Effects of Metals and Nucleotides on the Inactivation of Sea Urchin Sperm Guanylate Cyclase by Heat and N-Ethylmaleimide*

David L. Garbers† and Joel G. Hardman

From the Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

SUMMARY

Preincubation of sea urchin sperm guanylate cyclase at 35, 37, 40, or 43°C resulted in inactivation. Various metals were able to protect guanylate cyclase against heat inactivation. Estimation binary enzyme-metal dissociation constants for Mn^{2+}, Fe^{2+}, La^{3+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+} were 123, 36, 5.5, 69, 984, 335, 79, and 47 μM, respectively. Extrapolated rates of enzyme denaturation in the presence of saturating concentrations of metal divided by the rates of enzyme denaturation in the absence of metal gave values of 0.13, 0.08, -0.1, 0.30, 0.06, 0.28, and 0.42 for Mn^{2+}, Fe^{2+}, La^{3+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+}, respectively.

GTP, MgGTP, and SrGTP protected the enzyme only slightly against heat inactivation, but CaGTP and MnGTP protected substantially. Neither CaGTP nor MnGTP protected maximally, however, unless the metal concentration exceeded that of GTP. At fixed free Mn^{2+} or free Ca^{2+} concentrations, protection curves as a function of MnGTP or CaGTP appeared to be sigmoidal, suggesting multiple nucleotide binding sites. MnATP also protected against heat, but CaATP was virtually ineffective.

Sea urchin sperm guanylate cyclase was inactivated by N-ethylmaleimide; CaGTP and MnGTP were effective protectants with estimated binary enzyme-Me^{2+}-nucleoside triphosphate dissociation constants of 40 and 170 μM, respectively. MnGTP protected only slightly or not at all against N-ethylmaleimide.

These results suggest that: (a) sea urchin sperm guanylate cyclase binds free metal, (b) the binding of free metal is required for protection by nucleotides, and (c) the enzyme contains multiple nucleotide binding sites.

Sea urchin sperm guanylate cyclase activity is detectable in the presence of Mn^{2+} or Fe^{2+}, but not in the presence of Mg^{2+} or Ca^{2+} as the only metal (1). Because of sigmoidal kinetics with respect to Mn^{2+} at fixed GTP concentrations, it has been suggested that the enzyme binds free metal in addition to MnGTP (1, 2); enzyme inhibition by free GTP, however, also could explain these results. The enzyme displays positive cooperative kinetic patterns with respect to MnGTP at fixed free Mn^{2+} concentrations, which suggests that MnGTP serves as both an activator and a substrate for the enzyme (1). Guanylate cyclase activity from sea urchin sperm and other sources also can be increased by the addition of Ca^{2+} in the presence of Mn^{2+} (1, 3-5). The apparent complex interactions of metals and nucleotides with sea urchin sperm guanylate cyclase have been examined further in this study by subjecting the enzyme to denaturation conditions.

EXPERIMENTAL PROCEDURE

Methods

Enzyme Preparation—Particulate enzyme was prepared as described in another paper (6).

Triton X-100 previously has been shown to disperse and increase the apparent activity of guanylate cyclase from particulate fractions of sea urchin sperm and other sources (1-5). Triton-dispersed enzyme preparations were made by the following procedures: Triton X-100 (final concentration 1%) was added to suspensions of washed sperm particles prepared as described in another paper (9). The suspension then was sonicated (approximately 15 s) and centrifuged at 100,000 × g for 1 hour. Greater than 90% of the enzyme activity was recovered in the supernatant fraction. The specific activity of these preparations ranged from 50 to 100 nmol of cyclic GMP formed min⁻¹ mg of protein⁻¹ at 30°C and pH 7.8.

Assay—The usual assay mixture contained 32 mM triethanolamine (TEA) buffer at pH 7.8, 0.8 mM dithiothreitol, 8 mM theophylline, 8 mM sodium azide (NaN₃), 6.5 mM MnCl₂, and 0.32 mM GTP containing 1 to 3 × 10⁶ dpm of [³²P]GTP in a volume of 0.5 to 0.6 ml. Assays were done at 30°C, and the determination of the cyclic [³²P]GMP formed was carried out as described previously (6).

Preincubation Conditions—The conditions of each preincubation are described in the corresponding figure legend. Controls were run for all preincubation conditions. The control samples in heat denaturation experiments were preincubated at 0-2°C; those in N-ethylmaleimide experiments were preincubated at 0-2°C in the absence of N-ethylmaleimide or in the presence of N-ethylmaleimide with excess dithiothreitol. The volume of preincubation mixture (40 μl) transferred to the assay mixture represented 5 to 10% of the assay volume.

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1 The abbreviations used are as follows: cyclic GMP, guanosine 3',5'-monophosphate; TEA, triethanolamine.
**RESULTS**

**Inactivation of Enzyme by Heat**—Preincubation of sea urchin sperm guanylate cyclase at various temperatures resulted in initial first order rates of inactivation (Fig. 1). The values of \( t_{1/2} \) for inactivation were approximately 35, 14, 5, and 1.5 min at 35, 37, 40, and 43 °C, respectively. Arrhenius plots of the initial rates of inactivation between 35 and 43 °C were straight, suggesting that thermodynamic properties of the enzyme inactivation do not change between these temperatures. The inclusion of 1% Triton X-100 labilized the enzyme to heat, decreasing the \( t_{1/2} \) for inactivation at 37 °C from 11.5 to 4 min.

The rate of heat inactivation was also influenced by pH. The first order rates of inactivation at 37 °C at various pH values were as follows: pH 6.9, 0.006 min⁻¹; pH 7.2, 0.081 min⁻¹; pH 7.4, 0.115 min⁻¹; and pH 7.6, 0.141 min⁻¹. Thus, sea urchin sperm guanylate cyclase is more stable at slightly acid pH despite optimal catalytic activity at distinctly alkaline pH values (8).

**Protection against Heat Inactivation by Free Metals**—First order rates of enzyme inactivation also were observed in the presence of various metals. Guanylate cyclase from sea urchin sperm is dispersed by Triton X-100 and remains in the supernatant fraction after centrifugation at 100,000 × g for 1 hour (2, 8). Mn²⁺ protected Triton-dispersed guanylate cyclase against heat inactivation, but the degree of protection declined with repeated freezing and thawing (Fig. 2). When the data shown in Fig. 2 were replotted according to Equation 1, \( K_d \) was found to increase from 0.22 mM after one freeze-thaw to 0.44 mM after six freeze-thaws. When Triton-dispersed preparations were stored at −70 °C for 1 to 2 months, the estimated \( K_d \) for Mn²⁺ increased to 1.2 mM. These results suggest that conformational changes occur upon freezing, thawing, or storage of the Triton-dispersed enzyme preparations, such that affinity of the enzyme for Mn²⁺ decreases. Because of these effects of freezing and thawing, both the particulate and Triton-dispersed enzyme preparations were routinely frozen and thawed only one time.

Protective effects of Mn²⁺ and several other metals were compared in preliminary experiments in the presence or absence of Triton. There was no appreciable effect of Triton on metal protection except with La³⁺, which was the most protective metal tested in the absence of Triton but was completely ineffective in the presence of detergent (not shown).

\( K_d \) and \( k_d/k_1 \) values for several metals were determined in experiments carried out in the absence of Triton. An example of how metal protection data were treated is shown in Fig. 3, where Mn²⁺ was the protectant. The estimated \( K_d \) for Mn²⁺ was 123 μM, and \( k_d/k_1 \) was 0.13. Results of similar experiments done with other metals are compiled in Table I. All values were calculated from experiments using the same enzyme preparation, so direct comparisons of values are valid. La³⁺ was by far the most effective enzyme protectant studied, while Ba²⁺ and Sr²⁺ were the least effective.

Enzyme protection by Mn²⁺, Ca²⁺, and Mg²⁺ was studied...
Protection against heat inactivation of sea urchin sperm guanylate cyclase by metals or metal-nucleotide combinations

Preincubation mixtures contained in a volume of 0.52 ml, 38 mM TEA buffer at pH 7.8, 0.5 mM dithiothreitol, 80 μg of protein, and the Mn2+ concentrations shown in the figure in a final volume of 0.48 ml. The preincubation was for 20 min at 37°.

Values are the means of six determinations. Vertical lines represent standard deviations.

Fig. 3 Protection of sea urchin sperm guanylate cyclase against heat inactivation by Mn2+. Preincubation mixtures contained 50 mM TEA buffer at pH 7.8, 0.5 mM dithiothreitol, 80 μg of protein, and the Mn2+ concentrations shown in the figure in a final volume of 0.48 ml. The preincubation was for 20 min at 37°. The estimated Kd was 123 μM, and k2/k1 was 0.13.

Table I
Estimated values of k2/k1 and Kd for various metals from heat protection data

| Metal | k2/k1 | Kd μM |
|-------|-------|-------|
| Mn2+  | 0.13  | 123   |
| Ca2+  | 0.30  | 692   |
| Fe2+  | 0.08  | 361   |
| La3+  | -0.1  | 5.5   |
| Mg2+  | 0.00  | 335   |
| Ba2+  | 0.59  | 984   |
| Sr2+  | 0.13  | 130   |
| Co2+  | 0.28  | 79    |
| Ni2+  | 0.42  | 47    |

* The negative value for k2/k1 indicates activation of enzyme during preincubation at 37° in the presence of La3+. Estimates of k2/k1 and Kd for Sr2+ were not made because VA/Vo was greater than 0.9 for all Sr2+ concentrations tested. Constants calculated in the absence of dithiothreitol.

with several different enzyme preparations. Binary enzyme-metal dissociation constants from all experiments ranged from 123 to 211 μM for Mn2+, from 410 to 692 μM for Ca2+, and from 335 to 1375 μM for Mg2+. The large variations in estimates of the Kd for Mg2+ were due to the high k2/k1 value.

To determine if metals acted in an additive or synergistic manner to protect the enzyme against heat, Ca2+, Mg2+, and Ba2+ (0.05 to 1.5 mM) were tested in combination with 0.39 mM Mn2+, which protected the enzyme to a greater extent than did the highest concentrations of the other metals. There were no additive or synergistic effects of the metal combinations; in fact, the combined effects of the ions were either no greater or slightly less than that of Mn2+ alone, suggesting that the four metals interact at the same site.

Table II
Protection against heat inactivation of sea urchin sperm guanylate cyclase by metal-nucleotide combinations

| Metal addition | Experiment 1 | Experiment 2 |
|----------------|-------------|-------------|
|                | No nucleotide | GTP | ATP | No nucleotide | GTP | ATP |
| None           | 12           | 14   | 13  | 31            | 28  | 48  |
| Mn2+           | 38           | 54   | 56  | 50            | 98  | 99  |
| Ca2+           | 10           | 58   | 24  | 30            | 122 | 50  |
| Fe2+           | 39           | 28   | 34  | 30            | 55  | 62  |
| Mg2+           | 17           | 10   | 22  | 39            | 25  | 62  |
| Sr2+           | 13           | 15   | 20  | 24            | 30  | 48  |

* The values represent the means of four observations. The S.E. for the various means ranged from 0.2 to 3.

The difference between the protective abilities of CaGTP and CaATP apparent from Table II was not a fortuitous result of the concentrations selected. The protection by CaGTP and CaATP was studied further as a function of the calcium-nucleotide concentration at a fixed free Ca2+ concentration of 0.7 mM (Fig. 4).

These experiments clearly demonstrated a marked difference between the abilities of CaATP and CaGTP to protect the enzyme against heat. The apparent sigmoidicity of the CaGTP protection curve could indicate multiple binding sites for CaATP and CaGTP to protect the enzyme against heat. The apparent sigmoidicity of the CaGTP protection curve could indicate multiple binding sites for CaGTP and CaATP.

Similar experiments were done with MnATP and MnGTP at a fixed concentration of free Mn2+ (Fig. 5). The results with MnGTP were similar to those seen with CaGTP, in that a sigmoidal curve suggesting multiple binding sites was observed. MnATP provided slight protection at concentrations less than 20 μM.

Experiments were next designed to determine if free Mn2+ was required for protection by MnGTP. GTP was fixed at 0.43 mM (a concentration of MnGTP giving maximal protection in the presence of 0.28 mM free Mn2+), and the Mn2+ concentration was varied (Fig. 6). Complete protection by MnGTP occurred only...
Protection of sea urchin sperm guanylate cyclase against heat inactivation by CaGTP or CaATP. The preincubation volume of 0.56 ml contained 35 mM TEA buffer at pH 7.8, 7.4 mM NaNa, 0.7 mM dithiothreitol, 0.7% Triton X-100, 0.7 mM Ca\(^{2+}\) in excess of GTP or ATP, 100 \(\mu\)g of protein and the CaATP or CaGTP concentrations given in the figure. The preincubation was at 37° for 10 min.

![Diagram](http://www.jbc.org/)

Fig. 4. Protection of sea urchin sperm guanylate cyclase against heat inactivation by CaGTP or CaATP. The ordinate represents the per cent guanylate cyclase activity remaining after preincubation for 10 min at 37°. The preincubation volume contained 35 mM TEA buffer at pH 7.8, 7.4 mM NaNa, 0.78 mM dithiothreitol, 0.7% Triton X-100, 100 \(\mu\)g of protein and the CaATP or CaGTP concentrations shown in the figure.

Protection of sea urchin sperm guanylate cyclase against heat inactivation by MnATP and MnGTP. The ordinate represents the per cent guanylate cyclase activity remaining after preincubation for 10 min at 37°. The preincubation volume contained 35 mM TEA buffer at pH 7.8, 7.4 mM NaNa, 0.78 mM dithiothreitol, 0.7% Triton X-100, 100 \(\mu\)g of protein and the MnGTP or MnATP concentrations shown in the figure.

Protection against N-Ethylmaleimide-induced Inactivation by Metal-Nucleotides—CaGTP provided substantial protection against N-ethylmaleimide whereas MnGTP, in contrast to its effects with heat, protected only slightly, if at all (Fig. 7). CaGTP was also much more potent than MnGTP in protecting against N-ethylmaleimide. Protection against N-Ethylmaleimide—Sea urchin sperm guanylate cyclase was very susceptible to inactivation by N-ethylmaleimide or \(\beta\)-hydroxymercuribenzoate. Dithiothreitol in excess of either agent completely protected the enzyme against inactivation. Inactivation rates in the presence of N-ethylmaleimide concentrations greater than 20 mM followed pseudo first order kinetics at 0°.

In contrast to their abilities to protect guanylate cyclase against inactivation by heat, neither Ca\(^{2+}\) nor Mn\(^{2+}\) provided any detectable protection against inactivation by N-ethylmaleimide when the metals were present in concentrations from 0.1 to 3.0 mM.

Protection against N-Ethylmaleimide—CaGTP provided substantial protection against N-ethylmaleimide whereas MnGTP, in contrast to its effects with heat, protected only slightly, if at all (Fig. 7). CaGTP and CaGMP did not substantially protect against N-ethylmaleimide.

MnATP provided more protection against N-ethylmaleimide than did MnGTP (Fig. 8), and as observed with heat inactivation, MnATP did not further protect when Mn\(^{2+}\) concentrations exceeded the ATP concentrations. Both CaGTP and CaATP protected against N-ethylmaleimide in the absence of substantial free Ca\(^{2+}\), but CaGTP was more potent. CaGTP was also much more potent than MnGTP in protecting against N-ethylmaleimide (note scale difference on figure). Concentrations of MnGTP at least 1 to 2 orders of magnitude greater than those of CaGTP when the Mn\(^{2+}\) concentration exceeded the GTP concentration. Similarly, maximum protection by CaGTP was observed only when Ca\(^{2+}\) concentrations exceeded those of GTP (data not shown).

When ADP or ATP concentrations were fixed, various metals were added, and the enzyme protection then measured, the behavior was quite different from that seen with GTP (data not shown). Although the combination of ADP or ATP with Mn\(^{2+}\), Ca\(^{2+}\), or Mg\(^{2+}\) protected to a greater degree than did the free metals alone, there was no further increase in protection when concentrations of the metals exceeded those of ADP or ATP.
shown in the figure. After preincubation for 90 min at 0-2°C, 40-1 pg of protein, and the concentration of ATP, GTP, and metals were required to give equivalent protection. The estimated $K_d$ for CaGTP was 40 $\mu$m, and that for MnATP was 170 $\mu$m; no attempt was made to determine dissociation constants for CaGTP and MnGTP.

**Discussion**

Protection of an enzyme against heat implies enzyme-effector molecule interaction. While protection of membrane-bound enzymes by effector molecules could involve nonspecific membrane stabilization phenomena, the protection data presented here are consistent with kinetic and other data (1,7) and suggest that the enzyme stabilization patterns observed reflect direct molecular interactions with the enzyme. The estimated $K_d$ of 123 to 210 $\mu$m for enzyme-Mn$^{2+}$ agrees closely with estimates of dissociation constants of about 320 $\mu$m from kinetic data (9). This suggests that protection of the enzyme by Mn$^{2+}$ and activation of the enzyme by Mn$^{2+}$ involve the same binding site. The failure of Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ to act additively with Mn$^{2+}$ in protecting the enzyme against heat inactivation suggests that these metals bind at one site.

La$^{3+}$ was the only cation tested that showed entirely different protection patterns in the presence and absence of Triton. In other experiments, it also has been observed that La$^{3+}$ is a more potent inhibitor of guanylate cyclase in the absence than in the presence of Triton. These Triton-sensitive inhibitory effects of La$^{3+}$ on guanylate cyclase activity are consistent with the data showing a Triton-sensitive protective effect against heat.

There is no apparent relationship between $k_2/k_1$ and $K_d$ for the various metals tested. Conformational change leading to enzyme stabilization (or possible desaturation) would be best reflected by $k_2/k_1$, while $K_d$ the binary enzyme-metal dissociation constant, would reflect detectable enzyme-metal interaction. With at least one enzyme, pyruvate kinase, Ca$^{2+}$ and Mn$^{2+}$ compete for a binding site, yet only Mn$^{2+}$ elicits a protein difference spectrum (10).

Metal-induced conformational changes have been suggested for a number of enzymes, including pyruvate kinase (11, 12), enolase (13, 14), glutamine synthetase (15, 16), inorganic pyrophosphatase (17), and phosphoglucomutase (18). Mn$^{2+}$ also has been shown to be required for maintenance of the tetrameric structure of arginase (19). The induction of an enzyme conformational change by metal need not always correlate with resultant enzyme activity (18, 20), and certainly no correlation between the binary enzyme-metal dissociation constant and enzyme activity need exist (17, 18, 20, 21).

The lack of protection by MnGTP except when Mn$^{2+}$ concentrations were in excess of GTP suggests that either the enzyme binds Mn$^{2+}$ and MnGTP in an ordered fashion, with Mn$^{2+}$ binding first, or that MnGTP binds to but fails to protect the enzyme unless free Mn$^{2+}$ is available to bind to the enzyme. In another paper (9), it has been shown that kinetic patterns are consistent with the enzyme binding at least 2 MnGTP molecules and that Mn$^{2+}$ is bound to the enzyme before at least 1 of the MnGTP molecules. Therefore, although it remains possible that MnGTP could bind to but not protect enzyme until free Mn$^{2+}$ was bound, this seems unlikely because of the kinetic data (9).

CaGTP protection curves against N-ethylmaleimide were hyperbolic, and linear replots suggested that CaGTP was binding to a single site. Since CaGTP protection curves against heat indicated multiple enzyme binding sites for CaGTP, it may be inferred that an essential N-ethylmaleimide-reactive amino acid occurs at but one of the CaGTP binding sites.

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D L Garbers and J G Hardman

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