ACTIVE SITES OF t-RNA. I - OLIGONUCLEOTIDE INHIBITION OF AMINO ACID ATTACHMENT

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One of the most important aspects of protein synthesis lies in the processes leading to a highly selective attachment of a specific amino acid to a particular RNA. The recognition of each transfer RNA by the corresponding synthetase has been but little studied until recently. One approach to this problem has been described by Hayashi and Miura (1964) who found that oligonucleotides larger than trinucleotides competitively inhibited valine incorporation. Clearly, one of the various possibilities for this kind of inhibition lies in a specific interaction between active sites of the enzyme and a given sequence in an oligonucleotide corresponding to the recognition sequence in the sRNA. Another possible cause of general inhibition could simply be the interaction of a complementary oligonucleotide with a given sequence in the sRNA. In view of the failure to obtain complexes between sRNA and pure polynucleotide materials, one must postulate that such an interaction occurs at the enzyme surface.

If the specificity of the inhibition depends solely on the composition and sequence of the oligonucleotide, it may be possible to reveal the nature of the active site of the sRNA. We therefore began working on the inhibition by various polynucleotides in the charging of tRNA by phenylalanine (codon UUU, UUC), lysine (codon AAA, AAG) and glutamic acid (codon GAA, GAG), using E. coli synthetases. During the course of this work several publications appeared. The inhibition of rat liver synthetase is particularly effective by poly G and poly I (Deutscher, 1965), and Hayashi and Miura (1966) reported that with yeast synthetase the oligonucleotide complementary to the messenger codon corresponding to the amino acids cause competitive inhibition, whereas the codon sequence itself caused slight inhibition.

RESULTS :

Neither poly U nor poly A at 37° show marked inhibition of

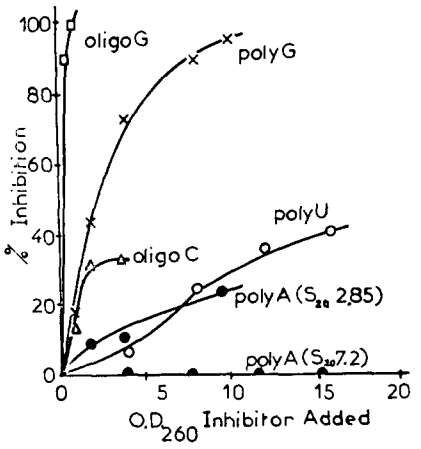

Fig.1 - phenylalanine

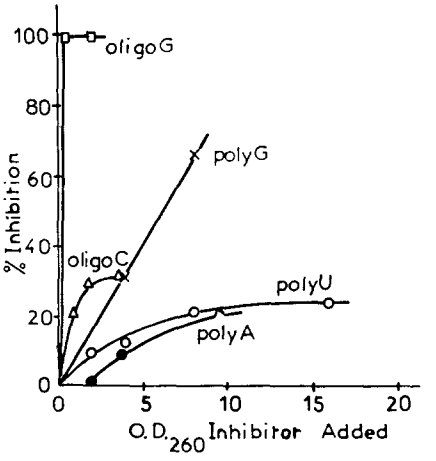

Fig.2 - glutamic acid

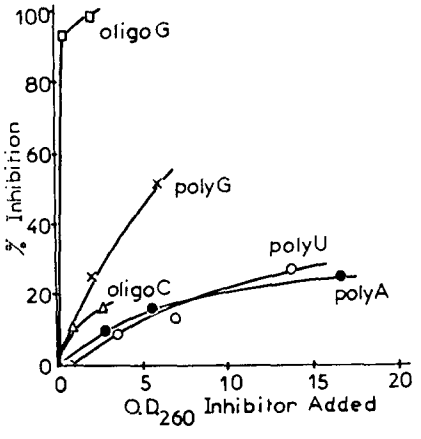

Fig.3 - lysine

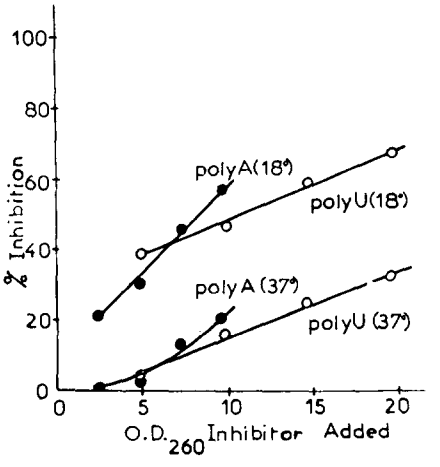

Fig.4 - lysine at 2 temperatures

Figures 1 - 4 : Effect of polymers and oligonucleotides on the charging of t-RNA : Incubation mixtures contained in 0.10 ml.: 100 mM Tris-HCl, pH 7.3; 10 mM $MgCl_2$; 0.28 mg. E.coli B sRNA (General Biochemicals Inc.); 0.3 mg disodium ATP; 0.03 mg CTP; 20,000-30,000 cpm of C^{14}-amino acid; and 20 µg protein. Incubation, 15 min. at 37°. E.coli B synthetases were prepared as reported by Hayashi and Miura (1964) but cells were broken by alumina grinding. Enzyme was stored at -90°. Polymers were prepared as described (Grunberg-Manago et al., 1956) with an average s of 9. Oligo G and oligo C were prepared by hydrolysis, 24 hrs in 7 N NH_4OH at 37°. After removing NH_4OH, the solution of oligo C was chromatographed on Whatman 3MM paper in n-propanol:water:NH_4OH (55:35:10). Oligonucleotides above a chain length of 6 were eluted with water. Assay for charging tRNA was by the method of Ingram and Pierce (1962).
In the experiments shown in fig.4, time of incubation was 5 min at 18° or 37° as indicated.

lysine, phenylalanine or glutamic acid incorporation by E.coli synthetases. Oligonucleotide mixtures obtained by partial alkaline hydrolysis showed no effect. Chemically synthesized oligonucleotides (Michelson, 1959) (A,C,G,U) all showed slight inhibition of lysine incorporation but were completely without effect on phenylalanine incorporation, in contrast with results described by Hayashi and Miura (1965). However, poly G of high molecular weight consistantly showed inhibition for all three amino acids. This inhibition was increased enormously (see Figs.1,2,3) by using alkaline degraded poly G.

A similar dependence on chain length is shown by polymers of cytidylic acid. Poly C itself causes no inhibition whereas oligo cytidylic acid significantly inhibits all three amino acids, although to a much lesser extent than oligo G. This is in contrast with the cases of poly A or poly U where relatively long chain length is essential for inhibition.

Kinetic studies suggested that inhibition of lysine charging by poly U is non-competitive. The results with poly G seemed to indicate both competitive and non-competitive inhibition.

In order to decide whether specific base pairing were involved, particularly between G and C residues which are more stable combinations than A and U residues, various analogues of poly G were examined with respect to lysine charging of t-RNA.

Table I - Effect of analogues of poly G on charging of t-RNA by lysine

Analogues	O.D.260 added	% of inhibition
Poly G s =9.2	2.1	17
Poly G s = 2	2.1	41
Oligo G	2.3	100
Poly I	2.7	0
Oligo I	0.3	42
Poly X	2.1	12
Oligo X	1.8	10
7-Methyl G	2.1	5
7-Methyl I	2.1	7
1,7-dimethyl I	2.1	0

Incubation mixtures were as described for Figs.1-3. Oligonucleotide mixtures of I and X were prepared by hydrolysis in NH_4OH, 7 N, 24 and 7 hrs, respectively. Methylated polymers prepared as described (Michelson and Pochon, 1966).

High molecular weight poly I itself was not inhibitory, but oligo inosinic acid strongly inhibited. Poly dimethyl-I showed no inhibition whatsoever, but 7-Me-G and 7-Me-I, both of which are capable of complexing with poly C with a much lower Tm than poly G, showed a slight inhibition; on the other hand, polyxanthylic acid (poly X), which does not complex with poly C but which does give a very stable complex with poly A, is inhibitory to some extent. No increase in inhibition was obtained by using oligo X, in contrast with oligo G and oligo I (Table I).

If hydrogen bonded base pairing is involved in the inhibition one would expect a striking temperature effect, particularly in the case of poly A, poly U and poly I, but not with poly G, in view of the stability of the respective complexes formed by these polymers. At 18° inhibition of lysine incorporation by either poly A or poly U was more than double as compared to that at 37°. (Fig. 4)

It may also be noted that marked inhibition by oligo G commences at the tetranucleotide, whereas with oligo I a pentanucleotide is necessary (Table II). This possibly reflects the difference in stability of the complex with poly C.

Table II - Effect of chain length and terminal PO_4 in lysine charging

	Nucleotide length	O.D.260 added	% of inhibition
Oligo-I	2	1.5	22
3'-phosphate	3	1.2	29
	4	1.2	17
	5	1.5	65
Oligo-I	2	1.5	22
5'-phosphate	3	1.6	32
	4	1.3	30
	5	1.7	80
Oligo-G	2	2.0	10
3'-phosphate	3	2.0	33
	4	2.0	58
	higher mol. wts.	2.0	88

3'phosphates of oligo I and G were prepared by NH_4OH hydrolysis of poly I and poly G for 24 hrs. 5'phosphates were obtained by a sheep kidney nuclease (Kasai, Grunberg-Manago, in preparation). Fractions of various nucleotide lengths were separated as described in Figs. 1-3.

Terminal PO_4 position (3' or 5') does not appear to play a role.

Since two reactions are catalyzed by synthetases, shown as follows :

E + ATP + AA ========= E-AA-AMP + PP

E-AA-AMP + tRNA ====== E + tRNA + AMP

it was of interest to decide at which step the inhibitory effect occured. No inhibition of PP-ATP exchange was observed, even with large amounts of oligo G, for phenylalanine, lysine or glutamic acid at 37° (Table III). Some effect was observed with poly A and poly U for lysine, particularly at 18°.

Table III - Effect of polymers on the amino acid stimulation of ATP-PP exchange (percent of inhibition)

	Poly A	Poly U	Oligo G
Phenylalanine (37°)	stimul. 19%	16	stimul. 27%
Glutamic acid (37°)	0	0	0
Lysine (37°)	11	5	10
Lysine (18°)	29	21	-

Incubation mixture contained in 0.5 ml (in mMolar concentrations): Tris-HCl, pH 7.8, 200; NaF, 20; $Na_4P_2O_7$, 4 (20,000-50,000 cpm P^{32}); disodium ATP, 4; $MgCl_2$ for lysine and phenylalanine, 10; $MgCl_2$ for glutamic acid, 30; amino acid, 20; protein, 20 µg. Approximately 9 O.D.260 of polymer and oligonucleotides were added. Incubation 15 min at temperatures indicated. Reaction was stopped by addition of 0.5 ml of 0.02 M of sodium pyrophosphate in 7% perchloric acid, followed by 40 mg of Norit A to adsorb ATP.

Hence the inhibitory effect of oligo G is associated only with the second step of the reaction, while with poly A and poly U, the situation remains ambiguous.

DISCUSSION :

Charging of tRNA by synthetases is inhibited by certain oligonucleotides -- at least in the case of the three amino acids studied in this report. While hydrogen bonding ability appears to be essential, the inhibition does not appear to be directly related to either the codon or the anticodon of the amino acid concerned. In all cases oligo G was the most effective inhibitor. The effect of chain length in the case of G or I is perhaps the consequence of the secondary structure of high molecular weight polymers which could tend to inhibit the effect of base pairing for stereochemical reasons. The effect of secondary structure has already been noticed by Deutscher.

Although it is clear, at least in the case of oligo G, that inhibition occurs at the step involving transfer of amino acid from AMP-Enzyme to tRNA, the nature of the mechanism is as yet obscure and further work with both purified synthetase and specific RNA is necessary.

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