RECONFIRMATION OF REPLACEMENT OF MAGNESIUM ION REQUIREMENT BY POLYAMINES IN ISOLEUCYL-tRNA FORMATION IN *ESCHERICHIA COLI*

Yoshifumi TAKEDA and Yuko OGISO

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka 565, Japan

Received 17 February 1976
Revised version received 28 May 1976

1. Introduction

Previously we reported that polyamines, such as spermine, spermidine and putrescine stimulate aminoacyl-tRNA formation in the absence of added Mg^{2+} [1] and that in the presence of polyamines, isoleucine-dependent PP_i–ATP exchange did not occur, whereas isoleucyl-tRNA formation did [2]. These findings were supported by other experiments in this laboratory [3–6] and elsewhere [7–9].

However, recently Thiebe [10] reinvestigated the effect of spermidine on aminoacylation and concluded that our results were due to contamination of the tRNA preparation with Mg^{2+}, since he claimed that the Mg^{2+} required in aminoacylation could not be replaced by spermidine. This conclusion was supported by the report of Chakraburtty et al. [11]. Santi and Webster [12] also claimed that the stimulatory effects of polyamines which we had reported were artifacts due to Mg^{2+} contaminating the tRNA preparation.

This paper reports that our previous finding that polyamines stimulate isoleucyl-tRNA formation in *Escherichia coli* in the absence of added Mg^{2+} is not due to contamination of our tRNA preparation with Mg^{2+}.

2. Materials and methods

Isoleucyl-tRNA synthetase of *E. coli* B was isolated and purified approx. 1000-fold by column chromatographies on hydroxylapatite and DEAE-Sephadex A-50 as described previously [3,4]. Examination of the purified enzyme preparation with an atomic absorption spectrophotometer (Hitachi, model 208) showed that it contained about 4.6 atoms of Mg^{2+} per enzyme molecule.

The standard reaction mixture for assay of isoleucyl-tRNA formation and the assay procedure were essentially as described previously [3,5,6]. The reaction mixture (0.1 ml) contained 0.1 M Tris-HCl, pH 7.8, 6 mM 2-mercaptoethanol, 1 mM ATP, 2.4×10^{-5} M [^{14}C]isoleucine (42.4 mCi/mmol, New England Nuclear Corp.) and 1.2 μg of the purified isoleucyl-tRNA synthetase. Cations and tRNA were added as specified in the table and figures. Reaction mixtures were incubated at 37°C for 60 min, and then a 0.075 ml sample of each was placed on a paper disc (25 mm in diameter), and the cold trichloroacetic acid-insoluble radioactivity was counted in a Beckman liquid scintillation spectrometer.

The tRNA used was prepared as follows. EDTA-treated tRNA: A solution of 20 to 40 mg of *E. coli* B tRNA (General Biochemicals) in 1 ml of distilled water was dialyzed successively against 2 liter volumes of 1 mM EDTA containing 2.0 M NaCl, 1 mM EDTA and then distilled water for at least 16 hours each at 4°C. This preparation was essentially the same as that used in our previous work [2–6, 13–17]. Phenol-treated tRNA: This was prepared as described by Thiebe [10]. Four mg of *E. coli* B tRNA (General (Biochemicals) were dissolved in 2 ml of buffer containing 1 M sodium chloride and 0.05 M EDTA, saturated with phenol at 4°C, and adjusted to pH 8.0. The solution was then dialyzed four times against 1 liter volumes of the same buffer at 4°C for 48 h,

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and then successively against 1 liter of 1 M sodium chloride for 3 h and 1 liter of distilled water for 5 h with several changes of the dialysate.

The Mg^{2+} contents of the tRNA preparations and other components were measured with an atomic absorption spectrophotometer, Hitachi model 208. After an appropriate dilution of the samples, various amounts of the standard Mg^{2+} solution were added as the internal standards. The concentrations of the internal standards were 2×10^{-2}, 4×10^{-2}, 6×10^{-2} and 8×10^{-2} ppm. The Mg^{2+} contents of the samples were calculated from the values of absorbance at 2852 Å of samples with and without the internal standards. A mixture of acetylene gas and air was used for the fuel. The final concentration of the tRNA preparations and other components analysed by the atomic absorption spectrophotometer were as follows: untreated tRNA, 5 μg/ml; EDTA-treated tRNA, 0.5 mg/ml; phenol-treated tRNA, 0.5 mg/ml; ATP, 5 mM; isoleucyl-tRNA synthetase, 0.24 μg/ml; spermine, 7.5 mM; 2-mercaptoethanol, 0.3 M; Tris-HCl buffer, 0.5 M. The whole reaction mixture was analyzed after 10-fold dilution.

When ATP was passed through a column of Chelex 100 (capacity 0.3 mmol/ml, 1 × 4 cm) as reported by Thiebe [10], it was found that its Mg^{2+} content became more than that of untreated ATP. A similar finding was reported by Chakraburrty et al. [11]. Thus, untreated ATP was used in this study.

3. Results

3.1. *Mg^{2+} contents of tRNA preparations*

The Mg^{2+} contents of various tRNA preparations were measured with an atomic absorption spectrophotometer and the results are shown in table 1. It was found that *E. coli* B tRNA contained 7.8 atom of Mg^{2+} per mole of tRNA, assuming that the mol. wt. of tRNA is 2.5×10^4. When this preparation was dialyzed against EDTA solution as described above (EDTA-treated tRNA), its Mg^{2+} content decreased to 0.08 atom per mole of tRNA. When tRNA was treated as reported by Thiebe [10] (phenol-treated tRNA), its Mg^{2+} content decreased to almost the same level as that of EDTA-treated tRNA.

Table 1
Mg^{2+} contents of various tRNA preparations

Sample	Mg^{2+} content (atom/mole of tRNA)
Untreated tRNA	7.8
EDTA-treated tRNA	0.08
Phenol-treated tRNA	0.10

Table 2
Mg^{2+} contents of the reaction mixture and of its various components

	Concentration of Mg^{2+} (mM)
Whole reaction mixture	
before the incubation	0.0177
after the incubation	0.0185
EDTA-treated tRNA, 400 μg	0.013
ATP, 0.1 μmol	0.00062
Ile-tRNA synthetase, 1.2 μg	0.00056
Spermine, 0.3 μmol	0.0011
Isoleucine, 2.4 nmol	0.00086
2-mercaptoethanol, 0.6 μmol	0.000033
Tris-HCl buffer, 10 μmol	0.00023

3.2. *Mg^{2+} contents of the reaction mixture*

The Mg^{2+} content of the standard reaction mixture was measured after incubation at 37°C for 60 min. As shown in table 2 (upper column), Mg^{2+} concentration of the reaction mixture was 0.0177 mM before the incubation. This amount of Mg^{2+} is not sufficient to stimulate isoleucyl-tRNA formation. The Mg^{2+} concentration did not increase significantly during the incubation, indicating that there was no significant release of Mg^{2+} from the glassware. The Mg^{2+} contents of various components of the reaction mixture were also measured, the results being shown in table 2 (lower column). It was found that more or less Mg^{2+} was contaminated in all components of the reaction mixture, among which the tRNA was most contaminated. The total concentration of Mg^{2+} derived from these components was calculated to be 0.016403 mM. This value corresponded well to that observed with the whole reaction mixture. In this calculation the mol. wt. of isoleucyl-tRNA synthetase was assumed to be 114 000, as reported by Arndt and Berg [18].

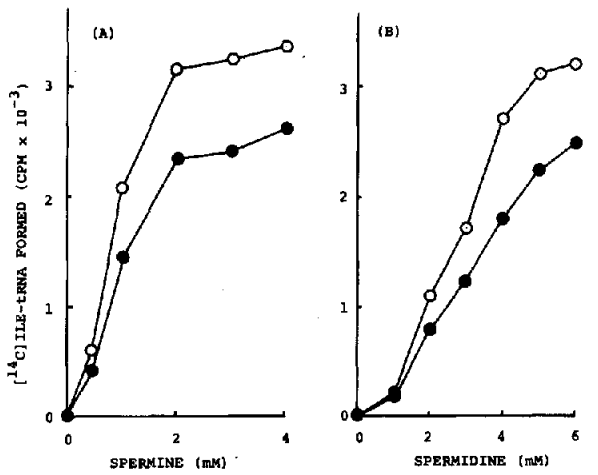

Fig.1. Stimulation of isoleucyl-tRNA formation by polyamines. The components of the reaction mixture and the assay procedure were as described in the text. Either spermine (A) or spermidine (B) was added as indicated. 400 μg of either EDTA-treated tRNA (○——○) or phenol-treated tRNA (●——●) (assuming that 1 A_{260} unit is equivalent to 40 μg of tRNA) were used. Values are averages of duplicate determinations. The control value (100 cpm), obtained without adding cations, was substracted from each value.

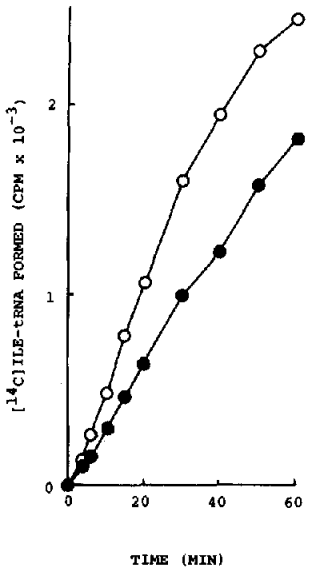

Fig.2. Kinetics of isoleucyl-tRNA formation in the presence of spermine. The experimental conditions were as described in the legend to fig.1 except that 3 mM spermine was added and the time of incubation was varied as indicated. (○——○), 400 μg of EDTA-treated tRNA; (●——●) 400 μg of phenol-treated tRNA.

3.3. *Stimulation of isoleucyl-tRNA formation by polyamines using EDTA- and phenol-treated tRNA*

The results in fig.1 show that isoleucyl-tRNA formation catalyzed by purified isoleucyl-tRNA synthetase was stimulated by polyamines, such as spermine and spermidine, with either EDTA- or phenol-treated tRNA. Without addition of any cations no isoleucyl-tRNA formation occurred. On adding polyamines only, EDTA- and phenol-treated tRNA were charged with [^{14}C]isoleucine at essentially the same rates. The kinetics of isoleucyl-tRNA formation in the presence of spermine was studied with either EDTA- or phenol-treated tRNA (fig.2). Similar results were obtained with both kinds of tRNA.

4. Discussion

Stimulation of aminoacyl-tRNA formation by polyamines has been reported from this laboratory [1–6] and elsewhere [7–9]. However, recent reports [10–12] have indicated that polyamine-stimulated aminoacylation is an artifact. Thiebe [10] reported that our previous results were due to contamination of our tRNA preparation with Mg^{2+}. Moreover, Chakraburtty et al. [11] concluded that the stimulation of aminoacylation by spermine was a function of the amount of tRNA used, because tRNA preparations contain bound cation, even after dialysis against 10^{-4} M EDTA. Similarly, Santi and Webster [12] ascribed the stimulatory effects of polyamines on aminoacylation reported previously as due to Mg^{2+} contaminating the tRNA.

As shown in table 1, the Mg^{2+} content of our tRNA preparation (EDTA-treated tRNA) was very low and less than that in tRNA prepared as described by Thiebe [10] (phenol-treated tRNA). The concentration of Mg^{2+} in the reaction mixture was 0.0177 mM, among which 0.013 mM was derived from 400 μg of EDTA-treated tRNA. This amount of Mg^{2+} is far less than the amount required to stimulate the reaction.

Moreover, we have recently found that when EDTA-treated tRNA is incubated with either spermine or spermidine and then filtered through a Sephadex

G-100 column, the tRNA preparation becomes freed from unbound spermine, spermidine and Mg^{2+}, if present, and that the tRNA can act as a substrate of aminoacylation without addition of any further cations [19].

From these observations we conclude that the stimulation of aminoacylation by polyamines we reported previously [1–6] is not due to Mg^{2+} contaminating the EDTA-treated tRNA used.

Thus the question arises of why Thiebe [10] and Santi and Webster [12] did not observe aminoacylation with their tRNA preparations in the presence of polyamines. As reported by ourselves [6] and by Chakraburtty et al. [11], polyamine-stimulated aminoacylation is inhibited by EDTA. We reported that this inhibitory effect of EDTA is due to chelation of ions which are tightly bound to the enzyme, and which are essential for its catalytic activity [6]. From the descriptions of Thiebe [10] and Santi and Webster [12], it seems possible that their reaction mixtures for aminoacylation were contaminated with EDTA. Thiebe [10] prepared crude aminoacyl-tRNA synthetase and dialyzed it against 10 mM EDTA to chelate possible Mg^{2+} contaminating the preparation. Santi and Webster [12] prepared tRNA by filtering it through Sephadex G-25 at 50°C using 150 mM EDTA. Thus it is possible that in their reactions small amounts of EDTA derived from these preparations inhibited the aminoacylation.

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