Reversible Inactivation of Guanylate Cyclase by Mixed Disulfide Formation*

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Highly purified preparations of guanylate cyclase from rat lung were inactivated by several disulfide compounds in a time- and dose-dependent manner. Cysteamine and cystine were the most potent disulfides tested. These disulfides, which included pantethine and oxidized coenzyme A, were also able to partially inactivate the enzyme. In addition to the decrease in basal activity (measured with either Mg$^{2+}$-GTP or Mn$^{2+}$-GTP), disulfide-inhibited enzyme was activated to a lesser extent by nitric oxide. Treatment with dithiothreitol or other reducing agents restored basal activity and increased the level of cGMP production following nitric oxide activation. Control enzyme samples exhibited a single GTP Km of 25 μM or 150 μM with Mn$^{2+}$ or Mg$^{2+}$, respectively. However, cysteamine-treated enzyme showed these same Km values as well as an additional GTP Km of 2 to 3 μM using either metal ion as cofactor.

When [35S]cystine was incubated with purified enzyme, radioactivity was incorporated into the trichloroacetic-acid-precipitable protein, and the counts were released following dithiothreitol treatment. In addition, [35S]cystine-labeled enzyme co-migrated with native guanylate cyclase on nondenaturing polyacrylamide gels. These data indicate that mixed disulfides can be formed between guanylate cyclase and certain naturally occurring compounds, and that disulfide formation leads to a reversible loss of enzyme activity.

Several laboratories have reported the purification of soluble guanylate cyclase (GTP-pyrophosphate-lyase (cyclizing), EC 4.6.1.2) from rat liver and rat lung (1-3). Although no preparation of the enzyme has shown convincing and relevant responsiveness to any naturally occurring hormone, many other compounds, including azide, nitric oxide, nitroprusside, nitrosamines, nitrosoureas, and unsaturated fatty acids, have been shown to activate partially purified or highly purified preparations of guanylate cyclase (2, 4-9). In addition, it has been reported that H$_2$O$_2$ can activate some crude preparations of rat lung enzyme (10), while agents such as dehydroascorbate can activate crude preparations of guinea pig spleen guanylate cyclase (11). Superoxide dismutase can also increase the activity of partially purified or highly purified enzyme (2, 12). In light of these data, it has been proposed that many of these compounds share a common pathway for enzyme activation via the formation of oxidizing free radicals (6, 12-15). The activating species may be nitric oxide in the case of the nitro and nitroso compounds (5, 16), or perhaps, under the appropriate conditions, the hydroxyl radical (12). The mechanism by which free radicals activate guanylate cyclase is not clear at present. However, the oxidation and reduction of some portion of the enzyme molecule is most likely, and has been suggested from studies with crude enzyme preparations.

Although the precise factors the regulate guanylate cyclase activity and guanosine 3',5'-monophosphate accumulation in vivo are unknown, the work cited above demonstrates that the enzyme is sensitive to a variety of oxidizing and reducing agents. These and other data suggest that changes in the oxidation-reduction state of cellular components, such as thiols, pyridine nucleotides, etc., may regulate guanylate cyclase activity and levels of cyclic GMP in intact cells. While it has been appreciated from studies with crude preparations that thiols and agents that modify sulfhydryl groups can alter either basal enzyme activity or activation by different agents (4, 16-19), the location and role of these thiols on guanylate cyclase or perhaps other regulatory proteins in such preparations are unknown, and can only be properly evaluated with highly purified preparations of the enzyme.

In studies aimed at assessing the role of thiols on guanylate cyclase activity, we have tested a variety of naturally occurring disulfides and thiols for their effects on very highly purified preparations of rat lung guanylate cyclase. We have found that a number of disulfides, including cysteamine, cystine, pantethine, and oxidized coenzyme A, can inhibit guanylate cyclase in a time- and concentration-dependent manner. This inhibition can be rapidly reversed by the addition of reducing agents such as dithiothreitol or β-mercaptoethanol. The inhibition by cysteamine is accompanied by the appearance of multiple Km values for GTP as opposed to control enzyme which shows a single Km for GTP. In addition, we have found that [35S]cystine can be incorporated into purified guanylate cyclase and that the bound radioactivity is removed from the enzyme upon addition of reducing agents and reversal of inactivation.

As shown here, disulfide compounds such as cysteamine and cystine have very marked effects on guanylate cyclase activity, and they may serve as useful probes for the study of this enzyme and its relationship to cyclic GMP metabolism.

MATERIALS AND METHODS

Soluble guanylate cyclase was purified from rat lung by a modification of procedures reported previously (2, 3, 15). The purification

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steps included isoelectric (pH 5) and ammonium sulfate precipitations, affinity chromatography on GTP-Sepharose, and either gel filtration chromatography on Sepharose 6B, or alternatively, preparative polyacrylamide gel electrophoresis. The purified enzyme was stored at \(-70^\circ C\) in 20 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 1 mM diithiothreitol, and 25% sucrose.

Samples of purified guanylate cyclase were incubated at 25°C without and with different thiols or disulfide compounds. Aliquots were withdrawn at various times and assayed for enzyme activity. Guanylate cyclase activity was assayed as described previously (2, 9). Incubations (100 µl for 10 or 15 min at 37°C) contained 50 mM Tris-HCl (pH 7.6), 4 mM MnCl₂ or MgCl₂, 1 mM GTP, 5 µg of bovine serum albumin, and 20 to 50 ng of enzyme protein. Nitric oxide gas (25 µl) was added to some incubations. GMP formed was measured by radioimmunoassay (20) as described previously (2).

Guanylate cyclase activity was assayed as described previously (2, 9). The percentage of control activity is derived from comparison to a control incubation with no disulfide added. Inactivation of guanylate cyclase by the addition of cystine was determined with the modified purification procedure and other properties of the protein will be published elsewhere. The most potent of these compounds was cystamine, which inhibited specific activities ranging from 438 to 60 mmol/mg of protein/min. The activities with Mn²⁺ are comparable to earlier reports of highly purified enzyme from rat lung (3) or liver (2). However, the activities with Mg²⁺ are somewhat higher than earlier reports. A high degree of purity of preparations was also suggested by the presence of a single protein band on polyacrylamide gel electrophoresis that migrated coincidentally with guanylate cyclase activity on parallel gels. Details of the modified purification procedure and other properties of the protein will be published elsewhere.

Table I summarizes the inhibitory effects of a number of disulfide compounds on guanylate cyclase activity. Cystamine (1) and cystine (1) were the most potent inhibitors of enzyme activity. Inactivation with Mn²⁺ progressed very rapidly with no apparent lag, and cystamine was faster than cystine. Within several minutes, one-half of the enzyme activity was lost, and little or no activity was observed. The common feature of the inhibitory disulfides was the presence of the cysteamine moiety (NH₂CH₂CH₂S⁻).

Fig. 1 summarizes the time course for the inactivation of guanylate cyclase by 1 mM cystamine and cystine. Inactivation proceeded very rapidly with no apparent lag, and cystamine appeared to inhibit the enzyme assayed with Mn²⁺-GTP somewhat faster than cystine. Within several minutes, one-half of the enzyme activity was lost, and little or no activity was observed.

![Graph](image-url)
apparent after 30 min of incubation with either agent. The addition of 5 mM dithiothreitol after 30 min of preincubation with 1 mM cystine completely restored enzyme activity, while partial restoration of activity that was inhibited with cystamine was observed (Fig. 1). Reversal of inactivation was very rapid, in that most of the recovered activity occurred within 1 to 2 min of dithiothreitol addition. The inhibition of guanylate cyclase and reversal was qualitatively similar when enzyme activity was assayed with Mg²⁺-GTP rather than Mn²⁺-GTP.

The dependence upon the concentration of cystine and cystamine and the time of preincubation for inhibition of enzyme activity are summarized in Fig. 2. At all concentrations tested, cystamine was a more effective inhibitor than cystine. It should be pointed out that this experiment was performed in the presence of 1 mM dithiothreitol, which was included during the purification of guanylate cyclase to stabilize the enzyme. As would be expected, the rate and extent of disulfide inactivation is a function of the dithiothreitol concentration in preincubations. Preparations of purifed enzymes that were dialyzed to reduce dithiothreitol to the micromolar range were less stable and much more sensitive to disulfide inhibition (data not shown). Other thiols were also tested for their ability to reverse the inhibition of guanylate cyclase activity by cystine (Table II). In addition to dithiothreitol, dithioerythritol and β-mercaptopoethanol restored full enzyme activity. Cysteamine and reduced glutathione restored some activity of enzyme inhibited with cystine. Cysteamine and reduced pyridine nucleotides had no effect in reversing inhibition by cystine.

The inactivation of guanylate cyclase by these disulfide compounds and the subsequent reversal of inactivation by reducing agents suggested that the decrease in enzyme activity was due to the reversible formation of mixed disulfides between free sulfhydryl groups on the enzyme and the added reagents. This interpretation is supported by our observation that at pH 9 to 9.5, the inactivation produced by these disulfides occurs more rapidly than at pH 7.6. Thus, the ionization of cysteine sulfhydryl groups on guanylate cyclase to the more reactive charged species may influence the rate of mixed disulfide formation and enzyme inactivation. The formation of mixed disulfides between guanylate cyclase and compounds such as cystine was also examined by the incorporation of radioactivity in the protein after incubation with [³⁵S]cystine. When guanylate cyclase was incubated with

TABLE II
Effect of thiol compounds and pyridine nucleotides on cystine-inactivated guanylate cyclase

<table>
<thead>
<tr>
<th>Addition (mM)</th>
<th>Percentage of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17</td>
</tr>
<tr>
<td>Dithiothreitol (1)</td>
<td>67</td>
</tr>
<tr>
<td>Dithiothreitol (5)</td>
<td>95</td>
</tr>
<tr>
<td>Dithiothreitol (10)</td>
<td>95</td>
</tr>
<tr>
<td>Dithioerythritol (10)</td>
<td>103</td>
</tr>
<tr>
<td>β-Mercaptopoethanol (10)</td>
<td>108</td>
</tr>
<tr>
<td>Glutathione (10)</td>
<td>83</td>
</tr>
<tr>
<td>Cysteine (10)</td>
<td>52</td>
</tr>
<tr>
<td>Cysteamine (10)</td>
<td>1</td>
</tr>
<tr>
<td>NADH (4)</td>
<td>18</td>
</tr>
<tr>
<td>NADPH (4)</td>
<td>12</td>
</tr>
</tbody>
</table>

[³⁵S]cystine, there was rapid incorporation of ³⁵S into the enzyme that reached a plateau between 15 and 30 min of incubation (Fig. 3). The amount of radioactivity incorporated was decreased when increasing concentrations of unlabeled cystine were included in the incubation, and all the bound radioactivity was rapidly released from the enzyme with the addition of 10 mM dithiothreitol. When enzyme treated with [³⁵S]cystine was applied to polyacrylamide gel electrophoresis under nonnaturating conditions, radioactivity was obtained in a protein band that migrated coincidentally with guanylate
cyclase activity on a parallel gel (Fig. 4A). In addition, when cystine-inactivated enzyme was subjected to polyacrylamide gel electrophoresis, no activity was measurable in the eluted gel slices under standard incubation conditions. However, if 10 mM dithiothreitol was included in the incubation, there was a large peak of guanylate cyclase activity in the same position as the native enzyme (Fig. 4B). Thus, cystamine, cystine, and presumably other disulfides tested react with free thiol groups on guanylate cyclase to form mixed disulfides, with loss in enzyme activity. The addition of reducing agents such as dithiothreitol cleaves the disulfide and restores enzyme activity.

Although nitric oxide can activate highly purified guanylate cyclase in the absence of other proteins or macromolecules (2, 9), the specific requirements and mechanisms of activation are not known. Fig. 5 shows that when Mg$^{2+}$-GTP was used in cyclase assays, basal activity was inhibited by both cystine and cystamine, but the time course of inhibition was somewhat slower than that measured with Mn$^{2+}$-GTP (Fig. 1). Control enzyme preincubated for several different times was activated 3- to 3.5-fold with nitric oxide, while cystine- and cystamine-treated samples were also activated, although to a slightly lesser extent (1.2- to 2.8-fold). Treatment of disulfide-inhibited enzyme with dithiothreitol led to partial restoration of basal activity, as well as a small increase in NO activation (2- to 3-fold).

As shown above, disulfide treatment was clearly decreasing the $V_{max}$ of guanylate cyclase activity, and it thus became important to also consider effects of disulfides on the $K_m$ for GTP. Fig. 6 and 7 show the Eadie-Hofstee transformations for the determination of $K_m$ using both Mn$^{2+}$-GTP and Mg$^{2+}$-GTP in control and cystamine-treated enzyme samples. Control enzyme gave a single GTP $K_m$ of 25 PM with Mn$^{2+}$ as cofactor, and a $K_m$ of 150 PM with Mg$^{2+}$ as cofactor. With Mg$^{2+}$-GTP (Fig. 7), there was also a suggestion of a second $K_m$ at very low GTP concentrations (1 to 2 PM). Moreover, when the enzyme was treated with cystamine, measurements with either Mn$^{2+}$-GTP or Mg$^{2+}$-GTP clearly showed more than one $K_m$. With both metal cofactors, the cystamine-treated enzyme exhibited one $K_m$ which was essentially the same as in control enzyme (30 PM for Mn$^{2+}$-GTP; 140 PM for Mg$^{2+}$-GTP), and there was also the very obvious appearance of another GTP site, with a $K_m$ of 2 to 3 PM with either cofactor. Thus, mixed disulfide formation decreases maximal velocity of the enzyme, and also alters the enzyme such that multiple GTP sites, either on the same or different enzyme molecules, now become apparent (see below).

**DISCUSSION**

Several laboratories have previously described the formation of mixed disulfides between proteins and thiols in normal tissues and tumors (23-25). There have also been several reports where the formation of mixed disulfides has been shown to modulate the activity of an enzyme. Horecker and co-workers (26, 27) have shown that the rabbit liver fructose-1,6-diphosphatase is activated by disulfide formation with cystamine, coenzyme A, and a number of other thiols and disulfides. Other workers have shown that rat liver glycerogen synthetase (28), guinea pig liver tyrosine aminotransferase (29), rat brain adenylyl cyclase (30), and rabbit liver phosphorylase phosphatase (31) are inactivated following mixed disulfide formation. In all these examples, the effects were somewhat specific for certain disulfide compounds and were reversed by the addition of reducing agents. These results suggested the possible importance of disulfide exchange as a regulatory mechanism for certain enzymes.

In this report, we have demonstrated the inhibition of very highly purified rat lung guanylyl cyclase following incubation with a number of disulfide compounds. Of the disulfides tested, cystamine and cystine were the most effective inhibitors of enzyme activity. The inhibitory effect was related to the structure of the disulfide, since other compounds containing the cystamine moiety (NH$_2$CH$_2$CH$_2$S$^-$), such as pantethine and oxidized coenzyme A, also gave considerable inhibition, while homeocystine, oxidized glutathione, and dibenzyl disulfide had little or no effect. Inhibition of enzyme activity was nearly complete by 15 to 30 min of preincubation with cystamine or cystine and was rapidly reversed by the addition of dithiothreitol or several other agents. On nonde-
naturizing polyacrylamide gels, cystine-inactivated enzyme migrated to the same position as native (untreated) enzyme, and the activity could only be measured when 10 mM dithiothreitol was included in the incubation. In addition, we have also been able to demonstrate the incorporation of \(^{35}\)S cystine into guanylate cyclase during preincubation. The incorporation of labeled cystine gave a time course similar to that of inhibition, was diminished by the addition of unlabeled cystine to the preincubation, and was rapidly reversed by the addition of dithiothreitol.

Treatment of guanylate cyclase with cystamine or cystine led to a lower rate of cGMP production following nitric oxide activation. This could suggest that either one or more free thiols involved in disulfide formation are required for nitric oxide activation, or that disulfide formation completely inhibits the enzyme (basal or NO-activated) and that the activation seen merely represents that population of enzyme which has not been inhibited. It is clear from these and other studies (4, 16-19) that free thiols are important components of guanylate cyclase activity, but the precise nature of their involvement is not known. As such, the relationship, if any, of sulfhydryl modification to hormonal regulation of guanylate cyclase remains an important unanswered question. However, answers to these and other questions about guanylate cyclase regulation should be forthcoming in the foreseeable future with increased quantities of purified enzyme and our recent development of monoclonal antibodies to the enzyme. Three data suggest more than one GTP site present in enzyme samples after cystamine treatment, although it is not possible at the present time to say whether this represents multiple GTP sites on individual enzyme molecules or a population of inhibited and uninhibited enzyme molecules, each with a single GTP site. It is interesting to note that following nitric oxide activation, there is also the appearance of multiple \(K_m\) values for GTP, \(^4\) although it is again difficult to say at present whether this represents multiple GTP sites or multiple populations of enzyme molecules. Binding studies and additional kinetic experiments are now under way to distinguish between these two possibilities.

The physiological significance of disulfide inhibition of guanylate cyclase is unclear at present. Several other enzymes have been shown to be altered following mixed disulfide formation (26-31), and at present, the specificity or generality of such an enzyme regulatory mechanism is not known. However, in the case of guanylate cyclase, certain previous observations concerning the enzyme make disulfide regulation an interesting possibility. A number of compounds, including reactive free radicals and oxidizing and reducing agents, are able to modulate guanylate cyclase activity. Therefore, a change in the oxidation-reduction state of intact cells and alterations in the intracellular levels of thiols and disulfides could regulate guanylate cyclase activity and cyclic GMP synthesis. Whether or not thiols and disulfides are involved in hormonal regulation of cyclic GMP accumulation in tissues is unknown. However, it is clear from the data presented here that the presence of free thiols and mixed disulfides in guanylate cyclase can influence either basal enzyme activity or the ability to activate the purified protein with some agents, and that disulfides such as cystamine and cystine may be useful tools to study the involvement of sulfhydryl modification in guanylate cyclase activity and regulation.

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REFERENCES


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\(^{4}\) H. Brandwein, J. Lewicki, and F. Murad, unpublished observations.