Seryl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* B. Enzyme–Substrate Interactions†

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ABSTRACT: Kinetic analysis of the [^{32}P]PP_i–ATP exchange reaction shows that the seryl-tRNA synthetase of *Escherichia coli* B displays ordered substrate addition, ATP first. ATP is active only as the monomagnesium salt. At high concentrations of magnesium, exchange is inhibited by dead-end complex formation between the ternary complex, E·ATP·Ser, and either free Mg^{2+} or $Mg_2P_2O_7$. ATP binding was measured by equilibrium dialysis; K_D is 0.02 mM and the stoichiometry is 0.93 mol/53,000 g. Serine is not bound in the absence of ATP ($K_D > 0.6$ mM). The formation of the ternary complexes E·ATP·Ser and E·Ser~AMP was measured by gel filtration under steady-state conditions; this stoichiometry, necessarily a measure of the number of active sites in the exchange reaction, is 0.97 mol/53,000 g.

The seryl-tRNA synthetase of *Escherichia coli* B is a dimer (mol wt 103,000) whose subunits (mol wt 53,000) are identical with respect to size and charge (Boeker *et al.*, 1973). The stoichiometry of the tRNA synthetase complex observed by Knowles *et al.* (1970) for the enzyme from *E. coli* K12 is 0.8 mol of tRNA/mol of dimer (mol wt 100,000) and the stoichiometry of the seryl adenylate synthetase complex observed by Bluestein *et al.* (1968) for the enzyme from yeast is 1 mol/89,000 g. These results suggest that seryl-tRNA synthetase has a single active site per dimer, a result difficult to reconcile with the finding that the subunits are apparently identical. We have therefore determined the stoichiometry of ATP binding by equilibrium dialysis and have reexamined the binding of seryl adenylate under steady-state conditions by means of a new technique. Our results show that there are in fact two active sites per dimer of *E. coli* B enzyme. Since the previous measurements were not made under equilibrium or steady-state conditions, it is likely that the stoichiometries obtained were underestimates.

To further characterize the enzyme–substrate interactions of this system, we have also analyzed the [^{32}P]PP_i–ATP exchange reaction kinetically. These experiments show that the preferred order of substrate addition is ATP (as the monomagnesium salt) first and serine second. This result is consistent with our failure to observe serine binding to the enzyme in the absence of ATP and Mg^{2+}.

Materials and Methods

Materials. Seryl-tRNA synthetase was purified from *E. coli* B according to Boeker *et al.* (1973); preparations used had a specific activity greater than 8 μmol/min per mg. Activated charcoal was treated with 6 N HCl, washed with distilled H_2O, and stored as a 10% suspension. ATP (disodium salt) was obtained from Sigma, L-serine from Mann, inorganic pyrophosphatase from Worthington, [8-^{14}C]ATP and [U-^{14}C]serine from Schwarz, and [^{32}P]PP_i from New England Nuclear. Gelatin was dialyzed exhaustively at 40° against distilled H_2O to remove traces of serine which otherwise interfered with velocity measurements at low serine concentrations. ADP (Sigma) was chromatographed according to Cohn (1957) to free it of ATP.

Exchange Reaction. The technique of Calendar and Berg (1966) was used to measure [^{32}P]PP_i–ATP exchange. The enzyme was diluted in 0.5 mg/ml of gelatin and 0.05 M Tris-Cl, pH 7.4 (37°); 25 μl was added to 0.975 ml of the same buffer containing 2.0 mM ATP, 1.0 mM [^{32}P]PP_i, 3.5 mM $MgCl_2$, and 0.4 mM L-serine. Aliquots (150 μl) were removed at appropriate intervals and added to 0.2 ml of 15% Cl_3CCOOH in 0.4 M $Na_4P_2O_7$. A 10% suspension (0.1–0.2 ml) of activated charcoal was added; the amount was determined empirically for each batch in order to obtain quantitative adsorption without quenching the scintillant. After 5 min, the suspension was rinsed onto Whatman No. 3 filter paper, washed five times with 5-ml portions of distilled water, and counted in 10 ml of scintillant (32 g of Beckman Fluoralloy TLA, 500 ml of Beckman Bio-Solv BBS-3, and 3 l. of toluene). Quench corrections were not necessary when the amount of charcoal was properly adjusted. Less than 15% of the limiting substrate was consumed during each measurement.

Equilibrium Dialysis. A 2-cm square piece of size 20 dialysis tubing was clamped between two circular chambers (1 cm diameter × 3 mm deep) cut in Lucite blocks. The chambers were filled through a 1 mm diameter channel, drilled radially from the outside of the block, with 100 μl of a solution containing 0.05 M Tris-Cl, pH 7.2, 0.01 M 2-mercaptoethanol, the appropriate ligand, and, on one side, seryl-tRNA synthetase. Cells were allowed to equilibrate for 14 hr at 6° with gentle rocking.

Steady-State Gel Filtration. A 0.5 × 7 cm column of Sephadex G-25 fine was poured in a Pasteur pipet plugged with glass wool. Two lengths of polyethylene tubing were sealed into the top with General Electric RTV-102; one was connected to a 20-ml Mariotte flask and the other used as a vent. The column was washed with 10 column volumes (14 ml) of 0.1 M Tris-Cl, pH 7.4, and then with 3 volumes of the eluent solution (containing 1.0 M Tris-Cl, pH 7.4, 2–40 μM [U-^{14}C]-serine (50 μCi/μmol), 0.15 mM ATP, 0.15 mM $MgCl_2$, 0.1 M 2-mercaptoethanol, and 4 μg/ml of inorganic pyrophosphatase). Seryl-tRNA synthetase (50 μl at 0.44–3.0 mg/ml) was added and elution carried out with the eluent solution. Fifty fractions of 50 μl were collected at 0.1 ml/min and

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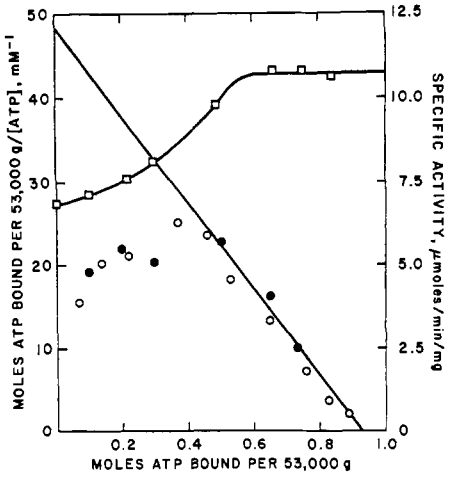

FIGURE 1: Equilibrium dialysis of ATP and seryl-tRNA synthetase. The free ATP concentration varied from 4.7 μM to 0.41 mM; the $MgCl_2$ concentration was 0.5 mM (○) or 10 mM (●) greater than the ATP concentration. Enzyme activity (□) was measured by tRNA acylation at maximum velocity (Boeker *et al.*, 1973); further details are in Materials and Methods.

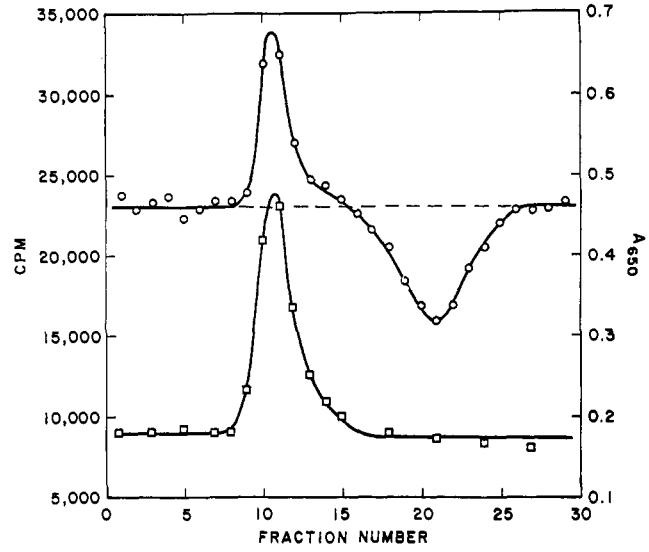

FIGURE 2: Binding of serine to seryl-tRNA synthetase in the presence of ATP. Experimental details are given in Materials and Methods. In this column only, a 75-μl sample was applied and 75-μl fractions were collected; 5-μl aliquots were counted in triplicate (○) and protein concentrations (□) were determined on duplicate 20-μl aliquots according to Lowry *et al.* (1951). Protein determinations were omitted in subsequent columns and the separation was improved by reducing both the sample and fraction volumes to 50 μl.

counted in 10 ml of the scintillant described above. The base line, obtained by averaging the counts per minute per fraction across the entire column, was used to calculate the free serine concentration. The amount of bound serine was obtained from the base line and the sum of the counts per minute per fraction across the peak. The amount of enzyme applied was taken as the total enzyme.

Results

ATP and Serine Binding. The binding of ATP to seryl-tRNA synthetase can be easily measured by equilibrium dialysis. Interpretation of the data is complicated by the fact that glycerol, which is generally present to stabilize the enzyme, is removed prior to the dialysis experiments in order to decrease the viscosity of the solution and thereby speed equilibration. The specific activity of the enzyme at the start of the experiment is decreased from 10 to 7 μmol/min per mg, and this inactivation is reversed by ATP binding (see Figure 1). Binding data for ATP expressed in the form of a Scatchard plot fit a straight line only at free ATP concentrations higher than 0.015 mM; at these levels the inactivation due to removal of glycerol is largely reversed. The deviations at lower ATP concentrations can be approximately accounted for by assuming that the inactive enzyme does not bind ATP.

From Figure 1, the ATP dissociation constant is 0.02 mM or less and the stoichiometry is 0.93 mol/53,000 g; binding is not affected by excess $MgCl_2$, as it would be if the enzyme bound two Mg^{2+}–ATP^{4-} species with different association constants or if an E–MgATP species bound additional Mg^{2+}. However, no binding was detected in the absence of $MgCl_2$; the concentrations of ATP and enzyme were such that a dissociation constant of 0.6 mM or less would have produced a 5% or greater difference between the dialysis chambers. Thus, ATP binds to seryl-tRNA synthetase only as a magnesium complex, presumably $MgATP^{2-}$.

Attempts to measure serine binding by equilibrium dialysis in the absence of ATP were unsuccessful. The results of experiments carried out in 0.05 M Tris, pH 7.4, and 0.01 M 2-mercaptoethanol were not affected by the addition of 10 mM $MgCl_2$, $MgCl_2$ and adenosine, $MgCl_2$ and AMP, or $MgCl_2$ and ADP. The concentrations of serine and seryl-tRNA synthetase were such that a dissociation constant of 0.6 mM or less would have produced a 5% or more difference between the two dialysis chambers.

Steady-State Measurement of Seryl Adenylate Binding. Aminoacyl adenylate–enzyme complexes can easily be isolated by gel filtration (Norris and Berg, 1964; Allende *et al.*, 1964). However, since these complexes are in rapid equilibrium with the free enzyme the stoichiometry obtained in such a non-equilibrium experiment will necessarily be a minimum value. Ordinary equilibrium binding techniques cannot be applied to this system since a chemical reaction occurs. We have therefore devised a gel filtration binding technique, based on that of Hummel and Dreyer (1962), for use under steady-state conditions. As will be shown later, the simplest reaction sequence for formation of the seryl adenylate–enzyme complex is

$$E \underset{k_2}{\overset{k_1(MgATP)}{\rightleftharpoons}} E\cdot ATP \underset{k_4}{\overset{k_3(Ser)}{\rightleftharpoons}} E\cdot ATP\cdot Ser \underset{k_6(PPi)}{\overset{k_5}{\rightleftharpoons}}$$

$$E\cdot Ser\sim AMP \underset{k_8(Ser\sim AMP)}{\overset{k_7}{\rightleftharpoons}} E \quad (1)$$

During gel filtration experiments, complex formation will be in the steady state if the concentrations of ATP, serine, PP_i, and seryl adenylate are constant. The first three conditions will be satisfied if the column is equilibrated and eluted with constant concentrations of ATP, serine, and an excess of pyrophosphatase, and if the enzyme-bound ligands are separated from the resulting area of lower concentrations. Since seryl adenylate is removed both by diffusion into the Sephadex matrix and by spontaneous hydrolysis, its concentration is assumed to be negligible; under these conditions, both $k_6(PP_i)$ and $k_8(Ser\sim AMP)$ can be neglected.

The results of a typical steady-state experiment are shown in Figure 2. Since serine is labeled the peak of enzyme-bound radioactivity is a measure of both the E·ATP·Ser and E·

TABLE I[a]

Substrate Addition	Denominator of Rate Equation
Amino acid first	$1 + \frac{K_a}{[A]} + \frac{K_p}{[P]} + \frac{K_{ia}K_m}{[A\cdot M]}$
ATP first	$1 + \frac{K_a}{[A]} + \frac{K_m}{[M]} + \frac{K_p}{[P]} + \frac{K_{ia}K_m}{[A\cdot M]} + \frac{K_a[M]}{K_{ia}K_{im}} + \frac{K_a[M]K_p}{K_{ia}K_{im}[P]}$
Rapid equilibrium random	$1 + \frac{K_a}{[A]} + \frac{K_m}{[M]} + \frac{K_p}{[P]} + \frac{K_{ia}K_m}{[A\cdot M]}$

[a] Equations are given in the form $v = V_m$/denominator; A = ATP, M = amino acid, P = PP_i; the catalytic constants, K_a, K_{ia}, etc., are defined following Cleland (1963a).

Ser~AMP complexes. From the partition equations for each enzyme species (King and Altman, 1956)

$$\frac{[E\cdot ATP\cdot Ser] + [E\cdot AMP\sim Ser]}{[E] + [E\cdot ATP]} = \frac{k_1k_3k_7[ATP][Ser] + k_1k_3k_8[ATP][Ser]}{\{k_2k_4k_7 + k_2k_5k_7 + k_3k_5k_7[Ser] + k_1k_4k_7[ATP] + k_1k_5k_7[ATP]\}} \quad (2)$$

Rearranging and defining the appropriate constants

$$\frac{[E\cdot ATP\cdot Ser] + [E\cdot AMP\sim Ser]}{[E] + [E\cdot ATP]} = \frac{1}{\frac{K_a}{[ATP]} + \frac{K_s}{[Ser]} + \frac{K_{ia}K_s}{[ATP][Ser]}} \quad (3)$$

By analogy to simple ligand binding, n is defined as the number of active sites and ν as the moles of serine bound per mole of enzyme. If the sites are independent

$$\frac{[E\cdot ATP\cdot Ser] + [E\cdot Ser\sim AMP]}{[E] + [E\cdot ATP]} = \frac{\nu/n}{1 - (\nu/n)} = \frac{\nu}{n - \nu} \quad (4)$$

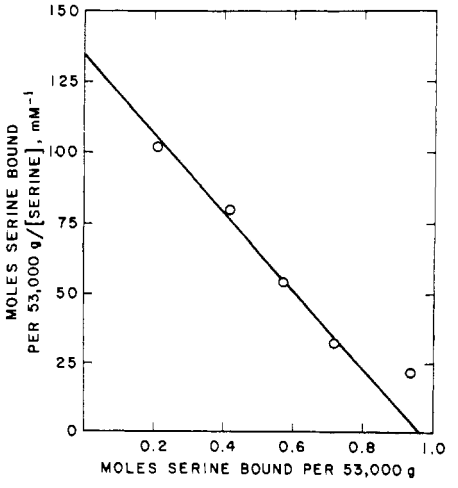

FIGURE 3: Concentration dependence of serine binding in the presence of ATP. The free serine concentration varied from 2.0 to 41 μM. Both the ATP and $MgCl_2$ concentrations were 0.15 mM; other details are given in Materials and Methods. The points at the two lowest serine concentrations are averages.

Substituting in eq 2 and solving for ν

$$\nu = \frac{n}{1 + \frac{K_a}{[ATP]} + \frac{K_s}{[Ser]} + \frac{K_{ia}K_s}{[ATP][Ser]}} \quad (5)$$

This result has the same form regardless of the number of ternary complexes in the mechanism. If the reaction sequence is ordered, amino acid first, a similar result can be obtained if ATP is labeled. However, if both substrates bind to the free enzyme (random addition) a term representing [E·Ser] must be added to the numerator of eq 2; the resulting equation cannot be rationalized. In general, the result in eq 5 is valid if substrate addition is ordered and the second substrate is labeled.

The binding data at a series of serine concentrations are presented in the form of a Scatchard plot in Figure 3. This presentation of the data is justified by rearranging eq 5 to

$$\nu = \frac{n}{1 + (K_a/[ATP])} - \frac{K_s[1 + (K_{ia}/[ATP])]}{1 + (K_a/[ATP])}\frac{\nu}{[Ser]} \quad (6)$$

The apparent value of the serine binding constant, K_s, is 7 μM and there is 0.97 mol of serine bound/subunit. No binding was observed in the absence of $MgCl_2$.

In theory, a similar experiment can be performed in which the ATP concentration is varied and a value for K_a obtained; n should remain the same. In practice, since serine must still be the labeled substrate and must also be present in large excess, this experiment requires large quantities of enzyme and has not been attempted; ATP binding has therefore been measured only by equilibrium dialysis as described above.

Substrate Addition Mechanism. A simple test of the ATP–amino acid addition mechanism of tRNA synthetases can be devised from consideration of the exchange reaction. Exchange rate equations, which have previously been presented in forms not immediately useful for ordinary kinetic analysis (Cedar and Schwarz, 1969; Cole and Schimmel, 1970a), are summarized for convenience in Table I. They differ completely in their dependence on the amino acid. If the addition mechanism is ordered, amino acid first, the only term in the rate equation which contains 1/[AA] is a cross term; the apparent K_{AA} will be unusually low and inversely proportional to the ATP concentration. For the ordered ATP first case the denominator of the rate equation contains terms in [AA]/K_{AA}' as well as K_{AA}/[AA]; the amino acid will be inhibitory at certain concentrations. If the compound kinetic constants involved differ sufficiently, plots of $1/v$ *vs.* 1/[AA] will be linear

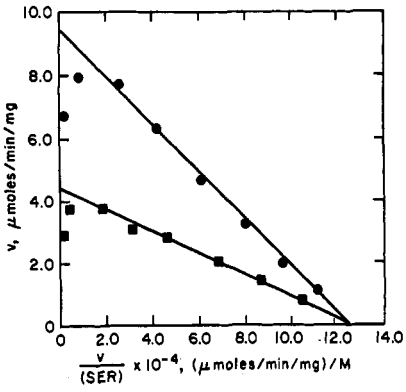

FIGURE 4: Initial rate of [^{32}P]PP_i exchange as a function of serine concentration under standard conditions. The range shown here is *ca.* 0.01–4.0 mM serine; higher concentrations are shown in Figure 5. The PP_i and ATP concentrations were 1.0 and 2.0 mM, respectively; the $MgCl_2$ concentrations were 3.5 mM (●, 0.5 mM excess) and 6.0 mM (■, 3.0 mM excess).

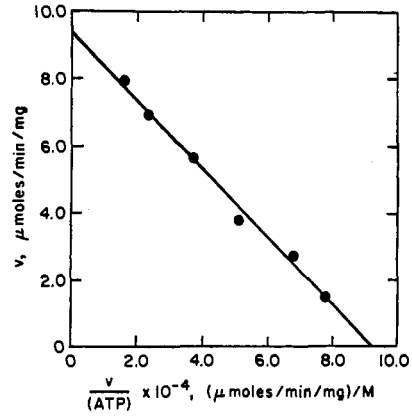

FIGURE 6: Initial rate of [^{32}P]PP_i exchange as a function of ATP concentration under standard conditions. The serine and PP_i concentrations were 0.4 and 1.0 mM, respectively; the concentration of $MgCl_2$ was 0.5 mM greater than the sum of the ATP and PP_i concentrations.

at low [AA] and plots *vs.* [AA] will be linear at high [AA]; alternately, there will be no rational plot. If ATP and serine add randomly and equilibrate with the E·AA·ATP complex, the rate equation shows neither effect; the apparent K_{AA} should behave normally under all conditions. If the reaction is random but the rapid equilibrium assumption not justified, the kinetics will not be hyperbolic in any case.

The results of measurements of the rate of pyrophosphate exchange as a function of serine, ATP, and PP_i concentrations are shown in Figures 4–7. Serine concentrations above 2 mM markedly inhibit exchange (Figure 4); the inhibition is linear when plotted appropriately (Figure 5). The serine concentration used in subsequent experiments is necessarily a compromise; the kinetic constants are approximations only. Neither ATP (Figure 6) nor PP_i (Figure 7) shows any deviation from hyperbolic behavior. The results are consistent with ordered addition, ATP first.

An alternate possibility is a rapid equilibrium random mechanism in which serine also acts as a substrate inhibitor; the pattern of PP_i inhibition of tRNA acylation strongly suggests that this is not the case (Boeker *et al.*, 1973).

Mg^{2+} Dependence. Unlike the rate of tRNA acylation, which is constant from 5 to 15 mM $MgCl_2$, the [^{32}P]PP_i–ATP exchange reaction is severely inhibited by excess Mg^{2+} (Figure 8). The analysis of Cole and Schimmel (1970b) shows that, when the $MgCl_2$ concentration is less than 2.5 mM (the sum of ATP and PP_i), only $MgP_2O_7^{2-}$ and $MgATP^{2-}$ increase as $MgCl_2$ increases; they must, therefore, be the active species. As $MgCl_2$ is increased beyond 2.5 mM, significant quantities of $Mg_2P_2O_7$ and Mg^{2+} are formed. The observed inhibition could be caused by dead-end complex formation between an enzyme–substrate complex and either of these species or by the disappearance of $MgP_2O_7^{2-}$ as $Mg_2P_2O_7$ is formed. In the latter case, a higher concentration of $MgCl_2$ should change K_{app}, but not V_{app}, when PP_i is the variable substrate; since this does not occur (Figure 7), the dead-end complex mechanism is to be preferred.

Using the second equation of Table I, the inhibition patterns resulting from dead-end complex formation between Mg^{2+} or $Mg_2P_2O_7$ and any of the enzyme–substrate complexes (*cf.* eq 1, above), taken singly or in combination, can be calculated. The observed pattern (Figures 4, 5, and 7) is identical only with that predicted if the E·ATP·Ser species forms dead-end complexes. Mg^{2+} is the more likely candidate for the second reactant since it is present at higher concentrations and would be expected to form complexes more readily. K_I, the equilibrium constant for dead-end complex formation, can be

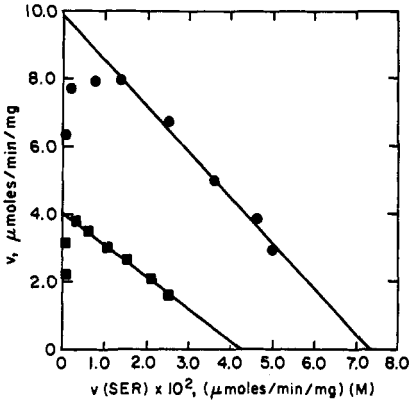

FIGURE 5: Initial rate of [^{32}P]PP_i exchange as a function of serine concentration. Conditions are the same as in Figure 4 except that the range of serine concentration is *ca.* 0.1–17 mM: (●) 3.5 mM $MgCl_2$ (0.5 mM excess); (■) 6.0 mM $MgCl_2$ (3.0 mM excess). Notice that this is not a standard Eadie plot; v decreases as the serine concentration increases.

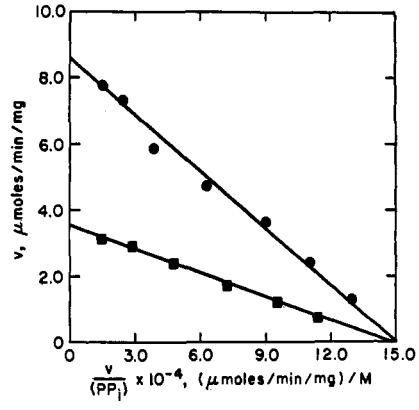

FIGURE 7: Initial rate of [^{32}P]PP_i exchange as a function of PP_i concentration under standard conditions. The serine and ATP concentrations were 0.4 and 2.0 mM, respectively; the concentrations of $MgCl_2$ were 0.5 mM (●) and 3.0 mM (■) greater than the sum of the ATP and PP_i concentrations.

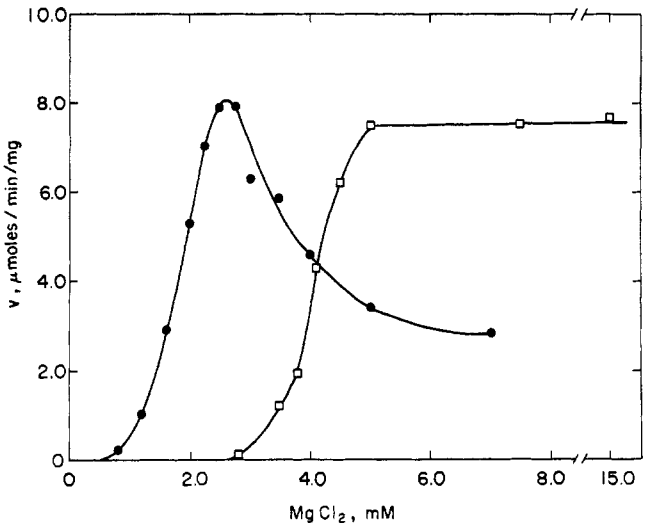

FIGURE 8: $MgCl_2$ dependence of [^{32}P]PP_i–ATP exchange (●) and tRNA acylation (□). The ATP concentration used in the acylation reaction (2.5 mM) is equal to the sum of the ATP (2.0 mM) and PP_i (0.5 mM) concentrations used for exchange. Other conditions are given in Boeker *et al.* (1973) and under Materials and Methods.

extracted from the numerator of the serine inhibition constant; assuming Mg^{2+} is the inhibitory species, K_I is *ca.* 2 mM.

Discussion

Amino acid dependent PP_i exchange was first demonstrated for tRNA synthesis in 1955–1956 (Hoagland, 1955; DeMoss and Novelli, 1955, 1956; Berg, 1955, 1956; Hoagland *et al.*, 1956). Although commonly used as an assay, it has been used only occasionally for investigating mechanisms (Rouget and Chapeville, 1968; Cedar and Schwarz, 1969; Cole and Schimmel, 1970a; Santi *et al.*, 1971); the simple test of substrate addition used here apparently has not been applied previously. Both this test and the binding studies show that the addition mechanism with respect to ATP and serine is ordered, ATP first; the association constant of the free enzyme for ATP is at least 30 times that for serine.

This conclusion is apparently consistent with that reached by Myers *et al.* (1971) on insufficient grounds for the *E. coli* K_{12} enzyme. These authors have attempted to distinguish the first two substrates of a bi-uni-uni-bi mechanism by varying each substrate at fixed levels of the other; while this procedure will distinguish the first two substrates from the third, it will not distinguish the first two from each other (Cleland, 1963b).

The *E. coli* K_{12} enzyme, however, does bind tRNA in the absence of the other substrates (Knowles *et al.*, 1970); it is possible therefore that these enzymes catalyze tRNA acylation by alternate reaction paths, one sequential, tRNA first, and the other ping pong, tRNA last.

The maximum velocity of [^{32}P]PP_i–ATP exchange, about 10 μmol/min per mg, is comparable to previously measured exchange rates (Calendar and Berg, 1966; Santi *et al.*, 1971) and slightly higher than that of tRNA acylation (Boeker *et al.*, 1973). At 0.4 mM serine and 0.5 mM excess $MgCl_2$, the apparent catalytic constants are, for ATP (K_a), 0.10 mM, for serine (K_m), 0.075 mM, for PP_i (K_p), 0.053 mM, and for serine inhibition ($K_{ia}K_{im}/K_a$), 8.1 mM. Barring isomerization, K_{ia} and K_{im} are the dissociation constants of the E·ATP and E·ATP·Ser complexes, respectively. K_{ia} is then 0.02 mM (Figure 1) and K_{im}, calculated from the serine inhibition constant, is 40 mM. This value suggests that at least one of the complexes, most likely E·ATP·Ser, must isomerize during the reaction.

Both the kinetics and the binding studies suggest that Mg–ATP^{2-} is the only ATP species bound by seryl-tRNA synthetase. This is, of course, a fully sufficient explanation for the Mg^{2+} dependence of acylation, exchange, seryl adenylate formation, and ATP binding. It has been shown in earlier studies from this laboratory (Bluestein *et al.*, 1968) that Mg^{2+} is not required at a later stage of the reaction sequence (tRNA acylation) since Mg^{2+} can be replaced by spermidine. Presumably, spermidine or divalent cations are required to stabilize the tRNA conformation. The inhibition of exchange is consistent with dead-end complex formation between Mg^{2+} and the species E·MgATP·Ser. It is apparent that this complex does not form during tRNA acylation (Figure 8); whether this is caused by the Mg^{2+} binding properties of tRNA or by a change in synthetase behavior when tRNA is present is unknown.

The gel filtration technique of Norris and Berg (1964) and Allende *et al.* (1964) has been used to isolate many aminoacyl adenylate–tRNA synthetase complexes. These complexes are unstable (DeMoss *et al.*, 1956; Berg, 1958); for example, the half-life of the yeast seryl adenylate–enzyme complex is 14 min at pH 7 and 30° (Bluestein *et al.*, 1968). Since the complex and the reactants are separated, this gel filtration technique cannot measure equilibrium or steady-state formation and the stoichiometry will necessarily be a minimum value. Nor can equilibrium dialysis be used; hydrolysis of the aminoacyl adenylate will, at true equilibrium, leave only AMP and free amino acid.

The steady-state binding technique used here is designed to demonstrate the true stoichiometry of binding and will do so if the ATP concentration is not limiting (eq 6). Although the values of K_{ATP} and K_{Ser} obtained for tRNA acylation, PP_i exchange, and complex formation are individually unrelated, examination of the microscopic rate constants shows that the ratio K_{ATP}/K_{Ser} should always be the same. This is essentially true for the acylation and exchange reactions; the values are 1.65 and 1.33, respectively. Thus, for complex formation, K_{ATP} must be on the order of 0.015 mM (twice K_{Ser}, see Figure 3) or 0.1 the concentration actually used.

The stoichiometry of both ATP and ATP·Ser plus Ser~AMP binding is 1 mol/subunit of mol wt 53,000, or two per seryl-tRNA synthetase dimer. The complex formation stoichiometry is necessarily a measure of the number of active sites which can function simultaneously in the [^{32}P]PP_i–ATP exchange reaction. Since the stoichiometry of 0.8 mol of tRNA bound per dimer was obtained for the *E. coli* K_{12} enzyme under nonequilibrium conditions (Knowles *et al.*, 1970), it seems likely that if the stoichiometry were to be measured under steady-state conditions two functional active sites could be determined in the acylation reaction as well.

Acknowledgments

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Studies on a Calf Thymus Ribonuclease Specific for Ribonucleic Acid–Deoxyribonucleic Acid Hybrids†

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ABSTRACT: Ribonuclease H, an enzyme that specifically hydrolyzes the RNA moiety of RNA–DNA hybrids, has been extensively purified from calf thymus. The enzyme is apparently extranuclear in origin. It appears to have a molecular weight of 74,000, and only one major subunit. It requires covalent cations for activity, and prefers Mn^{2+} over Mg^{2+}. The enzyme acts on poly(rAdT) as an endonuclease to produce mono- and oligoribonucleotides, terminated by a 5′-phosphate. No activity could be demonstrated on natural double- or single-stranded DNA, or on single-stranded RNA. The homopolymer hybrids tested either served as substrate for the enzyme or acted as an inhibitor. No single-stranded homopolymer was a substrate or an inhibitor, except poly(rA) which was an inhibitor. Double-stranded viral RNA and several of the homopolymer double-stranded ribonucleic acids are inhibitors of the reaction. The pattern of inhibition of calf thymus ribonuclease H by a number of different rifampicin derivatives has been investigated.

A ribonuclease (ribonuclease H) has been described in extracts of calf thymus that appears to hydrolyze specifically the RNA portion of RNA–DNA hybrids (Stein and Hausen, 1969; Hausen and Stein, 1970). The DNA portion of the hybrid remains intact. Single- and double-stranded DNA and double-stranded RNA have minimal substrate activity. Ribosomal RNA has less than 2% of the substrate activity of the enzymatically synthesized hybrids.

A similar enzyme activity has been described in preparations of avian myeloblastosis virus[1] (AMV) by Molling *et al.* (1971), in mouse KB cells (Keller and Crouch, 1972), and chick embryo extracts (Crouch, 1973[2]).

Such an enzyme is of interest to those concerned with the structure of nucleic acids from several points of view. The enzyme's presence and specificity, if confirmed, raise the question of the possible significance of the hybrids that are its physiologic substrate. Moreover, a ribonuclease specific for the RNA moiety of a RNA–DNA hybrid could be of great value as a tool for the characterization and identification of hybrid nucleic acids. Further information is, however, required regarding the enzyme's specificity and mechanism of action. The purpose of the work reported here has been to further purify and characterize the calf thymus ribonuclease H.

The enzyme from calf thymus has been purified more than 200-fold and its specificity for RNA–DNA hybrids has been confirmed. No activity was detected using double- or single-stranded DNA, or single-stranded RNA. Similar findings were observed with a number of homopolymer materials. The hybrid homopolymer combinations tested were found to be either substrates or inhibitors of the enzyme. Some preliminary characterization of the protein has been accomplished, and data related to size, subunit structure, and mode of activity are presented.

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[1] Abbreviation used is: AMV, avian myeloblastosis virus.

[2] Crouch, R. (1973), private communication.