The Role of Divalent Cations in the Reactions of Valyl Transfer Ribonucleic Acid Synthetase of Escherichia coli

EFFECTS OF SPERMINE AND ETHYLENEDIAMINETETRAACETATE*

(Received for publication, June 14, 1974)

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SUMMARY
We have analyzed the function of spermine in the aminoacylation of tRNAVal by the valyl-tRNA synthetase of Escherichia coli. Our results indicate that Mg²⁺ is required for the aminoacylation reaction as well as for the ATP-PPᵢ exchange catalyzed by this enzyme. The apparent stimulation by spermine is a function of the tRNA used, which appears to contain bound cations even after dialysis against 10⁻⁴ M EDTA. Higher concentrations of EDTA totally abolish spermine-stimulated esterification of tRNAVal.

In this paper we describe studies on the roles of cations in the reactions of the valine synthetase of E. coli.

MATERIALS AND METHODS

Crude Escherichia coli B transfer tRNA was purchased from Schwarz. Valyl-tRNA synthetase and tRNAVal were purified according to the procedures of Yaniv and Gros (7). The enzyme was purified 500-fold from the crude extract and tRNAVal had an acceptance of 1.3 nmol per absorbance at 260 nm. Spermine was purchased from Calbiochem. Chelex 100 was obtained from Bio-Rad.

Atomic emission and atomic absorption spectroscopy were carried out in a Perkin Elmer 219 atomic absorption spectrometer and in an ARL spectrographic analyzer, respectively. The formation of valyl-tRNAVal and ATP-³₂PPᵢ exchange were measured according to Yaniv and Gros (7). The concentrations of EDTA, MgCl₂, and spermine added were varied as indicated in the figures. The incubations were run at 37° for 10 min; the reaction was stopped with 10% cold trichloroacetic acid and the precipitate was collected on GF/A filter paper, dried, and counted in a toluene scintillation cocktail. The formation of ³₂P⁻ATP was determined by measuring the charcoal-absorbable radioactivity in a gas flow counter.

RESULTS
Mg²⁺ Requirement—All of the known amino acid-activating enzymes require divalent cations and are generally studied in the presence of Mg²⁺. Before examining the effects of cations, we studied the dependence upon Mg²⁺ concentration of both the partial reaction of ATP-PPᵢ exchange, and the net forward reaction, by following the formation of aminoacyl-tRNA. This dependence is shown in Fig. 1. The maximum rates of reaction are observed at different concentrations of added Mg²⁺.

The difference in response to Mg²⁺ concentration could indicate different functions for the cation in the two systems or a difference in the effective concentrations in the two reactions. Because independent studies in this laboratory (Midelfort et al. (6)) convinced us that a concerted mechanism does not apply to aminoacyl-tRNA synthetases, we have examined the evidence advanced by others.

* This work was supported by National Science Foundation Grant GB 27440 and National Institutes of Health Grant GM 13037.

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Mg\(^{2+}\) dependence of valyl-tRNA synthetase catalyzed ATP-\(^{32}\)PP\(_i\) exchange and valyl-tRNA\(^{\text{val}}\) synthesis. The assays were done according to Yaniv and Girox (7). The exchange reaction contained per ml: 40 pmol of Tris-HCl, pH 7.9, 72 pmol of NH\(_4\)Cl, 6 pmol of mercaptoethanol, 2 pmol of ATP, 200 µg of bovine serum albumin, 2 pmol of L-valine, 2 pmol of sodium pyrophosphate (\(^{32}\)PP) with a specific activity of 1500 cpm/nmol, and 5 units of enzyme. Incubations were carried out at 37°C for 10 min. \(^{32}\)P]ATP adsorbed on charcoal and counted with a gas flow counter. The reaction mixture for aminoacylation contained Tris-HCl, ATP, mercaptoethanol, NH\(_4\)Cl, and bovine serum albumin for the same concentrations as described above plus 10 to 20 µg of tRNA\(^{\text{val}}\), 0.2 pmol of \([^{14}\text{C}]\)valine, and 0.5 unit of enzyme per ml of assay. Incubations were stopped with 10% cold trichloroacetic acid; 0.5 mg of carrier RNA was added for complete precipitation. The concentrations of Mg\(^{2+}\) ion added were varied as indicated in the figure. C---C, ATP-\(^{32}\)PP\(_i\) exchange; O---O, \([^{14}\text{C}]\)valyl-tRNA\(^{\text{val}}\) formation; zero on the abscissa represents no added Mg\(^{2+}\).

FIG. 1. Mg\(^{2+}\) dependence of valyl-tRNA synthetase catalyzed ATP-\(^{32}\)PP\(_i\) exchange and valyl-tRNA\(^{\text{val}}\) synthesis. The reaction conditions are similar to those of Fig. 1. Five times as much enzyme was used in the experiments with spermine.

Effects of Spermine and EDTA—To eliminate the effects of contaminating metal ions, Pastuszyn and Loftfield (4) added \(10^{-4}\) M EDTA to their reaction mixtures; this decreased the observed rates. We first examined the effect of spermine alone; in our reaction mixtures, as shown in Fig. 2, spermine causes a small but consistent stimulation over a broad range of concentrations. However, when EDTA was added in the presence of \(10^{-3}\) M spermine, the reaction was inhibited as shown in Fig. 3. The complete inhibition suggests that the reaction, even in the presence of spermine, depends upon a cation that forms a complex with EDTA. Addition of more spermine does not reverse the inhibition.

An objection to the use of EDTA to demonstrate an obligatory role for metal ions is the hypothetical inhibition of the enzyme (in the absence of added Mg\(^{2+}\)). The reaction conditions are similar to those of earlier figures. The amount of enzyme used was 0.5 unit per ml.

FIG. 2. The effect of spermine (O---O) and Mg\(^{2+}\) (C---C) on the aminoacylation of tRNA\(^{\text{val}}\). The reaction conditions are similar to those of Fig. 1. Five times as much enzyme was used in the experiments with spermine.

FIG. 3. The effect of EDTA (tetrasodium salt) on the aminoacylation of tRNA\(^{\text{val}}\) in the presence of spermine \((10^{-3}\) M). The reaction conditions are similar to those of earlier figures. The amount of enzyme used was 0.5 unit per ml.
by free EDTA. To examine this possibility we have studied the effect of varying the Mgsup+ concentration in the presence of EDTA. At pH 8.0 in the presence of 10^-3 M EDTA and 2 x 10^-4 M ATP, low concentrations of Mgsup+ should form mainly an EDTA complex but over 10% should exist as MgATP, because the binding constant of ATP for Mgsup+ is approximately 10^7 that of EDTA at this pH (8). The data of Fig. 6 indicate that the rate of aminoacylation of tRNAVal is proportional to the concentration of MgATP. The absolute activity of tRNAVal synthetase was as described under "Materials and Methods." Mgsup+ concentrations were varied as indicated.

**Metal Ions Bound to tRNA**—The experiments described above indicated that our tRNA preparations contained activating cations. This appeared to be true even though precautions were taken to remove divalent ion contamination. All reagents were prepared with glass-distilled water, the enzyme was dialyzed against 10^-3 M EDTA and 10^-4 M ATP, tRNAVal was treated with 1 mM EDTA and then dialyzed against 10^-4 M EDTA. Analysis of reagents by atomic emission and atomic adsorption spectroscopy indicated all reagents including spermine contain much less than 10^-8 M Cu^{2+}, less than 10^-9 M Al^{3+}, Ca^{2+}, and Mg^{2+}. The absolute amounts of these contaminants were not determined. Analysis from the laboratory of Kayne and Cohn (9) indicated the contamination to be 0.77 g atom of Zn^{2+}, 0.94 g atom of Cd^{2+}, and small amounts of Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} per molecule of EDTA-dialyzed enzyme.

We attempted to remove these trace amounts of metal ions by passage through Chelex 100, which is known to be a very strong metal binder. Unfortunately, atomic emission spectroscopy indicated that the samples passed through such columns contain more metal contamination (Cu^{2+}, Al^{3+}, Ca^{2+}, and Mg^{2+}) than untreated samples.

The introduction of metal by tRNA is seen more easily when crude tRNA is used instead of the purified acceptor for a given amino acid. In Table I are shown rates of esterification of tRNAVal with crude and purified preparations. The amount of crude tRNA used was 27 times that of the purified material, in order to provide equivalent amounts of valine acceptance. The rate of reaction was approximately 1 order of magnitude greater when crude tRNA was used in the absence of added metal ions.

In the presence of 10^-4 M EDTA, spermine stimulated the crude system to one-third the maximum rate obtained with Mg^{2+}, whereas in the purified system only about 2% of the maximum activity was found in the presence of spermine, and even this reaction was essentially eliminated by 10^-3 M EDTA.

**Studies of Robison and Zimmerman** (9) on the cation dependence of the transfer reaction of tRNAVal from baker's yeast were interpreted as showing that spermine is more effective than Mg^{2+} in transferring phenylalanine from isolated Phe AMP enzyme complex to tRNAVal although the initial rate of transfer is higher with Mg^{2+}. The amount of spermine required to catalyze this reaction maximally was only 6 μM, in comparison to the 0.3 mM Mg^{2+} needed. This indicates a very high binding constant for spermine with transfer RNA. Spermine also affected the intensity of fluorescence more effectively than Mg^{2+} (9).

These observations led us to study the effect of spermine on the requirement for Mg^{2+} in the transfer reaction. The data of Fig. 5 show that spermine causes a significant stimulation of the rate of esterification when suboptimal concentrations of Mg^{2+} were added. The stimulation shown for 10^-4 M spermine was identical with that obtained with 10^-2 M spermine. However, as the Mg^{2+} concentration is increased, the stimulation decreases and the maximum velocity is not changed by the presence or absence of spermine.

**PPi Exchange and Pyrophosphorolysis of Val AMP in Presence of Spermine**—In the experiments described above spermine

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**TABLE I**

| tRNA | MgCl | Spermine | EDTA | Esterification velocity |
|------|------|----------|------|------------------------|
| Crude | 1 | 1 | 1 | 520 |
| | 2 | 1 | 0.1 | 5500 |
| tRNAVal (85% pure) | 2 | 1 | 1 | 4500 |
| | 1 | 1 | 0.1 | 162 |
| | 1 | 1 | 0.1 | 10 |
| | 1 | 1 | 0.1 | 20 |
| | 1 | 1 | 0.1 | 4200 |

**Fig. 4**. The effect of increasing concentrations of Mgsup+ on inhibition of aminoacylation by EDTA. Reactions at pH 6.9 (■) and pH 8.0 (C) in the presence of added EDTA, (■) and (C) represent reactions at pH 6.9 and 8.0, respectively, in the presence of 10^-4 M EDTA. The reaction conditions are as described under "Materials and Methods." Mgsup+ concentrations were varied as indicated.
J/ml of mercaptoethanol, 0.2 pmol of Na₄₃₂PZ₀₇ (3.7 to 3.9 x 10⁻⁵ cpm/pmol), 0.1 to 0.2 pmol of Val-AMP, magnesium acetate, 10⁻⁵ to 10⁻⁷ M.

with no spermine. The concentrations of Mg²⁺ were varied from 0.5 ml of a 30 mg/ml acid-washed charcoal suspension was added started by addition to an otherwise complete reaction mixture of tRNA synthetase-catalyzed pyrophosphate exchange observed in a G-M gas flow counter. Reaction mixtures lacking enzyme activity of discs dried under infrared irradiation was determined which was washed with an additional 50 ml of ice-cold water. Radioactivity of discs dried under infrared irradiation was determined in a G-M gas flow counter. Reaction mixtures lacking enzyme served as blanks. Under the conditions of these experiments, 1 and 13 mm Mg increased the activity by 3- and 5-fold, respectively, compared to controls without added magnesium. □—□, 13 mm Mg; ○—○, 1 mm Mg; ■—■, control, no magnesium.

did not stimulate the ATP-PP₁ exchange reaction whereas in another study (4) it actually inhibited the residual reaction still observed in the absence of added divalent metal ions. To explore further the role of spermine in the ATP-PP₁ exchange reaction it was desirable to study its effect on both the formation of the enzyme-Val-AMP complex from ATP and valine and on the reverse of this reaction. An attempt to study the forward reaction under the conditions of the over-all reaction but excluding the binding of nonreacting substrates met with considerable technical difficulties. On the other hand, pyrophosphorylase of Val-AMP in the presence of spermine could be followed readily. Due to the instability of Val-AMP in neutral or alkaline solution, our experiments were performed at pH 6.0. As seen in Fig. 6, compiled from several separate experiments, spermine also inhibited the pyrophosphorylase of Val-AMP. Inhibition was observed also in a similar experiment but in the presence of 1 mm EDTA (not shown). No effect of spermine on the reaction was observed in the presence of a considerably suboptimal or a usual Mg²⁺ concentration. The significance of the small but reproducible stimulation in 13 mm Mg is unclear. Together with the observation that pyrophosphorylase proceeded at an appreciable rate even in the absence of added magnesium ions this pattern of behavior resembles that of the over-all valyl-tRNA synthetase-catalyzed pyrophosphate exchange observed by Pastuszyn and Loftfield (4).

Theoretically, the possible roles of cations in aminoacyl-tRNA synthetase activity include the formation of a complex with ATP, conferring a necessary structure on tRNA and binding to the enzyme. In general, ATP serves as a substrate for all enzymes only as a metal complex, usually with Mg²⁺, although in many cases other divalent metal cations can be substituted. Holler (11) has shown that the complex of ATP with spermine does not react with the enzyme. Indications that polyamines can support aminoacylation of tRNA, thus, led to proposals of radically different mechanisms than had previously been accepted. The finding that all of the stimulation caused by spermine is eliminated by EDTA reduces the strength of these proposals; it is necessary to consider a fundamental role for divalent metal ions even in the presence of spermine.

The source of divalent cations in our system is primarily the tRNA. It is not clear why dialysis against EDTA is relatively ineffective in removing these ions. It can be estimated, however, that the quantity of purified tRNA used in esterification reactions, at least 10⁻⁹ M, would carry sufficient metal to support a significant rate of reaction. Increased amounts of crude tRNA necessary to provide amino acid acceptance equivalent to the purified species would provide much more metal (6). Since most of this bound metal can be displaced by 10⁻⁴ M spermine, the formation of metal-ATP can account for all of the stimulation observed on the addition of polyamines.

Independent evidence for a role for polyamines in the transfer of amino acid from an aminoacyl-adenylateenzyme complex to tRNA has been reported by Robison and Zimmerman (9). They correlated the rate of transfer with changes produced in the physical properties of the tRNA. It should be noted, however, that the rates of transfer reported are approximately 2 orders of magnitude less than that calculated from the turnover of the enzyme used. This discrepancy suggests that their observations did not deal with the rate of transfer but with another property of the system. In an unpublished study, preliminary evidence was obtained for slow attainment of equilibria in the transfer reaction. It is possible that the effects of various cations may be attributed to the displacement of equilibria.

The small differences observed in the concentration dependence of the partial and over-all reactions can be understood in terms of the distribution of cations in the different reaction mixtures. It is clear that a metal ion complex of ATP is a necessary reagent in all cases; it is not yet clear whether metal ions play another role, associating with enzyme or tRNA. Our failure to observe stimulation of ATP-PP₁ exchange reactions by polyamines can be explained by the absence of divalent metal ions from the reaction mixtures. From Pastuszyn and Loftfield's data (4) it appears that spermine actually inhibited this reaction as it inhibited the pyrophosphorylase of Val-AMP in our study. This could be due either to a decrease in the effective concentration of pyrophosphate or to a direct effect on the enzyme, as observed with the isoleucyl synthetase (9). It appears, therefore, that the residual PP₁-exchange activity still observed in the presence of spermine (4) is more likely to represent incomplete inhibition (as observed by Holler for isoleucyl-AMP formation (11)) rather than weak catalysis of the forward reaction.

The finding that stimulation of aminoacylation of tRNA by polyamines is an artifact eliminates the necessity to propose a concerted reaction mechanism for the aminoacyl-tRNA synthetase (2). This finding, however, does not distinguish between

1 A. H. Mehler, D. Kern, and J. P. Ebel, unpublished data.
alternative mechanisms and a more positive analysis by kinetic methods has been carried out to establish the role of aminoacyl-AMP as an intermediate (12).

The experiments of others that indicate a role for spermine as a substitute for Mg\(^{2+}\) were carried out with a variety of preparations under various conditions. In view of our results, we must conclude that each of these experiments was carried out under conditions that permitted the artifact described in this article to give a false impression that Mg\(^{2+}\) is not required by the synthetase studied. Since submission of this article we have been informed of the independent studies of Santi and Webster (13) that corroborate our conclusions. Also, Kayne and Cohn\(^2\), stated that spermidine will not stimulate the aminoacylation of tRNA\(^{38}\) by Escherichia coli isoleucyl-tRNA synthetase when the Mg\(^{2+}\) concentration is less than 5 \(\mu\)M.

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