pactamycin-induced inhibition. At high Mg^{2+} levels where pactamycin will stimulate peptide synthesis, addition of rubradirin abolishes the pactamycin-induced stimulation. As far as pactamycin is concerned, these results indicate that pactamycin definitely interferes somehow with the chain initiation process which, depending on the Mg^{2+} concentration present in the system, results in inhibition of this process at low Mg^{2+} levels, no interference at intermediate Mg^{2+} levels (see Cohen *et al.*, 1969), and enhancement at high Mg^{2+} concentration. Inhibition of polypeptide biosynthesis by rubradirin is not directly related to the Mg^{2+} concentration present. In addition, rubradirin does not potentiate pactamycin-induced inhibition at low Mg^{2+} levels and abolishes pactamycin-induced stimulation at high Mg^{2+} levels. This suggests that both antibiotics interact at the same functional level of the ribosome, namely the peptidyl site. Rubradirin does not interfere with the peptide bond formation step studied with the puromycin reaction. It is also unlikely that rubradirin affects the chain termination process. Synthetic messenger-directed amino acid incorporation leads to the accumulation of peptide chain products which remain bound to the ribosomes, and practically no release of peptides occurs under these conditions. Inhibition of chain termination would thus remain obscured in these test systems.

The results presented therefore indicate that rubradirin interacts somehow with the function of the peptidyl site of the ribosome resulting in inhibition of polypeptide biosynthesis.

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Isoleucyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* B. Effects of Magnesium and Spermine on the Amino Acid Activation Reaction†

Eggehard Holler‡

ABSTRACT: We have investigated the interaction of magnesium and spermine with Ile-tRNA synthetase under conditions in which catalysis of L-isoleucine activation is observed. We used a fluorimetric method and titration and stopped-flow techniques. The results support the previous findings by Cole and Schimmel ((1970), *Biochemistry 9*, 3143) that ATP reacts as the magnesium salt. At concentrations higher than 1 mM, magnesium becomes inhibitory with an inhibition constant of 3–5 mM. Spermine is found to inhibit accumulation of enzyme-bound L-isoleucyl adenylate. Inhibition proceeds *via* two routes; one is binding of spermine to an effector site and the other is the formation of catalytically inert spermine–ATP. Presumably, binding to the effector site is followed by a conformation change of the enzyme, leaving the Michaelis–Menten complex less reactive. Binding of the effector is associated with an enhancement of the fluorescence intensity of the reporter group, 2-*p*-toluidinylnaphthalene-6-sulfonate, which is complexed with the enzyme. This response is in contrast to the fluorescence quenching observed for binding of substrates and products. Spermine binds to ATP. The strength of the interaction is comparable with that for magnesium. Spermine–ATP appears to bind to the enzyme, however, ability to participate in the catalysis of the amino acid activation reaction is lost.

The catalysis of an amino acid specific ATP-[^{32}P]PP_i exchange reaction together with the isolation of an enzyme-bound aminoacyl adenylate were taken as strong evidence for the formation of an intermediate as part of the specific charging reaction of a cognate tRNA catalyzed by an aminoacyl-tRNA synthetase (Berg, 1958; Bergmann *et al.*, 1961; Berg *et al.*, 1961; Norris and Berg, 1964). The rate of exchange as well as the preparative accumulation of the intermediate have been found to possess a substantial magnesium dependence. A

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careful analysis of this dependence has been recently reported by Cole and Schimmel (1970). That the requirement is not universal has been shown for the Tyr-tRNA synthetase from *Escherichia coli* which catalyzes the formation of tyrosyl adenylate in the absence of divalent cations (Chousterman and Chapeville, 1971). Yarus and Rashbaum (1972) have shown recently that in the case of Ile-tRNA synthetase the transfer of the aminoacyl moiety does not require the catalytic participation of a divalent metal ion. More important, a series of papers report that catalysis of the tRNA-charging reaction is adequately maintained after substitution of magnesium with polyamines, in particular with spermine and spermidine (Takeda and Igarashi, 1969; Igarashi *et al.*, 1971; Steinmetz Kayne and Cohn, 1972). Surprisingly, the ATP-[^{32}P]PP_i exchange is blocked (Igarashi *et al.*, 1971; Steinmetz Kayne and Cohn, 1972), and there is evidence that the aminoacyl adenylate intermediate is not accumulated (Igarashi *et al.*, 1971; Pastuszyn and Loftfield, 1972).

We have recently reported a fluorimetric method (Holler *et al.*, 1971), which has been shown suitable for rapid kinetic investigation of the amino acid activation reaction of the L-isoleucine system of *E. coli* (Holler and Calvin, 1972). We report in this paper the effect of magnesium on the rate of formation of the enzyme bound aminoacyl adenylate and the inhibition by spermine. We have collected information about the sites and the strength of interactions.

Materials and Methods

The 300- to 350-fold-purified Ile-tRNA synthetase was obtained from *E. coli* B cells (Miles Laboratories) following the method of Baldwin and Berg (1966). TNS[1] was purchased from Sigma as lot 60C-5270. Care was taken that the dye preparation consisted of material fluorescing between 480 and 500 nm (excitation 366 nm). Spermine tetrahydrochloride was obtained from Nutritional Biochemical Corp., and 8-hydroxyquinoline-5-sulfonic acid as dihydrate from Aldrich. All other chemicals were reagent grade (Baker). Deionized and distilled water was used which had been boiled and cooled under nitrogen to remove oxygen and carbon dioxide.

Fluorescence Measurements. Methods and fluorimeter have been described previously (Holler *et al.*, 1971; Holler and Calvin, 1972). Kinetics were followed with use of a modified Durrum-Gibson stopped-flow spectrophotometer as has been described (Holler and Calvin, 1972). In a typical experiment, a solution containing 0.15 μM Ile-tRNA synthetase, 43 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol was rapidly mixed with a solution containing 0.88 μM L-isoleucine, 11 μM ATP, 43 μM TNS, 0.05 M Tris-HCl, 0.01 M 2-mercaptoethanol, and $MgCl_2$ plus spermine at various concentrations. When the effect of ionic strength was studied, NaCl was added to the substrate mixture. Procedures for evaluation are generally based on linear plots for saturation functions as has been described by Eadie (1942). Experimental errors are given as mean deviations for three to five determinations.

Results

Magnesium Ions. The rate constant for the formation of Ile-tRNA synthetase–L-isoleucyl-AMP complex was measured as a function of magnesium concentration in the range 0.01–11 mM at fixed concentrations of 0.88 μM L-isoleucine and 11 μM ATP (Figure 1). It can be seen that the plot is biphasic, indicating that more than one molecule of magnesium could bind to the Michaelis–Menten complex. At concentrations below 1 mM the interaction was indicated as an increase of the rate of catalysis whereas at concentrations above 1 mM catalysis was inhibited. We have ruled out the possibility that inhibition is caused by the increase of ionic strength. For instance, 20 mM NaCl in the presence of 1 mM $MgCl_2$ had little effect (only a 10% decrease) on the rate constant. Higher concentrations of NaCl (0.1 and 0.2 M) reduced the rate by 50 and 70%, respectively. The analysis of the rate constants as function of low and high concentrations of Mg^{2+} was processed in the form of linear plots (Eadie, 1942), as shown in Figure 1 (inset) and in Figure 3. The evaluation was based on the assumptions of separate 1:1 interactions between magnesium and enzyme or the Michaelis–Menten complex, respectively. Evaluation at low concentrations of magnesium was based on reaction scheme 1. Magnesium, substrates, and

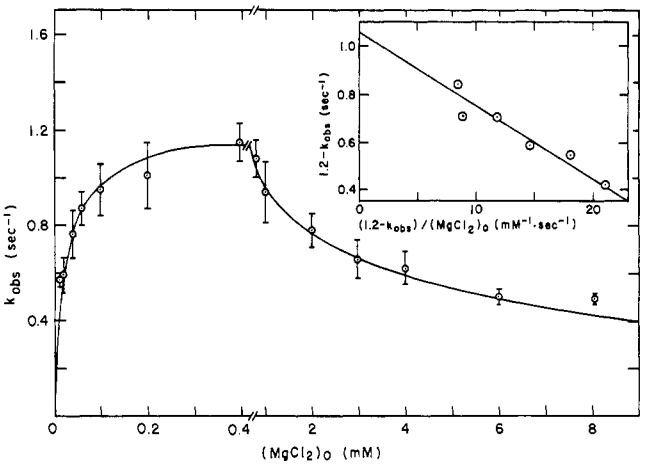
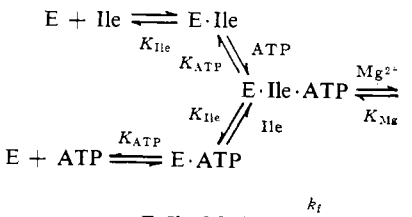


FIGURE 1: Formation of Ile-tRNA synthetase–L-isoleucyl-AMP complex at pH 8.0, 25°. The rate constant is measured as a function of the concentration of magnesium ions. At concentrations above 1 mM, catalysis is inhibited. Data in the inset are plotted according to a linear form of eq 5 for concentrations higher than 1 mM. The value $k_0 = 1.2$ sec^{-1} had been determined from the intercept for line a in Figure 3. Initial concentrations were 0.05–0.15 μM Ile-tRNA synthetase, 43 μM TNS, 0.88 μM L-isoleucine, 11 μM ATP, 0.05 M Tris-HCl, and 0.01 M 2-mercaptoethanol.



$$E \cdot Ile \cdot MgATP \overset{k_f}{\rightleftharpoons} E \cdot Ile \sim AMP + MgPP_i \quad (1)$$

enzyme come together to form the Michaelis–Menten complex, $E \cdot Ile \cdot Mg^{2+} \cdot ATP^{4-}$. It is not certain whether the sequence of substrate addition to the enzyme is ordered or random; however, as we have pointed out recently, reaction 1 can be handled as if it were a random mechanism provided the substrates Ile and ATP are of dilute concentrations [$(Ile)_0 < K_{Ile}$, $(ATP)_0 < K_{ATP}$] (Holler and Calvin, 1972). The quaternary Michaelis–Menten complex turns over to enzyme–L-isoleucyl adenylate complex, E·Ile∼AMP, associated with the release of magnesium pyrophosphate. The reverse reaction

[1] Abbreviation used is: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

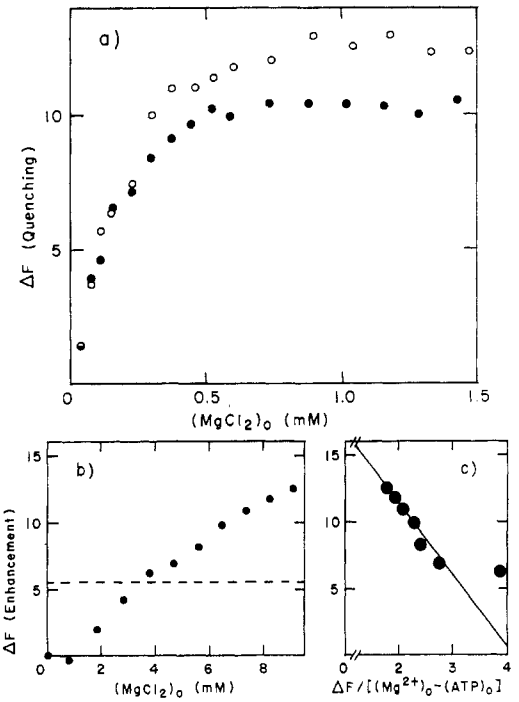


FIGURE 2: Fluorimetric titration of Ile-tRNA synthetase–ATP complex with Mg^{2+} ions at pH 8, 25°. (a) Fluorescence quenching upon titration with ATP (○) and ATP plus $MgCl_2$ (1:1) (●). Initial concentrations were 0.35 μM enzyme and 8 μM TNS. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, plus emission cutoff filter 430 nm. (b) Fluorescence enhancement upon titration with $MgCl_2$ at a fixed (2.2 mM) concentration of ATP. The broken line indicates fluorescence intensity before addition of ATP. Initial concentrations were 1 μM enzyme and 5 μM TNS. (c) Data from b are replotted according to the method by Eadie (1942), assuming that fluorescence enhancement is associated with the binding of a second Mg^{2+} ion. The dimension of the abscissa is mM^{-1}. The term $(ATP)_0$ is added in order to correct for the amount of magnesium bound to ATP.

is not considered because dilute solutions of enzyme and substrates were used ($(Ile)_0 < K_{Ile}$, $(ATP)_0 < K_{ATP}$, $(PP_i)_0 \ll K_{PP_i}$ (Holler and Calvin, 1972). Magnesium is thought to bind to the Michaelis–Menten complex. Since we consider reaction scheme 1 as random, it may as well bind to free ATP. Under the conditions of Ile and ATP in excess of enzyme, and magnesium in excess of ATP, the expression for the rate constant is given by

$$k_{obsd} = k_0 \frac{(Mg)_0}{K_{Mg} + (Mg)_0} \quad (2)$$

The symbol K_{Mg} refers to the dissociation constant for binding of the first magnesium ion. The parameter k_0 refers to complete saturation by Mg^{2+} ions and is defined by

$$k_0 = k_f \frac{(Ile)_0 \cdot (ATP)_0}{K_{Ile} \cdot K_{ATP}} \quad (3)$$

as has been previously derived by Holler and Calvin (1972). The symbols K_{Ile} and K_{ATP} refer to dissociation constants for binding of L-isoleucine and ATP, respectively. The subscript zero marks initial concentrations of reactants. The value for K_{Mg} was determined from the slope of line a in Figure 3 to be (19 ± 1) μM.

In the case of high concentrations of magnesium, evaluation is tentatively based on a mechanism in which the second magnesium combines with free enzyme or with the Michaelis–Menten complex *via* an effector site. Once the effector has been bound, the decomposition of the Michaelis–Menten complex into products may be blocked or proceed at a lower rate constant k_{fi}. The same model will be used for the inhibition by spermine in the case of saturating concentrations of magnesium. The following assumptions underly our further considerations. (1) The binding of the substrates is not impaired. (2) The formation of the enzyme-bound isoleucyl adenylate should induce a similar quenching of fluorescence as observed in absence of effectors. From titration of enzyme with L-isoleucine at high concentrations of magnesium (>10 mM) or in the presence of spermine (Figure 6) we have indication that fluorescence quenching would not be much different.

When again the substrates Ile and ATP are at low concentrations $[(Ile)_0 < K_{Ile}, (ATP)_0 < K_{ATP}]$ but in excess of enzyme, and when magnesium or spermine is in excess of ATP $[(Mg)_0, (spermine)_0 \gg (ATP)_0]$, then the expression for the rate constant is given by

$$k_{obsd} = \left[\frac{(Mg)_0 \cdot k_0}{(Mg)_0 + K_{Mg}}\right] \frac{K_i}{(I)_0 + K_i} + \frac{k_{fi}}{k_f} \frac{(I)_0}{(I)_0 + K_i} \quad (4)$$

The concentration of effector is indicated by the symbol $(I)_0$. K_i refers to the dissociation constant for binding to the effector site. For evaluation, $k_0 - k_{obsd}$ was plotted as the function of concentration as shown for high concentrations of magnesium in Figure 1, inset. When high concentrations (>1 mM) of magnesium were used, the first binding site for the metal ion was considered as being already saturated ($K_{Mg} \ll (Mg)_0$). Consequently, the difference between rate constants, $k_0 - k_{obsd}$, follows a saturation function according to

$$k_0 - k_{obsd} = \frac{(I)_0 k_0 \left(1 - \frac{k_{fi}}{k_f}\right)}{(I)_0 + K_i} \quad (5)$$

This relation was made linear and the data in Figure 1 (inset) were plotted accordingly. With use of the value $k_0 = 1.2$ sec^{-1} which had been obtained from the intercept of line a in Figure 3, we have determined the values for k_{fi} and K_i from the intercept and the slope, respectively, to be $k_{fi} \simeq 0$ and 3 mM.

We have further evidence for a specific interaction at high concentrations of magnesium ions from the following titration experiments. When enzyme was titrated with a solution containing ATP and $MgCl_2$ in at 1:1 concentration ratio, fluorescence was quenched to approximately the same extent as for ATP alone (Figure 2a). When a solution of enzyme plus ATP was titrated with magnesium at concentrations in excess of ATP, the intensity of the fluorescence was enhanced as the concentration of magnesium increased, until, finally, the original level of fluorescence (before addition of ATP) was exceeded (Figure 2b). The control experiment where enzyme–TNS was titrated with magnesium up to 10 mM while ATP had been omitted from the solution, indicated that fluorescence enhancement was not due to a direct interaction with the enzyme–TNS system.

Figure 2c presents the titration data in form of a linear plot (Eadie, 1942), where the concentration of Mg^{2+} ions has been corrected for the amounts bound to ATP. The value for the dissociation constant which is supposed to describe the bind-

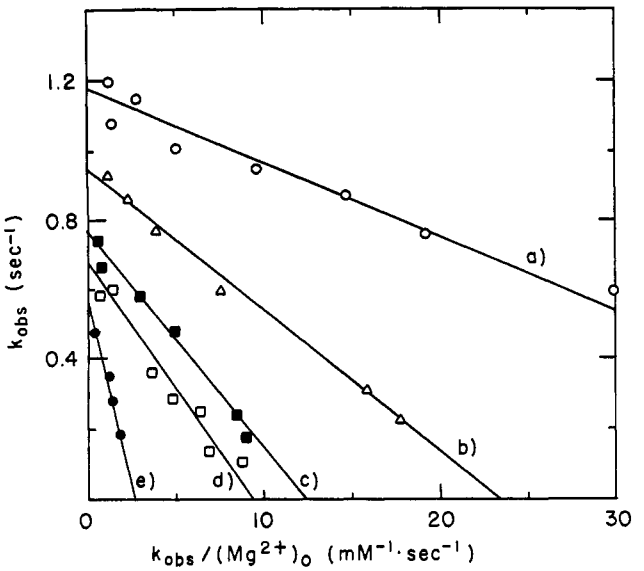


FIGURE 3: Inhibition of the L-isoleucine activation reaction by spermine at 25°, pH 8.0. Linearized plots (Eadie, 1942) for k_{obsd} as determined from stopped-flow experiments as function of concentration of Mg^{2+} ions at various fixed concentrations of spermine. Initial concentrations were 0.075 μM Ile-tRNA synthetase, 43 μM TNS, 0.88 μM L-isoleucine, 11 μM ATP, (a) zero, (b) 0.1 mM, (c) 0.316 mM, (d) 0.645 mM, and (e) 1.45 mM spermine. Solutions contained 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol. Concentration of $MgCl_2$ was varied between 0.01 and 1 mM. Excitation wavelength 290 nm, slit 2 mm, emission cutoff filter, Corning 373.

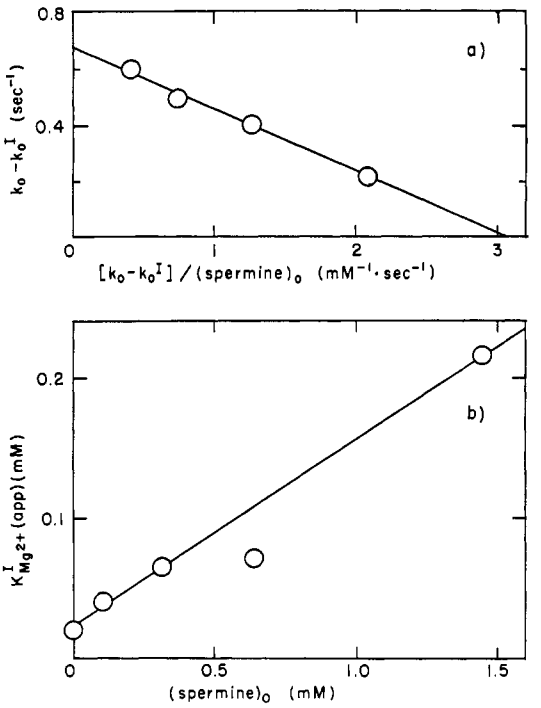


FIGURE 4: Evaluation of the data in Figure 3. (a) Linear plot for inhibition at saturating concentration of Mg^{2+} ions. The lines in Figure 3 intersect with the k_{obsd} axis at various values k_0^1 and k_0 in presence and absence of spermine, respectively. The difference $k_0 - k_0^1$ is plotted as function of the concentration of spermine. The linear arrangement is consistent with a saturation fraction for spermine binding to the Michaelis–Menten complex. The inhibition constant K_i is determined from the slope. Note that saturation is not associated with complete loss of activity. (b) The Michaelis–Menten constant, K_{Mg}(app) for magnesium is plotted as function of the concentration of spermine. We have assumed that Mg^{2+} ions and spermine compete for ATP. The theoretical relation is given by the expression $K_{Mg}(app) = K_{Mg}[1 + (spermine)_0/K_{I\cdot ATP}]$. The value for the inhibition constant $K_{I\cdot ATP}$ is determined from the slope using a value $K_{Mg} = 25$ μM (Paetzold and Amoulong, 1966).

ing of the second magnesium to the enzyme–$MgATP^{2-}$ complex, is determined from the slope of the line to be 5 mM. This value is in fair agreement with the value determined kinetically.

Spermine. When spermine (3 mM) was added to a substrate mixture containing 1 mM magnesium, formation of L-isoleucyl ~AMP was strongly inhibited. We have measured the rate constant of amino acid activation as a function of magnesium concentration at various fixed levels of spermine, as already described. The titration curves are presented as linear plots in Figure 3. It is seen that spermine inhibited catalysis *via* both parameters, first by decreasing the maximum velocity, and second by increasing the Michaelis–Menten constant. We have replotted the difference between the observed maximum rate constant and the maximum rate constant in the absence of inhibitor as a function of spermine concentration in Figure 4a according to a similar equation as derived from eq 5. In Figure 4a the symbol k_0^I refers to k_{obsd}, where the concentration of spermine is varied and the concentration of magnesium is extrapolated to infinity. Infinite concentration of magnesium refers to a condition where the first binding site (free or bound ATP) is saturated as indicated by the low-concentration phase in Figure 1. The inhibition constant of spermine was determined to be $K_i = 0.22$ mM. From the ordinate intercept it is clear that amino acid activation would not have been completely prevented by saturation of the effector site with spermine. From the value 0.67 sec^{-1} the ratio of the rate constants k_{fi}/k_f was determined to be 0.44, according to the definition of the intercept $= k_0(1 - k_{fi}k_f)$ and $k_0 = 1.2$ sec^{-1}. We believe that this inhibition is induced by binding of spermine to an enzyme effector site, in a manner similar to that we have discussed for the second magnesium ion. In order to test this hypothesis, we titrated a mixture of enzyme plus TNS in absence of substrates and Mg^{2+} ions (Figure 5). We obtained a typical titration curve associated with an enhancement of fluorescence intensity. Control titration of a solution containing only TNS indicated no significant change. In a second control we titrated a solution of 0.012 mM lysozyme and 27 μM TNS with up to 1 mM spermine without discovering any effect on the fluorescence properties. TNS is known to interact with lysozyme as indicated by a strong enhancement of the fluorescence intensity (Barel *et al.*, 1971; E. Holler, unpublished results). We take this observation as evidence that the titration of Ile-tRNA synthetase is not an artefact caused by direct interaction with TNS.

The dissociation constant for binding of spermine was determined from the slope of the linear plot (Figure 5 inset) to be (0.25 ± 0.03) mM. This value is in excellent agreement with the value determined from the spermine dependence of the maximum rate constant (Figure 4a). Furthermore, the interaction of spermine does not interfere with binding of L-isoleucine. This is shown by titration of Ile-tRNA synthetase with L-isoleucine in the presence of 1.7 mM spermine (Figure 6). The value for the dissociation constant for L-isoleucine as evaluated from the slope is (9 ± 1) μM in agreement with the value (5.8 ± 0.8) μM reported recently (Holler *et al.*, 1971). Moreover, the maximum effect of quenching, 44%, is close

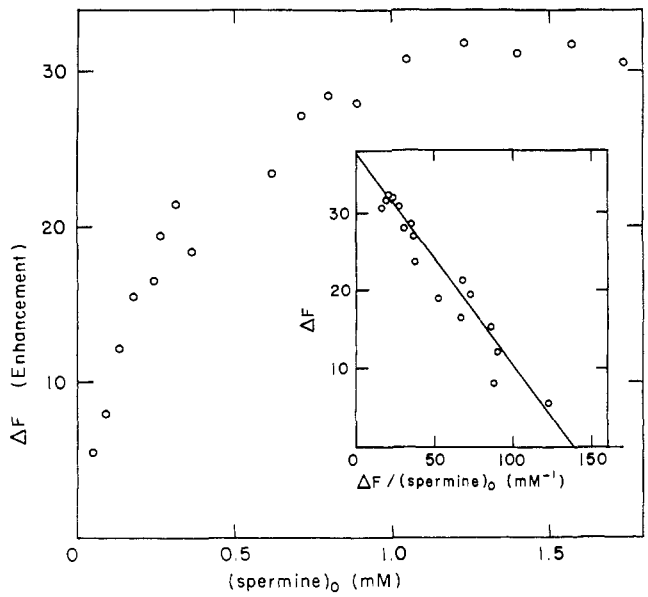


FIGURE 5: Binding of spermine to Ile-tRNA synthetase at pH 8.0, 25°. Titration is associated with an enhancement of fluorescence intensity. The dissociation constant for the formation of enzyme–spermine was determined from the slope of the line in the inset. The maximum enhancement of fluorescence intensity corresponds to 37% of the initial fluorescence intensity (corrected for background). Initial concentrations were 0.18 μM enzyme, 10 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, emission cutoff filter 430 nm.

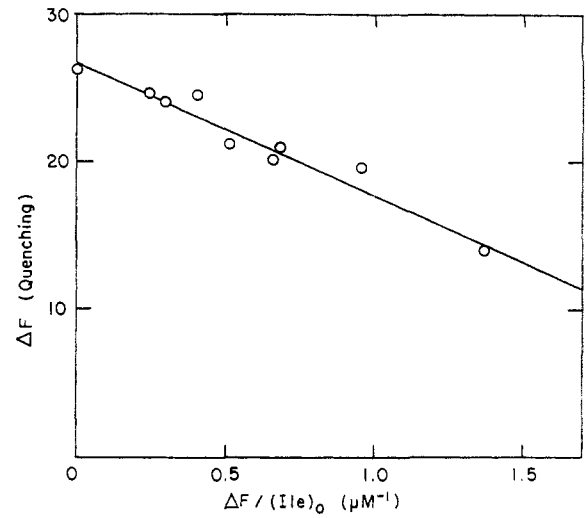


FIGURE 6: Binding of L-isoleucine to Ile-tRNA synthetase–spermine complex at pH 8.0, 25°. Titration is associated with quenching of fluorescence intensity as described (Holler *et al.*, 1971). Initial concentrations were 0.14 μM enzyme, 10 μM TNS, 1.7 mM spermine, 10.2 μM to 2.6 mM L-isoleucine, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, plus cutoff filter 430 nm.

to (37 ± 6)% when determined in absence of the effector. In contrast, the titration experiments with ATP indicated that binding of spermine and ATP was not independent (Figure 7). This observation then seemed to be related to the effect of spermine on the slope of the lines in Figure 3, indicating that the affinity for magnesium to form the magnesium–ATP complex decreased with increasing concentration of spermine. In particular, the following observations were made. (1) Addition of ATP to the solution containing Ile-tRNA synthetase, TNS, and spermine was associated with fluorescence quenching. The degree of quenching was dependent on concentration of ATP in the form of a saturation function. The concentration for saturation shifted to higher values as the concentration of spermine was increased. (2) Maximum quenching was dependent on the initial level of spermine concentration. The maximum degree of quenching, as indicated at high ATP concentration, was of the same size as the degree of fluorescence enhancement originally obtained upon addition of spermine to the enzyme–TNS mixture.

A coupling for spermine and ATP is expected when, for instance, the formation of the complex spermine–ATP is considered.

We have undertaken measurements to determine whether or not spermine binds to ATP and to evaluate the dissociation constant of the ATP–spermine complex. We have used the fluorescence technique to follow the binding of Mg^{2+} ions to 8-hydroxyquinoline-5-sulfonic acid (Schachter, 1961) as a function of the partition between ATP and the dye. Experiments and evaluation were processed as described for Figure 8. It is found that spermine binds to ATP with a dissociation constant $K_{I\cdot ATP}$ = 0.082 mM.

The titration curves in Figure 7 are consistent with the formation of spermine–ATP; however, we have to assume that spermine–ATP, when bound to Ile-tRNA synthetase, does not induce a change of the fluorescence intensity. This follows from the following consideration. The titration curves in Figure 7 return to the same level of fluorescence intensity upon increasing concentrations of ATP as observed for the absence of ATP and spermine. We have calculated the relative amount of spermine which is bound to ATP at a condition when this level of fluorescence has been attained using

$$(I\cdot ATP) = {}^1/_2[(I)_0 + (ATP)_0 + K_{I\cdot ATP}] - {}^1/_2[(I)_0 + (ATP)_0 + K_{I\cdot ATP}]^{1/2} - 4(I)_0(ATP)_0^{1/2} \quad (6)$$

The symbol I refers to spermine. Equation 6 is derived for conditions, where $(ATP)_0 \gg (E)_0$, $(spermine)_0 \gg (E)_0$, and $(ATP)_0 \simeq (spermine)_0$. We have calculated the following relative amounts of ATP-bound spermine: (a) $(I\cdot ATP)/(I)_0$ = 93% at $(ATP)_0$ = 1.5 mM, $(I)_0$ = 0.415 mM; (b) 91% at $(ATP)_0$ = 1.1 mM, $(I)_0$ = 0.248 mM; (c) 99% at $(ATP)_0$ = 0.8 mM, $(I)_0$ = 0.155 mM. We see that according to $(ATP)_0 - (I)_0 > K_{ATP}$ = 0.25 mM, Holler *et al.*, 1971), the concentration of ATP should in all cases be high enough to form substantial amounts of enzyme–ATP complex. Formation of the complex should have been associated with a further decrease of the fluorescence intensity (Holler *et al.*, 1971). This is not observed in Figure 7, indicating that spermine–ATP must be competing with ATP for Ile-tRNA synthetase and that spermine–ATP does not induce a change in fluorescence intensity upon binding to the enzyme.

The kinetic data in Figure 4b were also evaluated on the basis of competition between magnesium and spermine for ATP. The values for the apparent Michaelis–Menten constants as measured upon variation of the concentration of magnesium were replotted as a function of the concentration of spermine and were evaluated as described in Figure 4b.

The value for the inhibition constant was found to be 0.15 mM, in fair agreement with the value determined for the dissociation constant for spermine–ATP.

TABLE I: Kinetic and Equilibrium Parameters for Binding of Magnesium and Spermine.[a]

Effector	k_0[b,c] (sec^{-1})	k_{fi}/k_f[d]	K_i[e] (mM)	K_{Mg}[f] (μM)	$K_{I\cdot ATP}$[g] (μM)	Exptl Source and Determination
Magnesium						
$(Mg)_0$, 1 mM	1.2			19 ± 1[h]		Kinetically; Figure 3a; eq 2
				25		Titration of ATP; Paetzold and Amoulong (1966)
$(Mg)_0$, 1 mM		0	3			Kinetically; Figure 1 (inset); eq 5
			5			Fluorimetric titration of enzyme–MgATP complex; Figure 2b,c
Spermine		0.44	0.22			Kinetically; competition experiments with Mg^{2+}; Figures 3 and 4a; eq 5
			0.25 ± 0.03			Fluorimetric titration of enzyme Figure 5
					150	Kinetically; competition experiments with Mg^{2+}; Figures 3 and 4b
					82	Titration according to the procedure described in Figure 8

[a] For 25°, most of the experiments were done in 0.05 M Tris-HCl buffer in the presence of 10–43 μM TNS. [b] As defined by eq 3. [c] As measured for conditions referring to Figure 1. [d] As defined by eq 1 and 4. [e] Defined as the dissociation constant for binding of magnesium or spermine to an effector site of the enzyme. [f] As defined by eq 1. [g] Defined as the dissociation constant for the spermine–ATP complex. [h] Experimental errors are given as mean deviations for three to five determinations.

In conclusion, we have shown that two molecules of spermine can react with the Michaelis–Menten complex of the L-isoleucine activation reaction. One binds to an effector site, decreasing the maximum rate of catalysis, the other combining with ATP to form an unproductive spermine–ATP complex. The overall expression for the rate constant in the presence of varying amounts of magnesium and spermine is given by

$$k_{obsd} = \frac{(Mg)_0}{K_{Mg}\left(1 + \frac{(I)_0}{K_{I\cdot ATP}}\right) + (Mg)_0} \times \left[\frac{K_i}{(I)_0 + K_i} + \frac{k_{fi}}{k_f}\frac{(I)_0}{(I)_0 + K_i}\right] \quad (7)$$

for $(Mg)_0 \geqq 1$ mM. Experimental conditions are the same as outlined for eq 1–4. Values for the parameters k_0, k_{Mg}, K_i (for spermine), $K_{I\cdot ATP}$, and k_{fi}/k_f (for spermine) are summarized in Table I.

Nature of the Effector Site. Binding of spermine to the effector site is associated with an enhancement of fluorescence. Upon saturation, the wavelength for maximum emission is shifted to 432 nm (excitation at 366 nm, spectra not corrected for instrument properties). Comparison is made with the maxima 443 nm for enzyme–TNS, and 438 nm for enzyme–TNS–substrate complexes (excitation at 340 nm).

We have observed that the maximum rate constant (at infinite concentration of magnesium) of the amino acid activation reaction decreased when spermine was binding to the effector site of Ile-tRNA synthetase. We interpret this by assuming that spermine induces a conformation change at the active site, rendering a less active enzyme. A slow process (k_{obsd} = 12 sec^{-1}) which is characterized as a fluorescence enhancement has been observed when a solution containing 6.4 mM spermine plus 43 μM TNS was rapidly mixed in a stopped-flow apparatus with a solution containing 0.15 μM enzyme, 2.82 μM L-isoleucine, 39.6 μM ATP, 43 μM TNS, and 20 mM EDTA (pH 8.0, 25°). The same process was observed when 1.34 mM $MgCl_2$ had been added instead of EDTA. The slowness of the process, although at saturation concentration of spermine, is in accord with the suggestion that the effector induces a conformation change at the active site, when the interconversion step is rate limiting.

It was of interest to test whether or not spermine and Mg^{2+} ions compete for the same effector site. In a competition experiment where an enzyme (0.19 μM enzyme–12 μM TNS, pH 8.0, 25°) containing 2 mM $MgCl_2$ was titrated with spermine, a value for the apparent dissociation constant was measured which was twice the value in the absence of magnesium. This finding suggests that both ions might compete for the same site. Furthermore, titration of an enzyme solution

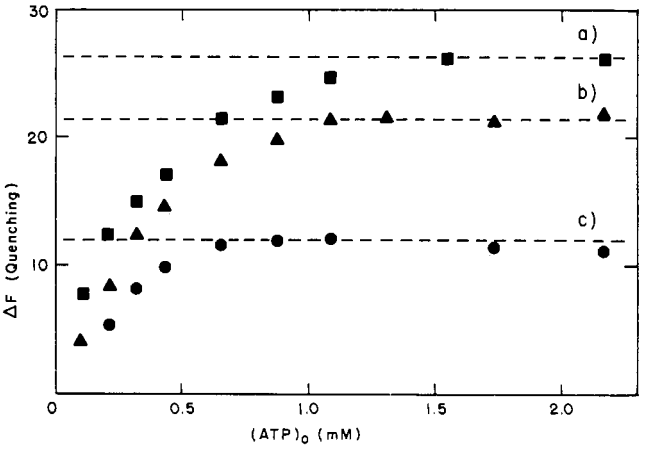


FIGURE 7: Titration of a mixture of Ile-tRNA synthetase plus spermine with ATP. Concentration of spermine was (a) 0.415 mM, (b) 0.248 mM, and (c) 0.155 mM. Initial concentrations were 0.19 μM enzyme, 12 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 10 nm, emission 470 nm, and slit 10 nm. Broken lines indicate the original levels of fluorescence for Ile-tRNA synthetase–TNS complex.

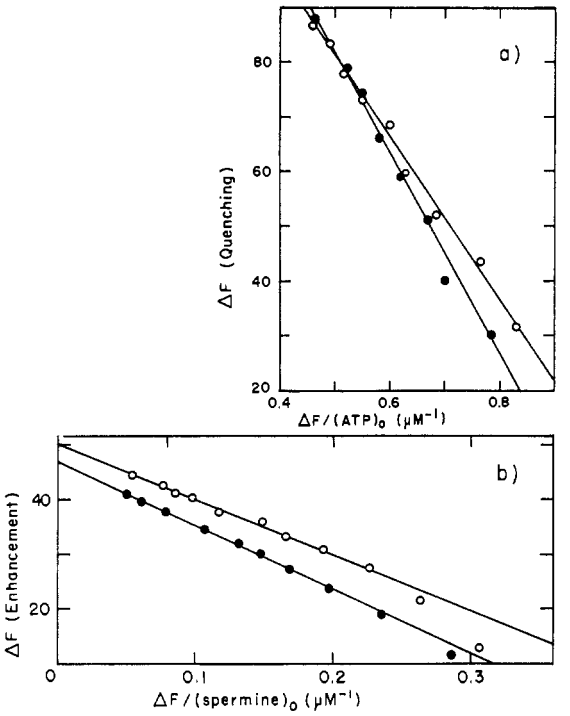


FIGURE 8: Determination of the dissociation constant of spermine–ATP, $K_{I\cdot ATP}$, at pH 8.0, 25°. The fluorescence intensity of 8-hydroxyquinoline-5-sulfonate was measured as a function of various concentrations of ATP (a) and of spermine (b). An average value for the dissociation constant $K_{I\cdot ATP}$ was determined from the slopes for the two sets of data in b according to the relation $K_{I\cdot ATP} = [-\text{slope}][1 + (ATP)_0/K_{Mg\cdot ATP}(app)]^{-1}$. The value for $K_{Mg\cdot ATP}(app)$ has been determined according to $K_{Mg\cdot ATP}(app) = -\text{slope}$ from the linear plot a as a function of ATP concentration. The procedure is valid for conditions when $(spermine)_0 >> (ATP)_0 > (Mg)_0 << K_{Mg\cdot ATP}$ and $(8\text{-hydroxyquinoline-5-sulfonate})_0 >> (Mg)_0$. (a) A mixture of dye plus $MgCl_2$ was titrated with ATP. Initial concentrations were 1.53 mM 8-hydroxyquinoline-5-sulfonate, 2.5 μM $MgCl_2$, and 0.019–0.19 mM ATP. (b) A mixture of dye, $MgCl_2$, and ATP was titrated with spermine. Initial concentrations were 1.53 mM 8-hydroxyquinoline-5-sulfonate, 2.5 μM $MgCl_2$, 57.2 μM ATP, and 41 μM to 0.8 mM spermine. Excitation wavelength was 390 nm (slit 8 nm), emission wavelength 520 nm (slit 10 nm), plus 430 nm cutoff filter in the emission path.

containing 0.4 and 0.8 mM spermine with a solution of $MgCl_2$ indicated an initial quenching of the fluorescence. However, since effects from increasing ionic strength interfere with the measurement, the problem cannot be definitely solved at present.

Discussion

Cole and Schimmel (1970) have shown in a careful analysis that magnesium interacts with ATP and pyrophosphate and that only the monomagnesium salts are the reactive species. Our results support their findings. At low concentration of Mg^{2+} ions (<1 mM) the rate of formation of enzyme-bound L-isoleucyl adenylate depends strictly on the concentration of $MgATP^{2-}$. The reaction rate versus magnesium concentration profile follows the value 19 μM for a dissociation constant, in agreement with the value 25 μM reported for the dissociation of $MgATP^{2-}$, at 25°, 0.07 M tetrabutylammonium bromide (Paetzold and Amoulong, 1966). Presumably, magnesium can combine with either free or enzyme-bound ATP, but the possibility cannot be excluded that the interaction with bound ATP is less strong. This alternative could exist because the observed dissociation constant would reflect the strongest interaction between magnesium and ATP, similar as it is found for unproductive binding of substrates (Rupley, 1967).

The inhibitory effect of high concentrations of Mg^{2+} ions (>1 mM) has not been reported. Under conditions of ATP–[^{32}P]PP exchange this effect could be masked by the extent of formation of unreactive dimagnesium pyrophosphate which is reported to follow a dissociation constant 10^{-2} M (Cole and Schimmel, 1970). We found that under our conditions the variation of ionic strength, necessarily associated with variation of high concentration of magnesium, cannot account for the inhibition. Furthermore, we have observed that Mg^{2+} ions which bind at the level of the enzyme–ATP complex follow a similar concentration dependence. Our results from competition experiments with spermine indicate evidence that the site of interaction for the second magnesium ion might be close or identical with that for spermine.

Spermine interacts *via* two routes with the Michaelis–Menten complex of the amino acid activation reaction, both being inhibitory. One route is binding to the effector site of the enzyme and the other route is the formation of unreactive spermine–ATP. Binding to the enzyme is associated with a decrease of the maximum reaction rate. Inhibition as a function of the effector concentration follows a value for an apparent dissociation constant that is identical with the value determined from direct titration of Ile-tRNA synthetase. Binding of spermine to the enzyme–effector site presumably induces a conformation change which results in a decreased catalytic ability of the active site of the enzyme. The enhancement of fluorescence associated with the binding is in contrast to the fluorescence quenching associated with binding of substrates and inhibitors. The enhancement together with the blue shift of the maximum wavelength of emission indicate that the environment of the reporter group, TNS, has become more hydrophobic (McClure and Edelman, 1966; Stryer, 1965) when spermine was bound to the enzyme. This finding is in contrast to the observation that fluorescence is quenched when Ile-tRNA synthetase is titrated with L-isoleucine, ATP, or pyrophosphate, and yet the wavelength of maximum emission is shifted to the blue, but to a smaller extent. It appears likely that the ligands do not only induce different, more lipophilic environments, but also promote interaction between the fluorophor and a quencher differently. We have found that the relative degree of L-isoleucine-induced quenching is not changed by binding of spermine. This observation indicates that the binding of spermine has less, possibly a reverse, effect on the interaction between TNS and the quencher than for binding of L-isoleucine. We are, of course, aware of alternative mechanisms. Further experiments are underway for elucidation of the mechanism.

The spermine-induced conformation change apparently leaves the site for L-isoleucine intact. We have no evidence as to what extent the properties of the ATP site are changed. We have shown (Figure 8) that magnesium and spermine compete with comparable affinities for ATP. Because of this property, binding of ATP and spermine to the enzyme are coupled reactions. As we have mentioned, there is some evidence from the titration data in Figure 7 that spermine–ATP is capable of binding to the enzyme but that the ability to induce a change of the fluorescence intensity is lost. As for the kinetics, we have shown that simple competition between magnesium and spermine may account for the variation of the Michaelis–Menten constant at various fixed concentrations of spermine (Figures 3 and 4b).

There is no evidence about the location of the effector site which might accommodate both spermine and magnesium. In this connection, it is interesting that Steinmetz Kayne and Cohn (1972) have reported recently that 0.94 g-atom of Zn^{2+} and 0.77 g-atom of Ca^{2+} were bound per molecule enzyme. We think that these cations might interact with the site which we have called "effector" site.

In conclusion, we have found that the capability of Ile-tRNA synthetase to catalyze ATP-[^{32}P]PP_i exchange is lost in the presence of spermine because the effector binds to the enzyme and to ATP (and presumably to PP_i) to leave a less reactive enzyme and an unreactive spermine–ATP. We find that enzyme bound L-isoleucyl adenylate is not accumulated when magnesium is substituted by spermine. The fact that the overall reaction, that is, the specific aminoacylation of $tRNA^{Ile}$ is still catalyzed by the enzyme, leaves the question open of whether only the accumulation of the intermediate is avoided (its generation could be rate limiting) or whether catalysis follows a completely different pathway.

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