Guanylate cyclase is dephosphorylated in response to the interaction of egg peptides with a spermatozoan surface receptor (Suzuki, N., Shimomura, H., Radany, E. W., Ramarao, C. S., Ward, G. E., Bentley, J. K., and Garbers, D. L. (1984) J. Biol. Chem. 259, 14874–14879). Here, the phosphorylated form of guanylate cyclase was purified to apparent homogeneity from detergent-solubilized spermatozoan membranes by the use of GTP-agarose, DEAE-Sephadex, and concanavalin A-Sepharose chromatography. To prevent dephosphorylation of the enzyme during purification, glyceral (35%) was required in all buffers. Following purification, a single protein-staining band of Mr, 160,000 was obtained on sodium dodecyl sulfate-polyacrylamide gels. The final specific activity of the purified enzyme was 83 μmol of cyclic GMP formed/min/mg of protein at 30 °C, an activity 5-fold higher than that observed with the purified, dephosphorylated form of guanylate cyclase. A preparation containing protein phosphatase from spermatozoa, or highly purified alkaline phosphatase (from Escherichia coli), catalyzed the dephosphorylation of the enzyme; this resulted in a subsequent decrease in guanylate cyclase activity and a shift in the Mr, from 160,000 to 150,000. The phosphate content of the high Mr, form of the enzyme was 14.6 mol/mol protein whereas the phosphate content of the low Mr, form was 1.6 mol/mol protein. All phosphate was localized on serine residues. The Mr, 160,000 form of guanylate cyclase demonstrated positive cooperative kinetics with respect to MnGTP while the Mr, 150,000 form displayed linear, Michaelis-Menten type kinetics. The phosphorylation state of the membrane form of guanylate cyclase, therefore, appears to dictate not only the absolute activity of the enzyme but also the degree of cooperative interaction between catalytic or GTP-binding sites.

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.) exists in both soluble and particulate fractions of cells, its distribution varying dependent on the cell type (1). Following homogenization of sea urchin spermatozoa, the enzyme appears to exist completely in particles with most or all of the activity associated with plasma membranes (2–5). The sperm enzyme is a phosphorylated protein (6, 7) and when specific egg peptides bind to spermatozoan surface receptors a rapid dephosphorylation of guanylate cyclase oc-

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Phosphorylated Guanylate Cyclase

and Raday et al. (6). All purification steps were performed at 0-4 °C. Spermatozoa collected from Arbacia punctulata were diluted in 10 volumes of a buffer containing 25 mM MES, pH 6.2, 0.5% (v/v) Lubrol PX, 35% (v/v) glycerol, 10 mM NaF, 10 mM benzamide HCl, 5 mM dithiothreitol, and 5 mM MnCl2 (Buffer A). The sperm suspension was sonicated for 3 × 10 s and the resultant solution was centrifuged at 4 °C for 20 min at 90,000 × g to remove unbroken spermatozoa and cell debris. The supernatant fluid was then mixed with 15 ml of GTP-Sepharose which had been equilibrated previously with Buffer A. After 1 h the GTP-Sepharose was washed with 2 volumes of Buffer A (30 min each wash), and then enzyme was eluted with 25 ml of a solution containing 25 mM triethanolamine, pH 7.5, 35% glycerol, 0.1% Lubrol PX, 50 mM NaCl, 10 mM NaF, 10 mM benzamide HCl, and 5 mM dithiothreitol (Buffer C). Concanavalin A-agarose was subsequently washed with 5 volumes of Buffer C and guanylate cyclase was eluted with 10 ml of a solution containing 25 mM MES, pH 6.2, 25% glycerol, 10% ethylene glycol, 1% Lubrol PX, 10 mM NaF, 10 mM benzamide HCl, 5 mM dithiothreitol, and 500 mM α-methyl mannospyranoside. The eluted enzyme was concentrated by several centrifugation steps at 5,000 × g for 30 min using Centricon microconcentrators and 2 volumes of Buffer B containing 0.2 M NaCl (30 min each wash). Guanylate cyclase was then eluted from the ion exchange resin by the addition of 20 ml of Buffer B containing 0.5 M NaCl. The solution containing the enzyme was mixed with 5 ml of Concanavalin A-Sepharose (12 h) that had been previously equilibrated with a solution containing 25 mM triethanolamine, pH 7.5, 35% glycerol, 0.1% Lubrol PX, 50 mM NaCl, 10 mM NaF, 10 mM benzamide HCl, and 5 mM dithiothreitol (Buffer C). Concanavalin A-agarose was subsequently washed with 5 volumes of Buffer C and guanylate cyclase was eluted with 10 ml of a solution containing 25 mM MES, pH 6.2, 25% glycerol, 10% ethylene glycol, 1% Lubrol PX, 10 mM NaF, 10 mM benzamide HCl, 5 mM dithiothreitol, and 500 mM α-methyl mannospyranoside. The eluted enzyme was concentrated by several centrifugation steps at 5,000 × g for 30 min using Centricon microconcentrators and stored at -20 °C until use. Guanylate cyclase was stable for at least 1 month under these conditions.

Protein Determination—Protein was estimated by Peterson's (17) modification of the Lowry et al. method (18) with bovine serum albumin used as the standard.

Gel Electrophoresis—Gels were run as described by Ward and Vacquier (19) with modifications described later (8). The gels were subsequently stained by the method of Morrissey (20).

Separation of Protein Phosphatase Activity from That of Guanylate Cyclase—A. punctulata spermatozoa were collected and diluted with 10 volumes of a solution containing 20 mM trithanolamine buffer, pH 7.5, containing 5 mM dithiothreitol. The sperm suspension was homogenized (3 × 10 s sonication) and centrifuged at 100,000 × g for 2 h. The supernatant fluid, shown in preliminary experiments to contain a majority of the apparent protein phosphatase activity, was added to a DEAE-Sephalocel ion exchange column that had been equilibrated with a solution containing 25 mM triethanolamine buffer and 0.1% SDS. At the end of the chromatography the enzyme was mixed with 5 ml of Concanavalin A-Sepharose (12 h) and 5 ml of a solution containing 25 mM MES, pH 6.2, 25% glycerol, 10% ethylene glycol, 1% Lubrol PX, 10 mM NaF, 10 mM benzamide HCl, 5 mM dithiothreitol, and 500 mM α-methyl mannospyranoside. The eluted enzyme was concentrated by several centrifugation steps at 5,000 × g for 30 min using Centricon microconcentrators and stored at -20 °C until use. Guanylate cyclase was stable for at least 1 month under these conditions.

Effect of Glycerol on the Stabilility of Guanylate Cyclase—A major problem with respect to purification of the M, 160,000 (phosphorylated) form of guanylate cyclase was its rapid and apparent irreversible conversion to a low M, (dephosphorylated) species upon homogenization of spermatozoa. Within 1 h after homogenization at 22 °C and within 12 h at 4 °C the enzyme was totally converted to a low M, form. A large variety of added protein phosphatase and protease inhibitors were not effective at preventing the M, conversion during purification. However, for reasons not yet understood, the addition of glycerol to buffers used for homogenization resulted in a stabilization of enzyme activity (Fig. 1). Therefore, although guanylate cyclase activity of extracted spermatozoa was reduced by 75% in the absence of glycerol (30 min at 30 °C), the inclusion of 50% glycerol resulted in only a 15% decrease in enzyme activity. At 0-4 °C in the presence of 50% glycerol, guanylate cyclase activity was reduced by only 20% after 12 h (data not shown). The prevention of a decrease in guanylate cyclase activity by glycerol was directly related to retention of the enzyme in the high M, form (Fig. 2). Therefore, all buffers during purification contained 35% glycerol. A percentage of 55% was routinely used since this concentration

![FIG. 1. Effect of glycerol on guanylate cyclase activity in sperm homogenates.](image-url)
Phosphorylated Guanylate Cyclase

Fig. 2. The effect of 35% glycerol on the M, shift of guanylate cyclase. To 300 µl of A. punctulata spermatozoan extract prepared as in the legend to Fig. 1, an equal volume of 70% glycerol or water was added. At 0, 30, and 60 min after addition of glycerol or water, 200-µl aliquots were prepared for SDS-polyacrylamide gels as described (8). The gel was silver-stained as described (19). Approximately 10 µg of protein were added per lane. Lanes A, C, and E are sperm extracts in the presence of 35% glycerol. Lanes B, D, and F are without glycerol. Lanes A and B are at 0 min, lanes C and D at 30 min, and lanes E and F at 60 min of incubation at 30 °C. The position of the phosphorylated form (M, 160,000) and the dephosphorylated form (M, 150,000) of guanylate cyclase are indicated by the arrows. The M, 140,000 band is an unidentified protein.

allowed convenient handling of the solutions during chromatography and still prevented the dephosphorylation of guanylate cyclase.

Purification and Recovery—The specific activity of guanylate cyclase obtained from a typical enzyme preparation is presented in Table I. In different preparations, the specific activities have ranged from 70 to 110 pmol of cyclic GMP formed/min/mg of protein at 30 °C. The specific activity was about five times higher than that of guanylate cyclase previously purified from sperm membranes (2, 5) or from rat lung (14), and was nearly 45 times higher than the enzyme purified from rat adenocortical carcinoma cells (15).

Homogeneity—After SDS gel electrophoresis (150–200 ng of protein applied per gel) a single protein-staining band was visible which had an apparent molecular weight of 160,000 (Fig. 3A). A faint silver-stained band was visible around 50,000 daltons. The purified enzyme comigrated with the M, 160,000 form of guanylate cyclase normally present in intact cells (Fig. 3B). It will be noted that some slight staining is observed as a smear between 160,000 and 150,000; this appears to represent the formation of a small amount of the various dephosphorylated forms of the enzyme.

Metal Ion Requirement—Guanylate cyclase from a large variety of tissue sources preferentially uses Mn2+ as a cofactor; Mg2+ is generally 5–20% as effective as Mn2+, and Ca2+ is even less effective (1). The M, 160,000 form of guanylate cyclase also was highly dependent on Mn2+ for activity (Table II). Activity in the presence of Mg2+ and Ca2+ was low (approximately 0.5% of the activity observed in the presence of Mn2+). No detectable activity was observed in the presence of other divalent cations such as Ba2+. The general properties of the high M, form of guanylate cyclase, therefore, are similar to those of the low M, enzyme (3) and to the unpurified particulate or detergent-dispersed enzyme preparations from sea urchin spermatozoa (24, 25).

Loss of Activity and Conversion to the Low Apparent M, Form—In detergent extracts of spermatozoa, guanylate cyclase activity decreased in a time-dependent manner, both at 0 and 22 °C (9). This loss of enzyme activity was associated with a loss of phosphate and conversion of guanylate cyclase to the low M, form. A protein phosphatase that causes conversion of guanylate cyclase from the high M, form to the low M, form was obtained from the 100,000 × g supernatant fluids of extracted spermatozoa (see “Experimental Procedures”). When the protein phosphatase was incubated with guanylate cyclase, there was a time-dependent loss of guanylate cyclase activity (Fig. 4). Approximately 70% decreases in guanylate cyclase activity were seen within 30 min at 30 °C in the presence of the protein phosphatase; the decrease in enzyme activity coincided with the M, conversion (not shown).

Effect of Bacterial Alkaline Phosphatase on the Purified High M, Form of Guanylate Cyclase—Incubation of the purified, phosphorylated form of guanylate cyclase with E. coli alkaline phosphatase resulted in rapid decreases in enzyme activity. The addition of protein phosphatase inhibitors, 200 µM sodium orthovanadate (26) or 50 mM NaF (27), to the reaction mixture resulted in protection of guanylate cyclase from alkaline phosphatase. The phosphate content of the two M, forms of guanylate cyclase were estimated to be 1.5 mol of phosphate/mol of low M, form and 14.6 mol/mol of high M, form. These values are in basic agreement with earlier estimates made from gel slices of the crude enzyme (22).

Kinetic Behavior—Positive cooperative kinetics as a function of MeGTP represent a common characteristics of the membrane form of guanylate cyclase from various tissues (2, 28–30). When the sea urchin sperm enzyme was previously purified to homogeneity, normal Michaelis-Menten kinetics were observed (3). It was argued that since loss of cooperativity did not seem to involve a major molecular weight change of the enzyme, the loss of an essential low molecular weight cofactor(s) or partial denaturation of the enzyme might account for the change. Highly purified forms of guanylate cyclase from rat lung (14) or rat liver (31) also demonstrate normal Michaelis-Menten kinetics even though the enzyme in initial cell extracts shows positive cooperativity (16).

The purified phosphorylated form of the enzyme from spermatozoa displayed positively cooperative kinetic behavior as a function of MnGTP. The apparent Vmax was 92 µmol of cyclic GMP formed/min/mg of protein and the S0.5 = 40.7 µM (Fig. 5, left). When the enzyme was treated with partially purified protein phosphatase for 30 min at 30 °C and then

| Table I |

<table>
<thead>
<tr>
<th>Protein</th>
<th>µg</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm homogenate</td>
<td>41,250</td>
<td>4.6</td>
<td>0.11</td>
<td>-fold</td>
<td>100</td>
</tr>
<tr>
<td>30,000 g supernatant</td>
<td>5,520</td>
<td>4.4</td>
<td>0.79</td>
<td>7</td>
<td>96</td>
</tr>
<tr>
<td>GTP-Sepharose eluate</td>
<td>470</td>
<td>2.4</td>
<td>5.1</td>
<td>46</td>
<td>53</td>
</tr>
<tr>
<td>DEAE-eluate</td>
<td>110</td>
<td>1.9</td>
<td>17.6</td>
<td>160</td>
<td>43</td>
</tr>
<tr>
<td>Concanavalin A eluate</td>
<td>1.09</td>
<td>0.09</td>
<td>83.1</td>
<td>753</td>
<td>2</td>
</tr>
</tbody>
</table>
was electrophoresed on 12.5% SDS-polyacrylamide gel and silver-stained (19). Panel A, approximately 10 μg of total sperm protein was loaded on lane 1. Purified enzyme was loaded on lane 2. The M₅ standards used were myosin (200), β-galactosidase (116), phosphorlyase b (92), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), and lysozyme (14.4).

Panel B, approximately 10 μg of sperm protein were loaded on lanes 1 and 2. Lane 1, control sperm homogenate (guanylate cyclase in the high M₅ form). Lane 2, resact (Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH₂)-treated sperm homogenate (guanylate cyclase in the low M₅ form). Lane 3, purified enzyme (approximately 300 ng). To differentiate between high and low M₅ forms of guanylate cyclase, it was necessary to run the gels for 6 h at 45 mA/slab gel. Only a part of the gel is shown.

**TABLE II**

<table>
<thead>
<tr>
<th>Metal (3 mM)</th>
<th>Relative activity</th>
</tr>
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<tbody>
<tr>
<td>Mn²⁺</td>
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</tr>
<tr>
<td>Mg²⁺</td>
<td>0.43</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>ND</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.5</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>ND</td>
</tr>
<tr>
<td>CO³⁻</td>
<td>ND</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detectable.

Assayed for activity, the kinetic pattern changed to Michaelis-Menten (Fig. 5, right), with an apparent Vₘ₉ of 19 μmol of cyclic GMP formed/min/mg and an apparent Kₘ = 160 μM. Conversion to the low molecular weight form in crude detergent extracts also resulted in a loss of positively cooperative kinetic behavior with respect to MnGTP concentrations (Fig. 6, left and right).

In 1983, Ward and Vacquier (6) showed that a plasma membrane protein of _A. punctulata_ sea urchin spermatozoa was rapidly dephosphorylated in response to egg jelly. Coincident with a loss of ³²P, the M₅ changed from 160,000 to 150,000. Since the M₅ of the protein appeared similar to a previously purified plasma membrane protein, guanylate cyclase (3, 5), studies were initiated to determine if the phosphoprotein, in fact, was guanylate cyclase. Subsequent research not only identified guanylate cyclase as the phosphoprotein (7, 8) but also identified the active component of egg conditioned media as resact (8), a small egg peptide with chemotactic properties (32). Coincident with the loss of phosphate due to receptor occupation by resact, guanylate cyclase activity in detergent extracts also markedly decreased (9).

Many different agents are now known to elevate cyclic GMP concentrations in various cells (33). Atrial natriuretic peptides (ANP) have been of special interest of late (34-36). In neuroblastoma (N4TG1) cells, for example, ANP causes cyclic GMP concentrations to reach maximal intracellular concentrations in about 5 min; these concentrations then return to basal levels at around 30 min (37). Cyclic GMP concentrations in sea urchin spermatozoa, in contrast, rise rapidly to maximal concentrations (within 15 s) and return to near basal levels within 1 min (11, 12). There are reasons to believe that the transient elevations of cyclic GMP could be explained by the regulation of guanylate cyclase activity in both cases. First, in rat kidney membranes, guanylate cyclase...
activity is initially stimulated by ANP but then activity subsides until it is actually less than that of the nonstimulated enzyme (38). Second, early studies with cyclic AMP activation demonstrated an initial activation of guanylate cyclase that also subsides until it is actually less than that of the nonstimulated enzyme. The apparent transient activation of guanylate cyclase observed in other cellular systems also could be explained by phosphorylation/dephosphorylation mechanisms but this remains to be studied. The large amount of guanylate cyclase in spermatozoa offers significant advantages for such studies and therefore the sperm cell appears to represent an excellent cell model to study the regulatory properties of guanylate cyclase.

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REFERENCES