In our studies with purified soluble guanylate cyclase from rat lung, we have tested a number of guanosine 5'-triphosphate (GTP) analogues as substrates and inhibitors. 5'-Guanosilylimidodiphosphate (GMP-P(NH)P), guanylyl (β,γ-methylene) diphosphate (GMP-P(CH2)P), and guanosine 5'-O-(3-thiotriphosphate) (GTP-s) were found to be substrates for guanylate cyclase. GTPyS supported cyclic GMP formation at 20 or 75% of the rate seen with Mn2+-GTP and Mg2+-GTP, respectively. GMP-P(NH)P and GMP-P(Ch2)P supported cyclic GMP formation at 10-20% of the GTP rate with either cation cofactor. These analogues were found to have multiple Km values; one Km value was similar to GTP (150 μM with Mg2++, 20-70 μM with Mn2+), but an additional high affinity catalytic site (3 μM) was also observed. Guanosine tetrathosphate (Km = 10 μM), adenosine triphosphate (Km = 9 μM) and the 2',3'-dialdehyde derivative of GTP (dial GTP) (Km = 1 μM) were not good substrates for the enzyme; however, they were potent competitive inhibitors. These GTP analogues will be useful tools for the study of GTP binding sites on guanylate cyclase and they may also help elucidate the effects of free radicals and other agents on guanylate cyclase regulation.

Soluble guanylate cyclase (GTP-pyrophosphate-lyase (catalyzing) EC 4.6.1.2.) catalyzes the formation of cyclic GMP1 and has been purified by several laboratories (1-4). The holoenzyme (150,000 daltons) is a dimer of two similar sized subunits (72,000 daltons); however, it is not known if the individual subunits possess catalytic activity (4). The enzyme can be activated by fatty acids, hydroxyl radical, and a variety of nitroso compounds, including nitric oxide and sodium nitroprusside (5-10). Guanylate cyclase requires a cation cofactor for catalytic activity and exhibits a greater reaction velocity with Mn2+-GTP (Km = 25 mM) than with Mg2+-GTP (Km = 150 μM). However, upon activation, the enzyme utilizes Mg2+-GTP as effectively as Mn2+-GTP (11). Following activation with nitroso compounds or inactivation with the mixed disulfide cystamine (12), we have reported the appearance of a second, high affinity metal-GTP catalytic site (Km = 3 μM and 10-20 μM for Mn2+-GTP and Mg2+-GTP, respectively). This suggests that guanylate cyclase may contain several interactive nucleotide sites.

These complex kinetic characteristics prompted us to consider the effects of a number of nucleotide analogues on the enzyme. In this report, we summarize the properties of these analogues on purified guanylate cyclase. These analogues and inhibitors will be useful for further studies on the kinetic properties of soluble guanylate cyclase and may help elucidate the catalytic mechanisms of the basal and activated enzyme.

EXPERIMENTAL PROCEDURES

Guanylate cyclase was purified from rat lung by isoelectric and ammonium sulfate precipitations, followed by affinity chromatography on GTP-agarose and preparative polyacrylamide gel electrophoresis. The details of this procedure and characteristics of the purified enzyme have been reported previously (4).

Guanylate cyclase activity was assayed as described previously (2, 4). Incubations (100 μl for 10 min at 37 °C) contained 50 mM Tris-HCl (pH 7.6), 5 μg of bovine serum albumin, 4 mM MnCl2 or MgCl2 (0.06) to 1.0 mM GTP and/or analogues, and 10-20 ng of purified enzyme. Cyclic GMP formed was measured by radioimmunoassay (13). All values represent the means of duplicate or triplicate incubations from representative experiments. Protein was determined by the method of Lowry et al. (14).

Dial GTP was generated using a method described previously for the preparation of dial ATP (15). This protocol involved incubating 200 μmol of GTP with 240 μmol of NaIO4 on ice for 60 min. The resulting dial GTP was purified by chromatography on Sephadex G-10 column (2 x 20 cm) at 4 °C with degassed N2 purged water for the elution. The column fractions were monitored for absorbance at 258 nm and the leading half of the peak was pooled. Purity of the preparations was monitored by thin layer chromatography using polycrylamide imine cellulose plates and 3.2% NH4O2 as the solvent. Under these conditions, dial GTP and GTP have Rf values of 0.02 and 0.24, respectively. Concentrations of dial GTP were measured spectrophotometrically (258) = 14,900 cm-1 M-1 and aliquots were stored at -70 °C. GTP-S was from Boehringer Mannheim. Other nucleotides were products of Sigma and other materials were obtained as described previously (2, 4).

RESULTS

Purification of soluble guanylate cyclase from rat lung by the protocol outlined under "Experimental Procedures" resulted in a 3000-fold purification with specific activities under basal conditions of 250-400 nmol of cyclic GMP formed/min/mg and 24-60 nmol/min/mg with MnCl2 and MgCl2, respectively. The purified enzyme showed a single band of protein stain under a variety of electrophoretic conditions (4). We evaluated the ability of the purified enzyme to utilize several GTP analogues as substrates for the production of cyclic GMP. As shown in Table I, GTPyS supported cyclic GMP formation at a 20 or 75% rate of that seen with Mn2+-GTP and Mg2+-GTP, respectively. GMP-P(NH)P and GMP-P(Ch2)P were approximately 10-20% as efficient as equivalent concentrations of GTP with either metal. Guanosine tetra-
phosphate and dial GTP were not effective substrates for guanylate cyclase.

The Michaelis constants for the analogues were determined by monitoring cyclic GMP formation over a wide range of concentrations (see "Experimental Procedures"). Figs. 1 and 2 show Eadie-Hofstee transformations (16) of kinetic data for GTP, GTPyS, GMP-P(NH)P, and GMP-P(CH2)P. GTP plots showed a linear slope which corresponded to a $K_m$ of about 28 $\mu$M with MnCl$_2$ (Fig. 1). GTPyS yielded a curved plot with $K_m$ values of 70 $\mu$M and 3 $\mu$M (Fig. 1). As seen in Fig. 2, GMP-P(NH)P and GMP-P(CH2)P both exhibited curvilinear plots with the two most linear components giving $K_m$ values of 25-30 $\mu$M and 3 $\mu$M. Curvilinear plots were also observed with these analogues when Mg$^{2+}$ was used as the cation cofactor ($K_m$ values, 250-300 $\mu$M and 3 $\mu$M, data not shown).

The data in Figs. 1 and 2 were replotted by the method of Hill (17) and this is shown in Fig. 3. GTP yielded a Hill coefficient of 1, while values of 0.85, 0.6, and 0.45 were obtained from GMP-P(CH2)P, GMP-P(NH)P, and GTPyS, respectively. These kinetic data suggest two or more catalytic sites on soluble guanylate cyclase that appear to interact with negative cooperativity.

In Table II, inhibition of guanylate cyclase by various nucleotides is presented. Dixon plots (18) were used to derive the $K_i$ values and these data are not shown. Two GTP analogues, guanosine tetraphosphate and dial GTP, were good competitive inhibitors with $K_m$ values of 10 $\mu$M and 1 $\mu$M, respectively. The purine and pyrimidine nucleotide triphosphates were tested and the purines led to greater inhibition of the enzyme than any of the pyrimidines. ATP was the most potent inhibitor among the triphosphates, with a $K_i$ of 9 $\mu$M. CTP, UTP, and TTP were also inhibitory with higher $K_i$ values of 40-50 $\mu$M. It should be noted that with all these compounds, the Mn$^{2+}$-dependent activity was more sensitive to inhibition than the Mg$^{2+}$-dependent activity. This differential inhibitory effect was also observed for inorganic triphosphate ($K_i = 4 $ $\mu$M).

**Fig. 1.** $K_m$ determinations for GTP and GTPyS. Guanylate cyclase assays were performed as described under "Experimental Procedures" with 4 mM MnCl$_2$ and GTP or GTPyS at concentrations of 1 to 1000 $\mu$M.

**Fig. 2.** $K_m$ determinations for GMP-P(CH2)P and GMP-P(NH)P. This experiment was performed as described in the legend to Fig. 1, with 4 mM MnCl$_2$ and the indicated nucleotides.

**Fig. 3.** Hill coefficients for GTP and GTP analogues. The data from Figs. 1 and 2 were replotted by the method of Hill (17) and the indicated slopes ($n$) were calculated from the plots.

**Table I**

| Nucleotide | $M_g^{2+}$ activity | $M_n^{2+}$ activity | $M_n^{2+}/M_g^{2+}$ ratio |
|------------|---------------------|---------------------|--------------------------|
| GTP 0.1    | 18.0                | 260                 | 14.4                     |
| 0.5        | 36.0                | 317                 | 8.8                      |
| GTPyS 0.1  | 11.5                | 59.0                | 5.1                      |
| 0.5        | 27.5                | 65.0                | 2.4                      |
| GMP-P(NH)P 0.1 | 30.0              | 34.0                | 11.3                     |
| 0.5        | 3.0                 | 31.0                | 6.2                      |
| GMP-P(CH2)P 0.1 | 2.0               | 22.5                | 11.2                     |
| 0.5        | 7.5                 | 31.0                | 4.1                      |
| G-tetra-P 0.1 | 0.5               | 4.0                 | 8.0                      |
| 0.5        | 1.0                 | 4.0                 | 4.5                      |
| Dial GTP 0.1 | 0                  | 0.35                | 0.3                      |
| 0.5        | 0.5                 | 1.1                 | 2.2                      |

**Table II**

| Agent       | $M_g^{2+}$ activity | $M_n^{2+}$ activity | $K_i$ |
|-------------|---------------------|---------------------|-------|
| ATP 0.1     | 92                  | 38                  | 9     |
| 0.5         | 50                  | 9                   |       |
| ITP 0.1     | 110                 | 52                  | 2     |
| 0.5         | 75                  | 20                  |       |
| CTP 0.1     | 98                  | 66                  | 42    |
| 0.5         | 73                  | 30                  |       |
| UTP 0.1     | 97                  | 50                  | 8     |
| 0.5         | 96                  | 38                  |       |
| Deoxy TTP 0.1 | 68               | 38                  |       |
| 0.5         | 62                  | 38                  |       |
| G-tetra-P 0.1 | 81               | 49                  | 10    |
| 0.5         | 52                  | 17                  |       |
| Dial GTP 0.1 | 74                 | 40                  |       |
| 0.5         | 2                   | 1                   |       |
| P-P-P 0.1   | 71                  | 59                  | 4     |
| 0.5         | 38                  | 15                  |       |
DISCUSSION

A number of laboratories have recently purified soluble guanylate cyclase from several mammalian sources (1-4). However, no information is available on the stoichiometry of substrate binding or on the requirements of the guanine nucleotide binding site. To gain insight into these questions, as well as the complex kinetic characteristics of guanylate cyclase, we examined the effects of various nucleotide analogues on the enzyme.

Three GTP analogues, GTPyS, GMP-P(CH2)P, and GMP-P(NH2)P, were substrates for the enzyme and supported cyclic GMP production with either Mn2+ or Mg2+. Kinetic analysis revealed a single Km for Mn2+-GTP (28 μM), in good agreement with published values (4). However, with the three analogues, Eadie-Hofstee plots were curvilinear; one component exhibited a Km of 25-70 μM, while an additional component(s) corresponded to a Km of 3 μM. Hill coefficients were calculated while GTP gave a value of 1, values for the analogues were less than one. These data suggest that purified preparations of soluble guanylate cyclase have two or more guanine nucleotide catalytic sites. In the case of GTP, the sites appear to be equivalent and perhaps noninteracting, while with the GTP analogues, the sites appear to be nonequivalent and interactive with negative cooperativity. Interestingly, these data correlate with kinetic observations made for GTP following inactivation with mixed disulfides (12) or activation with sodium nitroprusside. It should be noted that the particular form of guanylate cyclase has also been shown to exhibit nonlinear kinetics, although in that instance the interaction was one of positive cooperativity (19, 20).

Although the data presented here demonstrate two or more sites for GTP or related guanine nucleotides on purified soluble guanylate cyclase, we cannot speculate whether these sites exist on the same or different enzyme molecules. As mentioned above, soluble guanylate cyclase is a dimer. While one could suggest that each monomer may contain one GTP site capable of allosteric interactions with the other GTP site under certain conditions, the alternate explanation of these multiple Km values representing a single site per enzyme dimer with different nonequivalent populations of enzyme molecules in the purified preparation cannot be ruled out at this time. Further studies aimed at assessing the stoichiometry of guanine nucleotide binding independent of catalytic activity are now underway.

Two other GTP analogues, guanosine tetraphosphate and dial GTP, were not utilized to any significant extent as substrates for the enzyme, but were found to be potent competitive inhibitors. Using cold and radiolabeled dial GTP and reducing conditions (sodium cyanoborohydride) we tried to irreversibly inactivate and label guanylate cyclase, but these attempts were unsuccessful. The irreversible inactivation of pyruvate carboxylase and adenylate cyclase has been reported following the reduction of the 2′3′ dialdehyde derivative of ATP (15, 21). This suggests, perhaps, a different structure for the guanine nucleotide binding site on guanylate cyclase as compared to certain ATP utilizing enzymes. Other purine and pyrimidine nucleotide triphosphates were also found to be competitive inhibitors, with ATP being the most effective. In addition, triplyphosphate was a very good inhibitor. From these data, it is evident that the guanine nucleotide binding site on guanylate cyclase contains at least two domains, one involved with purine base specificity and binding and one involved with binding phosphate moieties. Moreover, the addition of an extra phosphate group beyond the γ position, or the loss of the intact sugar ring on the nucleotide substrate, by conversion of vicinal hydroxyls to aldehydes, seems to diminish greatly the ability of the enzyme to perform its catalytic function.

The mechanisms by which guanylate cyclase can be regulated are important questions which touch upon enzyme regulation, as well as the possible functions of cyclic GMP in biological systems. It is clear that additional work is needed to understand more fully the kinetic mechanisms of guanylate cyclase activity and modulation, but it is felt that with large quantities of purified enzyme along with these substrate analogues more detailed information will be forthcoming.

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