer, pH 7.4, were incubated for 10 min at 37°C (11). Data reported are means of values from duplicate incubations.



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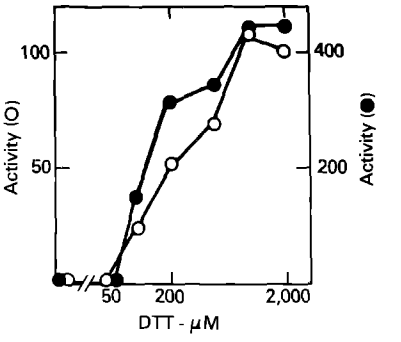

(2)

Figure 1: Inactivation of rat liver guanylate cyclase by PHMB. Samples (10 μl, 1.2 μg) of rat liver guanylate cyclase were incubated in a total volume of 15 μl with the indicated concentration of PHMB (and ~ 66 μM DTT introduced with the enzyme) for 5 min at 0°C. DTT, final concentration of 2 mM, (△,▲) or diluent (○,●) was then added in 5 μl and, after 5 min at 0°C, guanylate cyclase was assayed without (○,△) or with (●,▲) activator. Activity, nmol/min/mg protein.

Figure 2: Reactivation of PHMB-inactivated rat liver guanylate cyclase by DTT. This experiment was carried out as described in Fig. 1 except that all samples of enzyme (1.2 μg) were incubated for 5 min at 0°C with 200 μM PHMB. DTT was then added to the indicated concentration (~ 50 μM DTT and ~ 150 μM PHMB carried from first incubation). After 5 min at 0°C, guanylate cyclase was assayed without (○) or with (●) activator. Activity, nmol/min/mg protein.

Materials. GSH, GSSG, lipoic acid (oxidized), cystamine, phenylmethylsulfonyl fluoride, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), GTP (sodium salt), N-ethylmaleimide (NEM), and cysteine were purchased from Sigma Chemical Co.; DE52 from Whatman, Inc.; Agarose-hexane-guanosine 5'-triphosphate (GTP-Agarose) from P. L. Biochemicals; rabbit γ-globulin from Schwarz/Mann; DTT from Bethesda Research Laboratories; PHMB and diamide (1,1'-azobis (N,N-dimethylformamide)) from Aldrich Chemicals.

RESULTS

Reversible Inactivation of Purified Guanylate Cyclase. The purified rat liver guanylate cyclase was inactivated by PHMB (Fig. 1) and completely reactivated by DTT (Fig. 2). Although activities were higher when activator fraction was present in assays, the relative effects of PHMB and DTT were similar (Figs. 1 and 2).

Incubation of activator fraction with GSSG (or DTNB) did not alter the capacity of a maximally effective concentration of activator fraction to

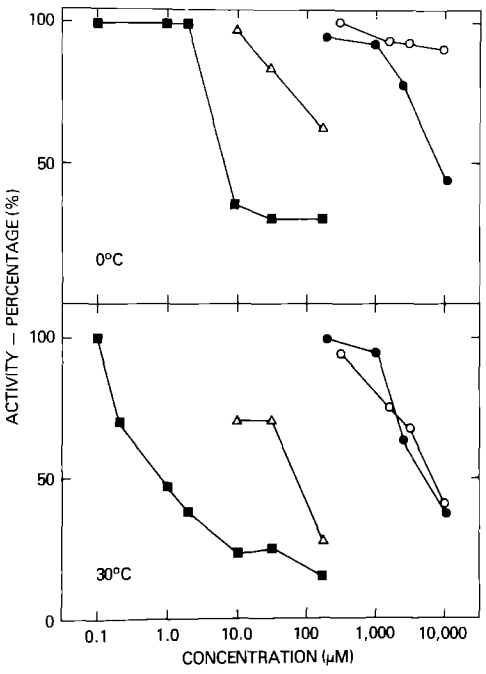

Figure 3: Inactivation of calf liver guanylate cyclase by sulfhydryl reagents. Samples (0.1 to 0.2 μg) calf liver guanylate cyclase purified as described in Methods (specific activity 100-300 nmol/min/mg protein) were incubated in a total volume of 40 μl for 10 min at 0°C or 30°C with the indicated concentration of (■) DTNB, (O) GSSG, (Δ) cystamine, or (●) lipoic acid before assay of guanylate cyclase. Activator (15 μg) was present in all samples to prevent the irreversible loss of activity independent of sulfhydryl reagents that occurred with these dilute solutions of enzyme, especially at 30°C. Activities are expressed relative to that of enzyme incubated in the absence of sulfhydryl reagent = 100.

increase guanylate cyclase activity (activity without activator, 30 pmol/min; with 27 μg activator, 72 pmol/min; with activator incubated with 0.5 mM DTNB followed by removal of free DTNB by chromatography on Sephadex G-25, 85 pmol/min).

GSSG (Fig. 3) as well as PHMB (data not shown) also inactivated purified calf liver guanylate cyclase. Inactivation persisted after removal of GSSG on Sephadex G-25; 2 mM DTT, GSH, or cysteine produced maximal reactivation, whereas ascorbate and nitroprusside had no effect (data not shown). DTNB and, to a lesser extent, cystamine were more potent than GSSG in inactivating guanylate cyclase; all were more effective at 30°C than at 0°C (Fig. 3). Inactivation by lipoic acid required relatively high concentrations and was seemingly

TABLE I

Reversible Inactivation of Guanylate Cyclase by Incubation of Liver Slices with Diamide, DTNB, or NEM

Exp. no.	Additions to slices	Guanylate Cyclase Activity			
		Buffer	10 mM DTT	20 mM DTT	20 mM GSH
		(pmol/min/mg protein)			
1	None	39 (100)[a]	40 (102)	49 (126)	54 (138)
	Diamide, 20 mM	18 (46)	18 (46)	28 (72)	48 (123)
	DTNB, 5 mM	18 (46)	45 (115)	47 (120)	-- --
	NEM, 1 mM	50 (128)	49 (126)	49 (126)	47 (126)
2	None	17 (100)	-- --	15 (88)	17 (100)
	DTNB, 5 mM	9 (53)	-- --	14 (82)	19 (112)
	NEM, 4 mM	9 (53)	-- --	14 (82)	15 (88)

Rat liver slices were washed briefly in Krebs-Ringer phosphate medium, then distributed (two slices each) to 25-ml Erlenmeyer flasks containing 3 ml of the same medium with bovine serum albumin, 30 mg/ml. After incubation for 10 min at 37°C, the indicated additions were made. In Exp. 1, 5 min later (DTNB, NEM) or 20 min later (control, diamide) slices were transferred to 3 ml of ice-cold 0.25 M sucrose/10 mM Tris/1 mM EDTA, pH 7.4. They were washed three times with 3 ml and homogenized in 1 ml of the same medium. In Exp. 2, incubation was continued for 5 min after additions were made, then flasks were set in ice and equivalent concentrations of DTT were added to those containing DTNB or NEM. After 3 to 5 min, slices were transferred to 6 ml of the cold sucrose/Tris/EDTA medium, washed three times and homogenized in 1 ml of the same medium. All homogenates were centrifuged for 60 min at 105,000 x g and samples of supernatants taken for assay of guanylate cyclase activity in the presence of DTT or GSH where indicated. Data are means of values from duplicate samples of slices.
[a] In parentheses, activity relative to control = 100.

less dependent on temperature (Fig. 3). Inactivation produced by cystamine (up to 250 μM) or DTNB (up to 50 μM) was completely reversed by DTT, that by lipoic acid only partially (data not shown).

Reversible Inactivation of Guanylate Cyclase in Liver Slices. Incubation of rat liver slices with 20 mM diamide, which has been reported to oxidize intracellular GSH to GSSG (13), for 20 min decreased guanylate cyclase activity assayed in the soluble fraction of homogenates by ∿ 50%. Activity was restored to levels equivalent to those from control slices by inclusion of 20 mM GSH in the assay; DTT was less effective than GSH (Table I). Similarly, activity from

slices incubated for 5 min with 5 mM DTNB or 4 mM NEM was decreased ~ 50%, and this inactivation was reversed by GSH or DTT. In most experiments (e.g., Exp. 2, Table I), DTT and GSH had little effect on the activity from control slices, although they sometimes increased activity < 40% (Exp. 1, Table I).

DISCUSSION

From studies in several laboratories (1-11), it has been inferred that sulfhydryl groups could play a role in guanylate cyclase activity. Using extensively purified preparations of the soluble enzyme from rat and calf liver, we have now directly demonstrated the temperature-dependent inactivation of guanylate cyclase by compounds capable of forming mixed disulfides or covalent mercaptide bonds. Inactivation produced by GSSG was rapidly reversed by GSH or CySH as well as by DTT which also reversed that produced by other compounds. Although some inactivation experiments were carried out in the presence of the guanylate cyclase activator, which was necessary for stabilization of the dilute enzyme (particularly at 30°C), reversible inactivation by PHMB at 0°C was also demonstrated in the absence of activator. In addition, exposure of activator to GSSG or DTNB (followed by removal of these reagents) did not alter its ability to enhance guanylate cyclase activity in the assay. Thus, it appears that disulfides and sulfhydryl reactive compounds alter the activity of the enzyme itself, not that of the activator. The fact that inactivation by GSSG was decreased in the presence of Mn^{2+} and guanosine 5'-(β-, γ-imino)triphosphate, a GTP analog and competitive inhibitor of the enzyme (data not shown), is consistent with this conclusion.

Guanylate cyclase activity was reduced in soluble fractions from homogenates of rat liver slices previously incubated with agents expected to decrease in different ways the intracellular content of GSH and other sulfhydryls. Activity was restored by addition of DTT or GSH to assays. It appears that the only other enzyme for which similar observations have been reported is the

indoleamine N-acetyltransferase in pinealocytes (14), although there are many enzymes whose activity in cell-free systems or purified preparations can be altered by disulfide exchange or sulfhydryl reagents. Glutathione-dependent control of protein disulfide/sulfhydryl content of rat liver, along with a diurnal variation in the SS/SH ratio, has been reported (15). These observations, together with the studies reported here, are consistent with the possibility that guanylate cyclase activity, as well as that of the N-acetyltransferase (14), in intact cells may be subject to regulation through reversible modification of critical sulfhydryl groups, e.g., by protein thiol:disulfide exchange involving glutathione (or other disulfides), either nonenzymatic or enzymatically catalyzed.

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