Kinetic Analysis of Sea Urchin Sperm Guanylate Cyclase†

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ABSTRACT: A steady-state kinetic analysis of the reaction catalyzed by sea urchin sperm guanylate cyclase was made. Both free Mn^{2+} and MnGTP were required for the formation of guanosine 3′,5′-monophosphate. Initial rate and inhibition data indicated that the enzyme contained a single site for free Mn^{2+} but multiple binding sites for MnGTP. Nucleotides (ATP, ADP, dGTP, dATP) that were inhibitors of guanylate cyclase at higher MnGTP concentrations functioned as activators at lower MnGTP concentrations, supporting a model with multiple enzyme binding sites for nucleotides.

The guanylate cyclase activity in sea urchin sperm is much higher than has been reported for any mammalian tissue (Gray *et al.*, 1970); in fact, the specific activity of the enzyme from sea urchin sperm particles is at least double the activity of 445-fold purified guanylate cyclase from soluble fractions of bovine lung (White *et al.*, 1972). The sea urchin sperm enzyme activity, which can be easily measured in the absence of detergent, is confined to particulate fractions, probably plasma membranes (Gray and Drummond, 1973), and appears to be similar to mammalian guanylate cyclase activities located in particulate fractions in its essential properties. These include a dependence on Mn^{2+} for activity (Gray, 1971; Chrisman *et al.*, 1974; Garbers *et al.*, 1974a), and positive cooperative kinetic patterns with respect to MnGTP[1] (Chrisman *et al.*, 1974; Garbers *et al.*, 1974a). Guanylate cyclase activity in soluble (generally 100,000*g* supernatant fluid) fractions from various mammalian tissues also depends strongly on Mn^{2+} as a metal cofactor (Hardman and Sutherland, 1969; White and Aurbach, 1969), but only classical hyperbolic kinetic patterns as a function of the MnGTP concentration have been reported for the soluble enzyme (Hardman and Sutherland, 1969; White and Aurbach, 1969; Böhme, 1970; Thompson *et al.*, 1973). The particulate guanylate cyclase from sea urchin sperm, therefore, can serve as a model for the enzyme of much lower specific activity located in particulate fractions from various mammalian cells.

This investigation includes determinations of initial rates of reaction at various concentrations of MnGTP and of free Mn^{2+}, as well as inhibition and activation experiments using nucleotides other than GTP.

Materials and Methods

Sea urchin (*Strongylocentrotus purpuratus*) sperm guanylate cyclase was prepared and assayed as described elsewhere (Garbers *et al.*, 1974b). The assay mixture usually contained 7 mM sodium azide, 7 mM theophylline, 40 mM triethanolamine buffer at pH 7.8, 0.7 mM dithiothreitol, 1–3 × 10^6 dpm of [^{3}H]GTP and the concentrations of Mn^{2+} or GTP given in the legends to the figures. Assays were also conducted at pH 7.0, and although incubations were generally at 30°, linear reactions for at least 5 min at 37° were observed. In all instances, enzyme behavior at pH 7.0 or at 37° was similar to that observed at pH 7.8 or at 30°. It should be emphasized that linear accumulation of guanosine 3′,5′-monophosphate (cyclic GMP) as a function of both time and protein was observed in all experiments in this report. As in other papers (Chrisman *et al.*, 1974; Garbers *et al.*, 1974a) Mn^{2+} concentrations were maintained at constant values in excess of the nucleotide concentrations, thereby avoiding kinetic artifacts due to varying percentages of Mn^{2+}–nucleotide complex. This approach has been suggested by other workers (Cleland, 1970; Plowman, 1972) where binding constants are very high, as is the case with Mn^{2+} nucleoside triphosphates or Mn^{2+} nucleoside diphosphates (O'Sullivan and Perrin, 1964; Jallon and Cohn 1970).

The materials used in this report were as described elsewhere (Garbers *et al.*, 1974b; Chrisman *et al.*, 1974).

Results

Mn^{2+} and MnGTP Kinetics. When the initial reaction velocities were determined with varying levels of either MnGTP or free Mn^{2+}, different types of reciprocal plots were observed. Figure 1 shows the effect of varying the MnGTP concentration at fixed levels of free Mn^{2+}. The reciprocal plots were concave upward with intersection on the ordinate. The lines were analyzed as suggested by Cleland (1970) and found to fit a 2/1 function. Parallel lines (n = 1.4) were observed when these data were fitted to a Hill plot (Figure 2). As the concentration of free Mn^{2+} was increased, the $S_{0.5}$ (substrate concentration at one-half V_{max}) value for MnGTP decreased. Plots of the reciprocal initial velocity against reciprocal free Mn^{2+} concentration at fixed levels of MnGTP were linear, with intersection to the left of the ordinate (Figure 3). The dissociation constant for Mn^{2+} (0.32 mM) was estimated from the point of intersection. Because of the greater number of MnGTP points, data shown in Figure 1 were replotted as velocity^{-1} against [Mn]$^{-1}$, and the slopes and intercepts of these data were replotted as shown in Figure 4. Both the slope and intercept replots were nonlinear, with the slope replot extrapolating to zero.

Proposed Kinetic Models. Mechanistic models involving the binding of two or more MnGTP molecules per catalytic site will be presented in this discussion. These initial rate data, however, do not preclude possible cooperative behavior between subunits, where all sites are catalytic. The intersection of the curves in Figure 1 on the ordinate indicates that Mn^{2+} binds to the enzyme in an equilibrium fashion relative to the

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[1] The charge on MnGTP has not been designated, but at pH 7.8 it exists primarily as $MnGTP^{2-}$.

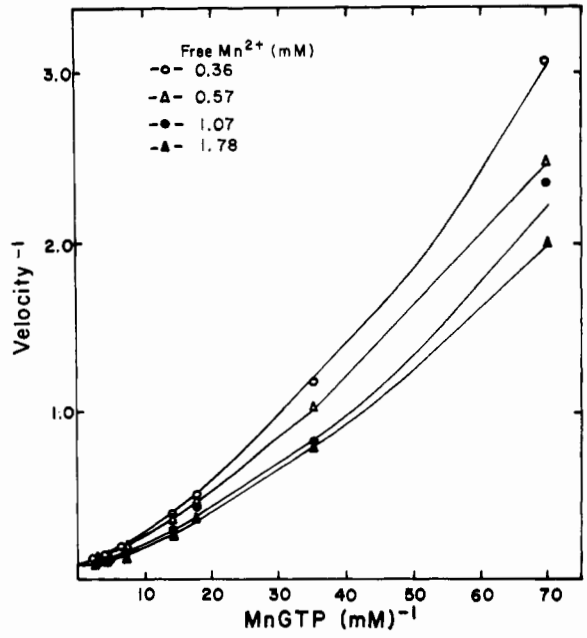


FIGURE 1: Reciprocal initial velocity as a function of reciprocal MnGTP concentration at fixed concentrations of free Mn^{2+}. Assays were as described in the Methods, with varying levels of Mn^{2+} and MnGTP as indicated in the figure. Velocities are expressed as the nmol of cyclic GMP formed 12 min^{-1} 80 μg of $protein^{-1}$.

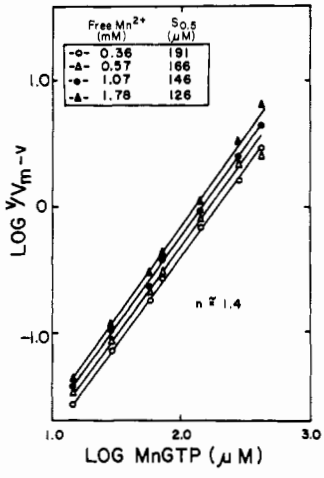


FIGURE 2: Hill plot of the data given in Figure 1.

binding of at least one MnGTP. The theoretical basis of these mechanisms which were first proposed by Segal *et al.* (1952) has been questioned by several groups (Dalziel, 1969; Orsi and Cleland, 1972; Wedler and Boyer, 1972). The absence of various substrate terms in the rate equation describing such systems was suggested to make the rate equations derived for such systems invalid (Dalziel, 1969). However, Rudolph and Fromm (1973) have presented theoretical arguments showing the validity of such mechanisms, and equilibrium-ordered mechanisms have also been suggested from experimental results for various metal-requiring enzymes (Cleland, 1970; McClure *et al.*, 1971; Morrison and Ebner, 1971a,b; Cleland *et al.*, 1972).

With sea urchin sperm guanylate cyclase, if only one MnGTP interacted with the enzyme per one free Mn^{2+}, the mechanism would be similar to the mechanism suggested for those enzymes. The nonlinearity of the MnGTP curves in Figure 1, however, indicates multiple interactions of MnGTP and free Mn^{2+} with guanylate cyclase.

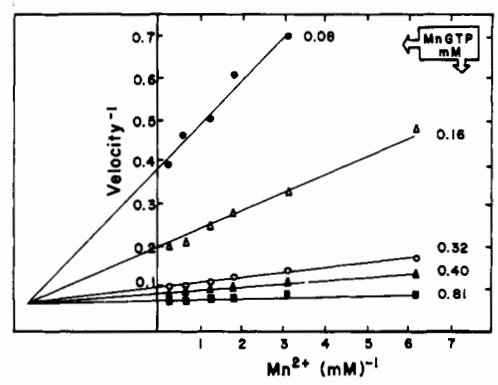


FIGURE 3: Reciprocal initial velocity as a function of reciprocal free Mn^{2+} concentration at fixed concentrations of MnGTP. Assays were as described in the Methods, with varying levels of Mn^{2+} and MnGTP as indicated in the figure. Velocities are expressed as the nmol of cyclic GMP formed 12 min^{-1} 80 μg of $protein^{-1}$.

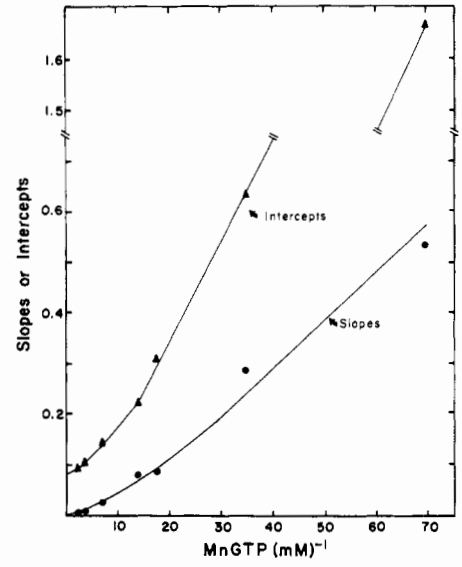


FIGURE 4: Replot of slopes and intercepts. Data given in Figure 1 were replotted as $[Mn]^{-1}$ against $velocity^{-1}$ and those slopes and intercepts were replotted as shown here.

Two possible mechanisms that can describe the initial rate data will be considered. The first is basically an equilibrium-ordered mechanism where the enzyme interacts with Mn^{2+} to form an active complex which then interacts with multiple MnGTP molecules allowing formation of product at a controlled rate. These interactions are shown in Scheme I, where E

SCHEME I

$$E + Mn^{2+} \overset{K_{Mn^{2+}}}{\rightleftharpoons} E-Mn^{2+}$$

$$E-Mn^{2+} + MnGTP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E-Mn^{2+}-MnGTP$$

$$E-Mn^{2+}-MnGTP + MnGTP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E-Mn^{2+}-MnGTP_2$$

$$E-Mn^{2+}-MnGTP_2 \xrightarrow{k_5} E-Mn^{2+}-MnGTP + \text{products}$$

= free enzyme. The binding of MnGTP in the above mechanism could also occur in a random order; in either case, the regulatory and catalytic binding sites would not be readily distinguishable.

The other possibility would involve random equilibrium binding of Mn^{2+} and one MnGTP with binding of an additional MnGTP to the ternary complex. This is illustrated in

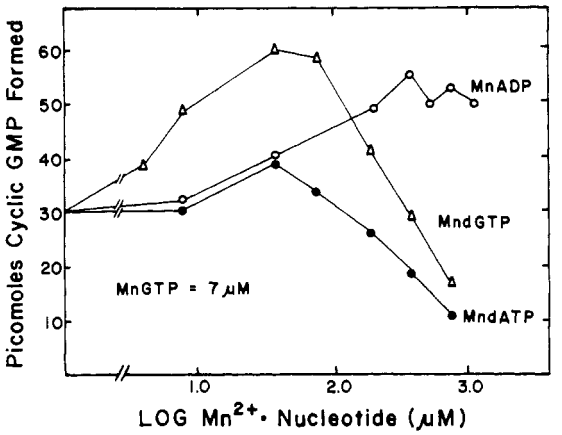
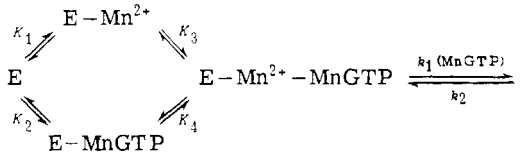


FIGURE 5: Activation of sea urchin sperm guanylate cyclase by MndGTP, MndATP, or MnADP. Assays were as described in the Methods with MnGTP and free Mn^{2+} concentrations equal to 7 μM and 1.6 mM, respectively. The ordinate represents the pmol of cyclic GMP formed 12 min^{-1} 80 μg of $protein^{-1}$.

Scheme II. It is assumed that the binding of Mn^{2+} and the first

SCHEME II



$$E\text{-}Mn^{2+}\text{-}MnGTP_2 \xrightarrow{k_3} E\text{-}Mn^{2+}\text{-}MnGTP + \text{products}$$

MnGTP is rapid relative to product formation. However, the mechanism will be a valid representation of the initial rate data even if substrate binding or product release is rate limiting to some extent (Rudolph and Fromm, 1971).

The same initial rate equation will describe either kinetic mechanism, and is presented below in the ϕ form suggested by Dalziel (1957). This equation is essentially identical with that derived by Fromm (1967) for a three substrate enzyme system which this system may be treated as if two GTPs and one Mn^{2+} bind to the enzyme.

$$E_0/V = \phi_0 + \frac{\phi_{[MnGTP]}}{[MnGTP]} + \frac{\phi_{[MnGTP]^2}}{[MnGTP]^2} + \frac{\phi_{[Mn^{2+}][MnGTP]}}{[Mn^{2+}][MnGTP]} + \frac{\phi_{[Mn^{2+}][MnGTP]^2}}{[Mn^{2+}][MnGTP]^2}$$

The ϕ's represent complex combinations of rate constants for either Scheme I or II. The important implications of the rate equation are the predictions of nonlinear double reciprocal plots with respect to MnGTP, linear plots with respect to Mn^{2+}, and an apparent Michaelis constant for Mn^{2+} of zero.

Activation Studies. The effect of various nucleotides on the formation of cyclic GMP when MnGTP concentrations were low (7 μM) and Mn^{2+} concentrations were high (1.6 mM) is shown in Figure 5. MnADP, MndATP, and MndGTP all served as activators of the reaction at low concentrations. At about 60 μM, however, MndATP and MndGTP began to inhibit the reaction, whereas MnADP continued to activate up to about 200 μM. The response to MnATP was similar to that seen with MndATP (Figure 6a). In the presence of 1 mM MnADP, neither MnATP nor MndGTP activated the enzyme (Figure 6a,b).

Although these data are compatible with either Scheme I or II, they suggest that nucleotides bind to a regulatory site before binding to the catalytic site. The activation of guanylate cyclase at low MnGTP concentrations by MndATP, MnATP, MnADP, or MndGTP could under these conditions be due to the binding of these nucleotides to a regulatory site. As the concentrations of MndATP, MnATP, or MndGTP increased, inhibition of enzyme activity was noted; this inhibition could be explained if at the higher concentrations these nucleotides competed with MnGTP for binding at the catalytic site. Since inhibition by MnADP was not observed, it may not effectively compete with MnGTP for binding to the catalytic site under conditions where MnGTP levels are low.

Inhibition Studies. As shown in Figure 7, when MnGTP levels were high, MnATP and MnADP inhibition data were nonlinear and partial when plotted according to Dixon (1953). These nonlinear types of inhibition have also been observed when using MnITP (data not shown). These types of inhibition patterns were observed for the adenine nucleotides at 30° or at 37° and at pH 7.0 or at pH 7.8.

The effects of nucleotide inhibitors were also evaluated using reciprocal plots. MnATP inhibition did not appear to be strictly competitive relative to MnGTP, since, as shown in Figure 8a, a small intercept effect was observed. Therefore, a third nucleotide site specific for adenine nucleotides could exist. Since the lines seem to converge at higher MnATP levels, the effect of MnATP may be on the equilibrium interactions. In other words, MnATP could bind to the enzyme at a regulatory site to form an enzyme-MnATP complex, which although active

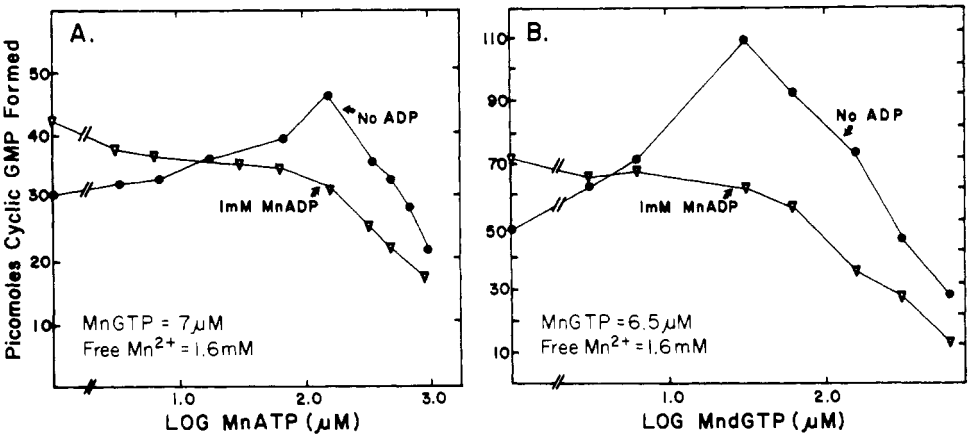


FIGURE 6: The effect of 1 mM MnADP on the activation of sea urchin sperm guanylate cyclase by MnATP or MndGTP. Assays were as described in the Methods with the MnGTP, free Mn^{2+}, MnATP, and MndGTP concentrations fixed as shown in the figure. The 12-min incubations were at 29° in A and at 30° in B, with all reactions containing 80 μg of protein.

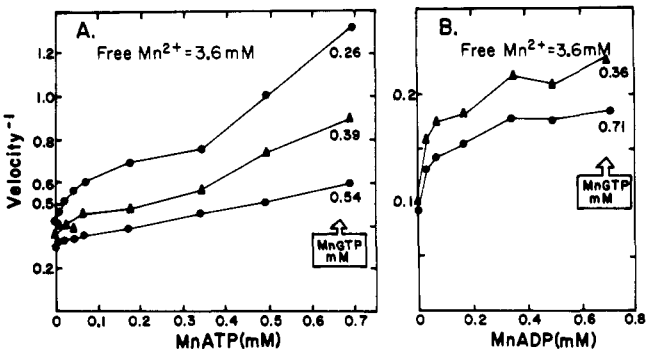


FIGURE 7: MnATP and MnADP inhibition patterns on Dixon plots. (A) Incubations were at pH 7.0 for 15 min at 30°, with the concentrations of free Mn^{2+}, MnGTP, and MnATP indicated in the figure. The velocities represent the nmol of cyclic GMP formed 15 min^{-1} 80 μg of $protein^{-1}$. (B) Assays were as described in A, except that incubations were at pH 7.8 and at 37°. The velocity represents the nmol of cyclic GMP formed 5 min^{-1} 80 μg of $protein^{-1}$.

would have a lower V_{max} than the enzyme-MnGTP complex. Further support for such a mechanism is the apparent linearity observed in the Lineweaver-Burk plots at high MnATP concentrations. This linearity suggests that the regulatory site is saturated and that the dependence of velocity on MnGTP is becoming a 1/1 function.

The effect of MnATP with regard to free Mn^{2+} is unusual. MnATP initially appears to be an uncompetitive inhibitor relative to Mn^{2+} (Figure 9a), but with increasing levels of MnATP, the reaction seems to become independent of the Mn^{2+} concentration. The initial uncompetitive inhibition is consistent with either proposed mechanism, if MnATP initially binds principally to the catalytic site. However, if MnATP binds to both the regulatory and catalytic sites, the inhibition is most consistent with Scheme I, unless it is assumed that MnATP is as effective an activator as MnGTP when it binds to the regulatory site. The declining dependence on free Mn^{2+} concentrations at high levels of MnATP can be attributed to an effective "locking" of Mn^{2+} on its site by high nucleotide levels. The equilibrium system can be shifted toward the higher order complexes, thus increasing the effective level of the enzyme-Mn^{2+} complex and allowing apparent independence on Mn^{2+} levels. This could occur with either Scheme I or II, since under these conditions the most common enzyme complex is likely to be E-Mn^{2+}-MnNTP. This effect may also be merely a consequence of the random binding interactions or higher order metal-nucleotide complexes.

Support for formation of a new activated complex also comes from MnADP inhibition studies. The inhibition by MnADP relative to MnATP is shown in Figure 8b. Since from activation studies and from the data shown in Figure 7b, MnADP appears to bind principally to the regulatory site, it is likely that MnADP causes a shift to another activated species (enzyme-MnADP) which has a lower V_{max} than the enzyme-MnGTP complex. Apparent linear patterns at high MnADP levels and a slight intercept effect (Figure 8b) are consistent with the MnATP data.

Previous work has indicated that MndGTP may serve as an alternate substrate for sea urchin sperm guanylate cyclase (Garbers *et al.*, 1974b), and the inhibition patterns shown in Figure 8c are strictly competitive, further suggesting that MndGTP binds to the same sites as does MnGTP. The inhibition patterns relative to Mn^{2+} were like those seen with MnATP (Figure 9b). The patterns were initially uncompetitive and dependence on free Mn^{2+} for enzyme activity seemed to disappear as MndGTP concentrations increased. MndGTP may be as effective an activator of sea urchin sperm guanylate cyclase as MnGTP, since linear reciprocal plots as a function of MnGTP were observed at relatively low MndGTP concentrations.

Discussion

Mn^{2+} and MnGTP may bind to sea urchin sperm guanylate cyclase by either Scheme I or II as described in the Results. Scheme I is the classical equilibrium-ordered model normally used to explain a slope replot passing through zero (Cleland, 1970). However, Scheme II will also describe the observed kinetic data in this case. The difference between the two mechanisms is the binding of MnGTP to the free enzyme in the case

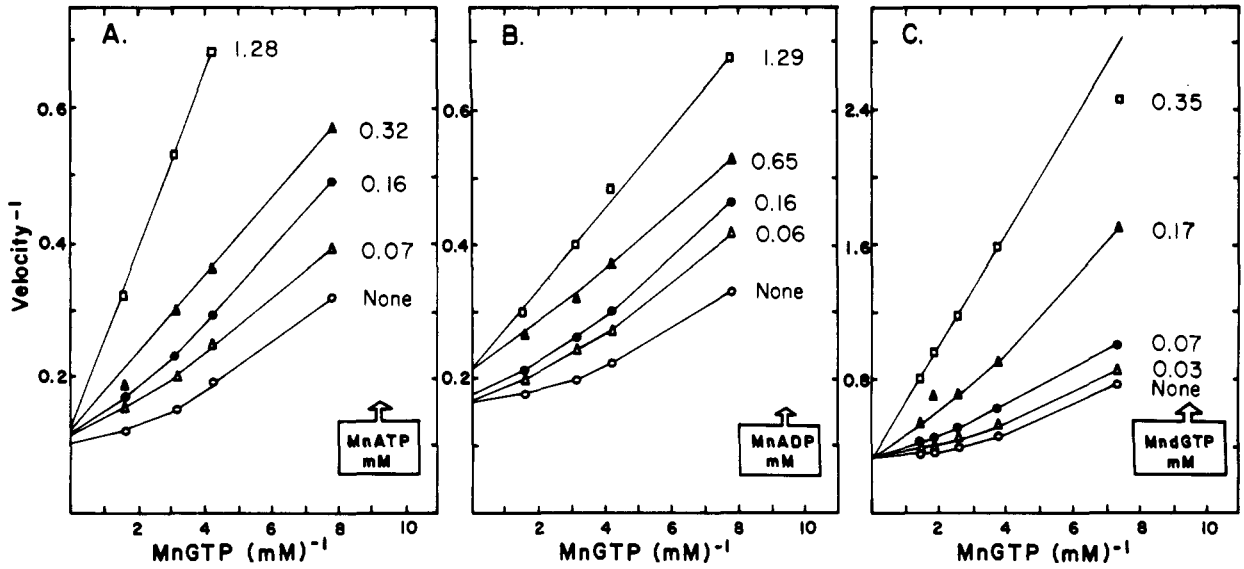


FIGURE 8: Reciprocal initial velocity as a function of reciprocal MnGTP concentration at fixed concentrations of MnATP, MnADP, or MndGTP. (A and B) Assay conditions were as described in the Methods, except that incubations were at 37°. The free Mn^{2+} concentration was 6.5 mM, and the velocity represents the nmol of cyclic GMP formed 5 min^{-1} 80 μg of $protein^{-1}$. (C) Incubations were at 30° for 20 min in the presence of 3.4 mM free Mn^{2+}. The velocities represent the nmol of cyclic GMP formed 20 min^{-1} 80 μg of $protein^{-1}$.

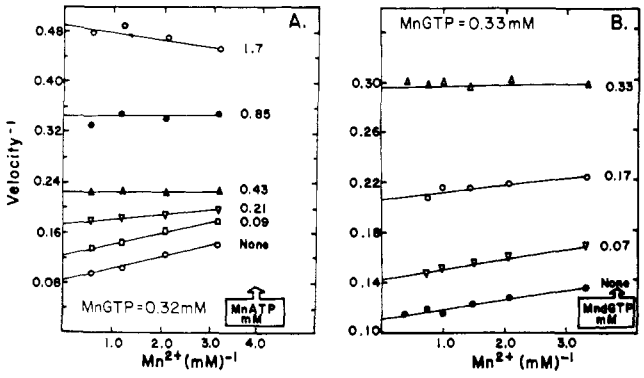


FIGURE 9: Reciprocal initial velocities as a function of reciprocal Mn^{2+} concentrations at fixed concentrations of MnATP(A) or MndGTP(B). (A) Assay mixtures were incubated in the presence of 0.32 mM MnGTP and the concentrations of free Mn^{2+} or MnATP indicated in the figure. Velocities are expressed as the nmol of cyclic GMP formed 5 min^{-1} 80 μg of $protein^{-1}$ at 37° and at pH 7.8. (B) Assay mixtures were incubated in the presence of 0.33 mM MnGTP and the concentrations of free Mn^{2+} or MndGTP indicated in the figure. Velocities represent the nmol of cyclic GMP formed 10 min^{-1} 80 μg of $protein^{-1}$ at 30° and at pH 7.8.

of Scheme II. Binding studies with purified enzyme will allow distinction between the two proposed mechanisms.

Binding studies have been indirectly accomplished in another paper (Garbers and Hardman, 1974) by measuring protection of enzyme activity against heat and *N*-ethylmaleimide denaturation. Although excess Mn^{2+} or excess Ca^{2+} over GTP was required for maximal protection against thermal inactivation, excess Mn^{2+} did not seem to be required for protection by MnATP or MnADP. In experiments involving protection against *N*-ethylmaleimide, CaGTP also seemed capable of protecting the enzyme in the absence of free Ca^{2+}. The binding of MnATP, MnADP, or CaGTP in the absence of free metal would be consistent with Scheme II.

In interpreting these data we have not considered the existence of nucleotide binding sites other than the proposed regulatory and catalytic sites. The slight intercept effects observed with inhibition by MnATP and MnADP may indicate other nucleotide binding sites. Furthermore, in contrast to the findings with MnATP or MnADP as protectors of enzyme activity, excess Mn^{2+} seemed to be required for maximum protection by MnGTP against denaturation (Garbers and Hardman, 1974). It should be emphasized that complex subunit interactions could give rise to the kinetic results observed but the simple mechanistic model described is sufficient to explain the data. Total resolution of this question will require detailed physical and mechanistic studies with purified guanylate cyclase.

The positive homotropic effects of MnGTP indicating multiple nucleotide binding sites on guanylate cyclase suggest a strong regulatory potential (Koshland, 1970). Particulate guanylate cyclase activity from rat lung also displays positive cooperative behavior with respect to MnGTP whereas the soluble enzyme shows classical hyperbolic behavior (Chrisman *et al.*, 1974). As observed with sea urchin sperm particulate enzyme, the Michaelis constant for Mn^{2+} approaches zero at high MnGTP levels with the particulate guanylate cyclase from rat lung (Chrisman *et al.*, 1974). It appears, therefore, that the gross cellular fractionation of soluble and particulate guanylate cyclase activities results in the separation of enzymes with different kinetic characteristics.[2]

A comparison of sea urchin sperm guanylate cyclase with adenylate cyclases from various tissues shows a number of similarities, and it is possible that basic enzyme mechanisms are similar for both cyclases. A free metal site on adenylate cyclases from various tissue sources has been postulated (Birnbaumer *et al.*, 1969; Drummond and Duncan, 1970; Drummond *et al.*, 1971; Flawia and Torres, 1972; Severson *et al.*, 1972), and the studies described here, as well as the binding studies in another paper (Garbers and Hardman, 1974), have confirmed the existence of a free metal site on sea urchin sperm guanylate cyclase. Nucleotides other than ATP have been shown to influence adenylate cyclase activity, and regulatory effects of nucleotides such as GTP (Rodbell *et al.*, 1971; Bockaert *et al.*, 1972; Krishna *et al.*, 1972; Swislocki *et al.*, 1973), AMP (Rossomando and Sussman, 1973), and other purines (Rodbell *et al.*, 1971; Wolff and Cook, 1973) have been suggested. Sea urchin sperm guanylate cyclase seems to have at least two nucleotide binding sites and ATP, dATP, ADP, or dGTP may stimulate guanylate cyclase activity at low GTP concentrations, presumably by binding to one of these sites.

Whether and how guanylate cyclase activity is regulated within the cell remains unanswered, but the kinetic mechanisms proposed in this report provide a basis for the evaluation of control mechanisms.

Acknowledgments

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Equilibrium Analysis of L-Phe-tRNA^{Phe} Complexes with L-Phenylalanyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* K 10†

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ABSTRACT: The interaction of tRNA^{Phe} and Phe-tRNA^{Phe} with L-Phe-tRNA synthetase has been investigated by equilibrium techniques. Dissociation constants were in the range of 1×10^{-7} M at pH 7.5. A 1:1 stoichiometry was demonstrated for tRNA^{Phe} by the equilibrium gel filtration method. Phe-tRNA^{Phe} was shown to be bound to the synthetase predominantly in a configuration wherein the L-phenylalanyl moiety is not in contact with the amino acid binding site. Using 2-*p*-toluidinylnaphthalene-6-sulfonate as a fluorescent reporter group, distinct steps of the synthetase-catalyzed reversal of the tRNA-aminoacylation could be followed as could the formation of the enzyme-bound adenylate and its breakdown to ATP and L-phenylalanine.

A mechanism of the catalytic action of aminoacyl-tRNA synthetases has been proposed recently for the L-isoleucine enzyme of *Escherichia coli* (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Schimmel, 1973). Accordingly, the reaction can be separated into: (1) activation of the amino acid by formation of aminoacyl adenylate from ATP and amino acid; and (2) transfer of the aminoacyl moiety to cognate tRNA. Based on measurements under conditions of pH and temperature quite different from those required for optimal activity, it was concluded that overall reaction is controlled by the rate of dissociation of the aminoacyl-tRNA–enzyme complex. Evidence that this may not be valid under other conditions and for other enzymes emerged recently from fast kinetic measurements (Pingoud *et al.*, 1973) and from aminoacylation kinetics substituting ATP by thio analogs (E. Holler and W. Eckstein, unpublished observations). Even though it has been shown that aminoacyl-tRNA and amino acid can bind simultaneously to the enzyme (Yarus and Berg, 1969; Eldred and Schimmel, 1973; Hélène *et al.*, 1971; Charlier, 1972), the existence of only one type of aminoacyl-tRNA complex has been presumed in the quantitative treatment of the aminoacyl transfer step.

The aim of the present publication is to provide new evidence for the existence of two types of complexes and to present a sensitive fluorimetric method for the investigation of the transfer reaction by rapid kinetic techniques.

Materials and Methods

Preparation of Enzyme and tRNA. L-Phe-tRNA synthetase (specific activity 53,600 nmol mg^{-1} hr^{-1}) was prepared from *Escherichia coli* K IO in the presence of phenylmethanesulfon-

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