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An Assessment of Polynucleotide Inhibition Studies of Aminoacyl-Transfer Ribonucleic Acid Synthetases*

Virginia Zewe Holten† and K. Bruce Jacobson

ABSTRACT: The nature of the interaction between an amino acid and its specific transfer ribonucleic acid (tRNA), as catalyzed by a specific aminoacyl-tRNA synthetase, was investigated using commercially available polynucleotide inhibitors. The basis for such an experimental approach rests upon the hypothesis that this inhibition could involve a specific interaction between the active site of the enzyme and a certain sequence of the polynucleotide corresponding to the recognition site in the tRNA. The results indicate that the data obtained from such experiments are equivocal and must be interpreted with caution. First of all, the degree of polynucleotide inhibition is strongly dependent upon the Mg^{2+} concentration, being much more pronounced under conditions when the cation concentration is limiting. This raises the question as to whether the polynucleotide is inhibiting by virtue of competing with the tRNA for the active site on the enzyme or by Mg^{2+} binding. Secondly, the inhibitory principle in the commercial polynucleotide preparations survived alkaline, acid, and snake venom phosphodiesterase destruction. Thirdly, the criterion of competitive inhibition between the tRNA and the polynucleotide inhibitor as evidence for binding at the active site of the enzyme is questionable, since the metal chelator EDTA also acts competitively with respect to tRNA. Thus, until the metal requirements of each of the components in the system are understood and satisfied, it is not possible to attribute inhibition by polynucleotides to binding at the active site.

A perplexing problem in the area of protein biosynthesis lies in an understanding of the process by which a specific amino acid is attached to a particular tRNA. At present, the nature of this interaction is little understood. One approach to the problem suggested by Hayashi and Miura (1964) involves the use of synthetic oligonucleotides as inhibitors of the aminoacyl-RNA synthetase reaction. A possible mechanism for this inhibition could involve a specific interaction between the active site of the enzyme and a certain sequence of the oligonucleotide corresponding to the recognition site in the tRNA. Thus, those oligonucleotides which function competitively with respect to tRNA would give some indication as to the nucleotide structure at the recognition site of the tRNA molecule.

Recently, several reports which utilize this approach

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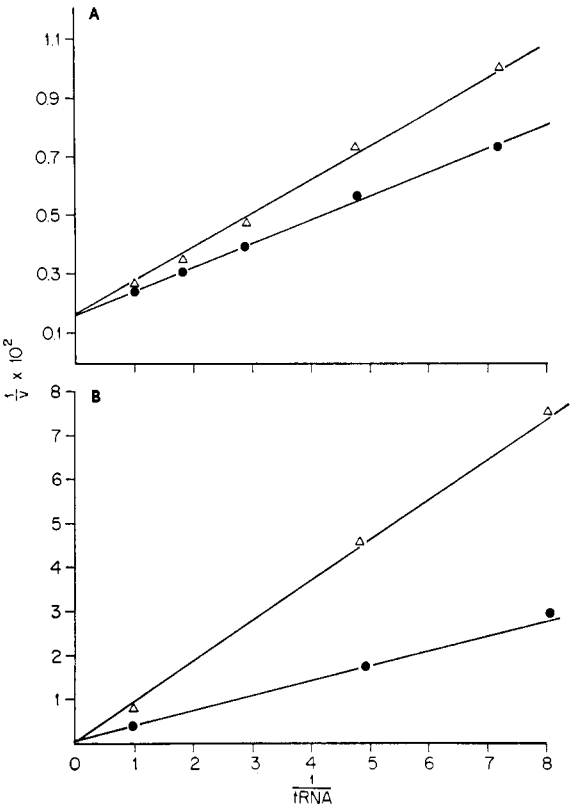

FIGURE 1: Plot of reciprocal of initial reaction velocity (counts per minute of [^{14}C]valine incorporated into tRNA^{Val} in 5 min) *vs.* the reciprocal of the tRNA concentration (expressed as A_{260} units per reaction mixture): in the absence of (●) and presence of (Δ) poly (A,C,U). (A) Mg^{2+} concentration is optimal. The reaction mixture samples contained 0.5 mM ATP, 5 mM magnesium acetate, 10 mM mercaptoethanol, 0.1 μc of [^{14}C]valine, tRNA^{Val} (0.14–1.05 A_{260} units), and 0.15 μg of enzyme in 0.05 M Tris-Cl buffer (pH 8.5). Where indicated, 9.7 A_{260} units of poly (A,C,U) was added. (B) Mg^{2+} concentration is limiting. The reaction mixture was the same as in 1A except that the magnesium acetate concentration was 0.5 mM. The tRNA concentration ranged from 0.125 to 1.25 A_{260} units/reaction tube. Where indicated, 4.8 A_{260} units of poly (A,C,U) was added.

have appeared. Deutscher (1965) has found poly G and poly I to be effective inhibitors of rat liver glutamyl-RNA synthetase. Hayashi and Miura (1966) reported that with aminoacyl-RNA synthetases from yeast, the oligonucleotide complementary to the messenger codon corresponding to a given amino acid caused competitive inhibition, whereas the codon sequence itself showed slight effect. Phenylalanyl-, glutamyl-, and lysyl-RNA synthetases from *Escherichia coli* were inhibited by certain oligonucleotides, although the inhibition did not appear to be related to either the codon or the anticodon of the amino acid concerned (Letendre *et al.*, 1966).

In the present series of investigations, various polyribonucleotides were tested on the valyl-RNA synthetase system from *E. coli*. The purpose of this communication is to relate some of the difficulties involved in interpretation when polynucleotides are used as inhibitors in synthetase reactions.

Methods

The valyl-RNA synthetase used in these studies was purified from *E. coli* B according to the procedure of Bergmann *et al.* (1961). Uniformly labeled [^{14}C]-valine (208.5 mc/mmole) was purchased from New England Nuclear Corp., and all polynucleotides[1] were Miles Chemical Co. products. All polynucleotides were dissolved in distilled water, and the pH was adjusted to 6.7. tRNA^{Val}, approximately 40% pure, was obtained by reverse-phase chromatography of *E. coli* tRNA (Kelmers *et al.*, 1965) and was dialyzed *vs.* distilled water before use. Aminoacyl-tRNA formation was measured according to the filter paper disk method of Bollum (1959) as modified by Mans and Novelli (1961), and samples were counted in a Packard Tri-Carb scintillation spectrometer.

Reaction samples in a final volume of 0.25 ml were incubated at 37°, and two 0.1-ml aliquots were withdrawn at intervals. In all instances, initial reaction velocity was measured.

The concentration of tRNA or polyribonucleotide was measured spectrophotometrically in 0.05 M Tris chloride buffer (pH 8.0) at 260 mμ in a cuvet of 1-cm light path. One A_{260} unit is that amount of RNA that gives an absorbance of 1.0 when measured under these conditions. The amount of tRNA or polyribonucleotide in an incubation mixture is given as A_{260} units added to a 0.25-ml volume. Thus the absorption at 260 mμ of the material in the incubation mixture would be four times the A_{260} units value.

Results

In the initial phase of the investigation, 50 polyribonucleotides of varied base composition were screened for their ability to inhibit the valyl-tRNA synthetase enzyme system. Fifteen polynucleotides containing various ratios[1] of A, C, or U (poly (A,C), poly (A,U), and poly (C,U)) inhibited the reaction from 10 to 20%. The reaction did not appear to be inhibited by the homopolymers A, C, U, I, and xanthylic acid nor by poly (I,U) (A,G) (3:1), (A,C,G,U) (1:7:1:1), or (G,U) (1:40). In these experiments, the final concentration of inhibitor in the reaction ranged from 2 to 33 A_{260} units, while the final tRNA concentration was 1.5 A_{260} units/reaction sample. Magnesium acetate concentration was 10 mM, and the magnesium:ATP molar ratio was 10:1.

[1] The nucleotide composition of the polynucleotides is specified by the manufacturer as the ratio of nucleoside diphosphate present at the initiation of polymerization by polynucleotide phosphorylase.

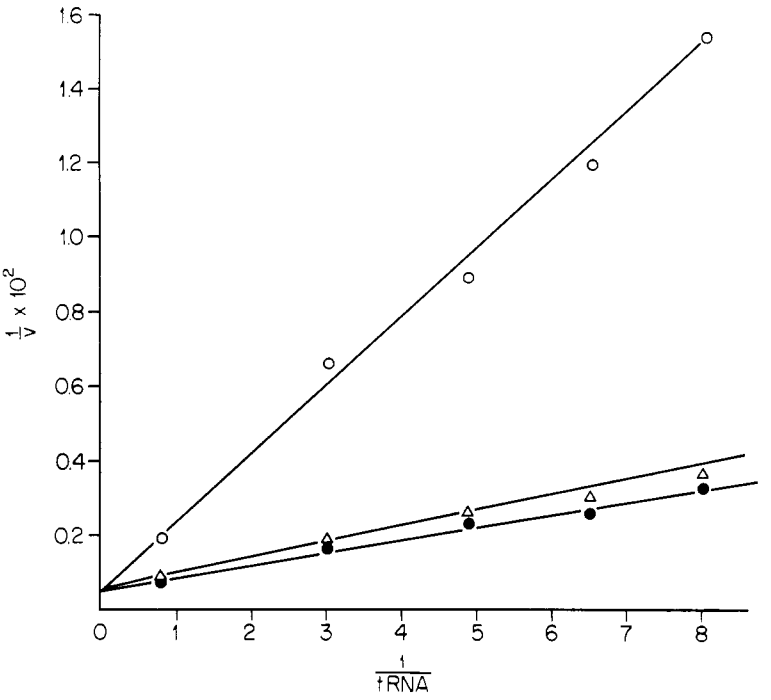

FIGURE 2: Plot of reciprocal of the initial reaction velocity *vs.* the reciprocal of tRNA concentration in the absence of and presence of EDTA. Magnesium acetate concentration was limiting. Reaction mixture samples contained 0.5 mM ATP, 0.5 mM magnesium acetate, 10 mM mercaptoethanol, 0.1 μc of [^{14}C]valine, tRNA^{Val} (0.125–1.25 A_{260} units), and 0.15 μg of enzyme in 0.05 M Tris-Cl buffer (pH 8.5). EDTA concentrations were none (●), 0.1 (△), and 0.2 mM (○). Samples were removed for assay at 14 min.

In an attempt to examine this interaction in more detail, poly (A,C,U) (1:1:1) was tested, since this polynucleotide would contain the various permutations of A, C, and U which appeared to inhibit the reaction in the initial screening. Examination of the Lineweaver–Burk (1934) plot shown in Figure 1A indicates that poly (A,C,U) appears to function as a competitive inhibitor with respect to tRNA.

In view of the recent report that magnesium may be of great importance in tRNA configuration (Gartland and Sueoka, 1966; Lindahl *et al.*, 1966), the valyl-RNA synthetase reaction was examined at various magnesium concentrations, while the ATP[2] was maintained constant at 0.5 mM. In the absence of Mg^{2+}, no reaction occurred; while at equimolar concentrations of magnesium and ATP, the reaction rate observed was about 40% of that observed at the optimal concentration of the cation (5 mM magnesium acetate and 0.5 mM ATP). At equimolar concentrations of Mg^{2+} and ATP (0.5 mM), poly (A,C,U) proved to be an extremely potent inhibitor of the valyl synthetase reaction, producing complete inhibition. Under optimal conditions, comparable amounts of polymer inhibited only 10–20%. The double reciprocal plot shown in Figure 1B reveals that when magnesium concentration is low, the inhibition by poly (A,C,U) is also competitive with respect to tRNA.

Since the metal ion concentration influences the degree of inhibition by poly (A,C,U), the effect of a metal chelator on the system was of interest. The results are shown in Figure 2. EDTA appears to function as a competitive inhibitor with respect to tRNA. Thus, the criterion of using competitive inhibition between polynucleotide and tRNA as proof of binding at the active site appears to be equivocal. The involvement of Mg^{2+}, not only in the ATP-requiring activation step but also in binding to tRNA or the polynucleotide, precludes any simple interpretation of the data.

Since exhaustive dialysis of the polynucleotide *vs.* EDTA is one of the steps in its isolation procedure (Miles Chemical Co. catalog), the possibility that enhanced inhibition at low magnesium concentration could be attributed to the tightly bound chelator was next considered. In the test system, the effectiveness of EDTA as a competitive inhibitor was assessed at increasing magnesium concentrations. At a magnesium:EDTA ratio of 10:1, the chelator was no longer an effective inhibitor. On the contrary, poly (A,C,U) inhibition could be decreased but not eliminated by increasing the magnesium concentration. Thus the possibility that all the inhibition caused by poly (A, C,U) could be traced to a small amount of residual tightly bound EDTA appears to be invalid.

The polynucleotide-dependent nature of the inhibition

[2] Abbreviations used: ATP, adenosine triphosphate; AMP, CMP, and UMP, adenosine, cytidine, and uridine monophosphates.

TABLE I: Effect of Hydrolysis of Poly (A,C,U) on the Inhibition of Valyl-RNA Synthetase.

HCl Hydrolysis[a]			KOH Hydrolysis[c]			Phosphodiesterase Hydrolysis[d]		
	Activity (cpm)			Activity (cpm)			Activity (cpm)	
tRNA A_{260} Units	Control[b]	Poly (A, C, U)	tRNA A_{260} Units	Control[b]	Poly (A, C, U)	tRNA A_{260} Units	Control[e]	Poly (A, C, (U)
0.125	18	0	0.132	28	0	0.125	262	71
0.208	26	0	0.44	68	5	0.208	370	92
1.25	93	0	1.32	251	6	1.25	807	486

[a] Reaction mixture samples contained 0.5 mM ATP, 0.5 mM magnesium acetate, 10 mM mercaptoethanol, 0.1 μc of [^{14}C]valine, indicated amounts of tRNA^{Val}, and 0.15 μg of enzyme in 0.05 M Tris-Cl buffer (pH 8.5). Where indicated, the final amount of hydrolyzed poly (A,C,U) in the samples was 7 A_{260} units. Poly (A,C,U) was hydrolyzed in 1 N HCl for 1 hr at 95°, chilled, and neutralized. The control tube was treated in a similar manner except that no polymer was added. Activity is expressed as counts per minute incorporated into tRNA^{Val}. [b] The difference in the control values for the HCl and KOH hydrolysis experiments is due to a salt inhibition of the synthetase reaction. The salt arose in the course of neutralization of the poly (A,C,U) and control tubes. [c] Poly (A,C,U) was hydrolyzed in 0.1 N KOH at 37° for 16 hr. The control was treated in a similar manner except that no polymer was added. Assay conditions were as indicated in footnote *a*. The final concentration of hydrolyzed poly (A,C,U) in the reaction was 10 A_{260} units. [d] Poly (A,C,U) was hydrolyzed in 0.05 M Tris-Cl (pH 8.5), 3.5 mM magnesium acetate, and 0.3 mg of Worthington snake venom phosphodiesterase at 37° for 18 hr (total volume, 0.9 ml). The control sample contained water instead of poly (A,C,U). Protein was precipitated by placing the reaction mixture in a boiling water bath for 20 min, followed by centrifugation. Assay conditions were the same as indicated in footnote *a*, except that the final concentration of poly (A,C,U) (hydrolyzed) was 10 A_{260} units. When the effect of hydrolyzed poly (A,C,U) was being assessed, no additional Mg^{2+} was added to the assay beyond that already present during the diesterase hydrolysis. The final concentration of magnesium (acetate) in the reaction mixture was 0.6 mM. [e] The high control values reported in the diesterase experiment are due to the following reasons: absence of the salt effect encountered in the HCl and KOH hydrolysis experiments, and the presence of an undetermined amount of magnesium in the snake venom phosphodiesterase preparation used for the hydrolysis.

was next investigated. Poly (A,C,U) was hydrolyzed by using acid, base, or snake venom phosphodiesterase and was then added to the synthetase assay system to ascertain if the inhibitory ability was still present. DEAE paper chromatography using 0.5 M ammonium bicarbonate as solvent (Jacobson, 1964) revealed that in all cases the polymer was completely hydrolyzed to the constituent mononucleotides in the case of alkaline and enzymic hydrolysis. No attempt was made to identify the acid hydrolysis products. Results are summarized in Table I and clearly indicate that in all instances the hydrolyzed polynucleotide preparation still contained inhibitory material. Consequently, the polynucleotide itself appears not to be totally responsible for the inhibition, since the inhibitor survives the destruction of the polymer. That the mononucleotide hydrolysis products were not functioning as inhibitors of the valyl-RNA synthetase reaction was shown by experiments in which 2′,3′-AMP, 2′,3′-CMP, and 2′,3′-UMP singly and in combination at a final concentration in the assay of 10–12A_{260} units did not decrease the formation of [^{14}C]valyl-tRNA. Similarly, the 5′-mono- and -diphosphate nucleosides had no effect on the enzymatic reaction. These results were obtained under conditions of both minimal and optimal Mg^{2+} concentration.

The acid-hydrolyzed polynucleotide was exhaustively dialyzed *vs.* distilled water and the nondialyzable material was examined for its inhibitory ability. The results above were corroborated, *i.e.*, the inhibitory material was still present, even after the destruction of the polynucleotide and the subsequent removal of the hydrolyzed products by dialysis. In these experiments, the nondialyzable material possessed negligible absorbance at 260 and 280 mμ, indicating that all nucleotide had been removed by dialysis. The nature of this inhibitory material is at present unknown.

Poly (A,U) (2:1), (A,C) (1:1), and (C,U) (1:1) were obtained and assayed in the standard valyl-RNA synthetase system. As was the case with poly (A,C,U), aminoacylation of RNA^{Val} was virtually nonexistent under conditions when the magnesium concentration was limiting, while when the cation concentration was optimal, inhibition was marginal.

Discussion

The results presented above indicate that caution must be exercised in the interpretation of polynucleotide inhibition studies of synthetase reactions. Because Mg^{2+} is involved in the activation step and perhaps also in tRNA or polynucleotide binding, it is essential to ascertain that the polynucleotide inhibition is not due simply to removal of the cation by the polymer.

Also, the sole criterion of competitive inhibition by polynucleotide with respect to tRNA as sufficient evidence for its binding at the active site of the enzyme is not satisfactory, as the EDTA experiment indicates. Because of the complications arising from metal binding by the tRNA, the polynucleotide, or the enzyme itself, no simple kinetic interpretation of the competitive-type Lineweaver–Burk plots can be made at this time.

Another problem of primary importance is the source and purity of the poly- or oligonucleotide. The poly (A,C,U) used in this laboratory consisted of four different commercial preparations bearing different lot numbers. These polymer preparations varied considerably among themselves in absorbancy units (A_{260}) per milligram. All solutions contained 4 mg/ml of solution, but the A_{260} units per milliliter were 55, 16, 12 and 77 (lot no. 185, 68, 225 and 238, respectively) for each of the four lots. When the high absorbancy poly (A,C,U) was dialyzed *vs.* distilled water, approximately 50% of the absorbancy was dialyzable.

Another problem which arose in connection with poly (A,C,U) was the inconsistency of the inhibition. The first three lots of poly (A,C,U) (which were used for all of the studies reported here) were inhibitory both before and after dialysis in the valyl-RNA-synthetase system, while the last preparation was not inhibitory even when the magnesium concentration was limiting. This seems to support the contention raised here that inhibition may result from some as yet unidentified contaminant introduced into the polymer preparation during its preparation or subsequent isolation. This does not preclude the possibility that inhibition by the polyribonucleotide may also be occurring under conditions where metal ion concentration is limiting. However, whether this is by virtue of competing with tRNA for an active site on the enzyme or is due to Mg^{2+} binding remains the crucial point. Since the formation of the aminoacyladenylate is dependent on Mg^{2+} and since binding of Mg^{2+} by tRNA and polyribonucleotides occurs, this point may be difficult to establish because different synthetases require different amounts of magnesium for optimal activity (Holten and Jacobson, unpublished observations).

The inhibitory activity of oligonucleotide fragments of RNA on aminoacyl-tRNA synthetase was shown by Hayashi and Miura (1964). Since the unhydrolyzed RNA was not inhibitory, it is unlikely that magnesium binding by the inhibitory fragments was the cause of inhibition. In their latter studies (Hayashi and Miura, 1966) pentanucleotides that had been synthesized chemically were shown to be inhibitory. In the studies of Deutscher (1965) polyribonucleotides, synthesized by polyribonucleotide phosphorylase, were shown to inhibit the synthetase reaction in a restricted and rather specific manner. Since tests were not made to exclude the possibility of the presence of an inhibitor that is stable to alkaline hydrolysis the question of the identity of the inhibitor is raised. We also have observed such selectivity among polyribonucleotides, although in our case an appreciable amount of inhibition was caused by a nonpolynucleotide contaminant. In view of the variable nature of the polynucleotide preparation and the fact that the synthetase was from rat liver one cannot say whether the inhibitor studies of Deutscher (1965) were affected or not by the alkali-resistant inhibitor. Those of Hayashi and Miura (1966) using chemically synthesized pentanucleotides most probably were not, yet for future explorations in this area it is well to recognize these problems.

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