Inhibition of an Aminoacyl Transfer RNA Synthetase by a Specific Trinucleotide Derived from the Sequence of Its Cognate Transfer RNA†

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ABSTRACT: In the previous article (Schoemaker, H. J. P., and Schimmel, P. R. (1977), *Biochemistry 16* (preceding paper in this issue) evidence was given suggesting that the uridine at the 8 position in tRNAs plays a role in synthetase–tRNA interactions. The sequence at the 8-9-10 position in $tRNA^{Ile}$ is U-A-G. It was found that the trinucleotide U-A-G is a potent inhibitor of the aminoacylation of $tRNA^{Ile}$. A number of other oligonucleotides, including the reverse sequence G-A-U, have no effect on the aminoacylation reaction. In addition, $tRNA^{Tyr}_2$, which has a different sequence than $tRNA^{Ile}$ at the 8-9-10 position, is not affected by the presence of U-A-G in the aminoacylation reaction with Tyr-tRNA synthetase. Other experiments show that U-A-G does not bind to $tRNA^{Ile}$ and that it does not inhibit isoleucyl adenylate formation. A kinetic analysis of the $tRNA^{Ile}$ aminoacylation reaction shows that U-A-G is a competitive inhibitor of $tRNA^{Ile}$ binding and has a dissociation constant at pH 7.5, 37 °C, of about 110 μM. Equilibrium dialysis experiments confirmed that U-A-G binds to Ile-tRNA synthetase. The uridine H-5 tritium exchange reaction of the trinucleotide in the presence and absence of enzyme was also studied. It was found that Ile-tRNA synthetase catalyzes tritium exchange into the U of U-A-G at a rate comparable to its effect on uridine-8 in $tRNA^{Ile}$. The combined results suggest strongly that U-A-G binds to Ile-tRNA synthetase at exactly the same site on the protein as does the corresponding sequence in intact $tRNA^{Ile}$.

In the preceding article, data were presented suggesting that the common uridine at the 8-position in transfer RNAs plays a role in synthetase–tRNA interactions (Schoemaker and Schimmel, 1977). Moreover, the data raise the possibility that a special kind of chemical interaction occurs between the bound enzyme and this base. If such an interaction does take place, one could imagine that a small oligonucleotide derived from the region around U8 might be able to interact with the enzyme at the site which is normally occupied by the U8 region of the transfer RNA. In the case of $tRNA^{Ile}$, the sequence at the 8-9-10 position is U-A-G. Several years ago, when conducting preliminary equilibrium dialysis experiments of oligonucleotides with Ile-tRNA synthetase, we observed binding of U-A-G to the enzyme (Jekowsky and Shimmel, unpublished). Having more recently established that this region of the $tRNA^{Ile}$ structure appears important for synthetase–tRNA interactions, we returned to explore in more depth the question of whether U-A-G interacts with the enzyme and if so whether the interaction has any significance as far as the mechanism of the synthetase–tRNA interaction is concerned.

We report here a study of the interaction of U-A-G with Ile-tRNA synthetase. It was found that this trinucleotide specifically inhibits the enzyme in the aminoacylation reaction while several other oligonucleotides, including the reverse sequence G-A-U, do not inhibit. Further characterization of the interaction suggests that U-A-G binds to the enzyme at the same site occupied by the corresponding trinucleotide sequence in the intact $tRNA^{Ile}$ structure.

Materials and Methods

Ile-tRNA synthetase and $tRNA^{Ile}$ were obtained as described in the preceding article (Shoemaker and Schimmel, 1977). Chromatographically pure oligonucleotides were kindly donated by Professor O. C. Uhlenbeck. The chromatographic purity of one of these (U-A-G) from one of the batches was confirmed by us. Other experimental details are given in the legends to the tables and figures.

Results

Inhibition by U-A-G of Aminoacylation of $tRNA^{Ile}$ and Specificity of Inhibition. We observed that, at concentrations of roughly 70 μM, U-A-G is a strong inhibitor of the aminoacylation of $tRNA^{Ile}$. The most immediate question is whether the inhibition by U-A-G is specific or whether other oligonucleotides also inhibit the enzyme. This question was approached by studying the effects of five other ologonucleotides, in addition to U-A-G. The results obtained are summarized in Table I. The table shows that significant inhibition is only achieved with U-A-G, and not with any of the other oligonucleotides. Of the other oligomers tested, the one of most interest is G-A-U. Not only does this oligomer serve as an ideal control because it has the reverse sequence of U-A-G, but it also corresponds to the anticodon trinucleotide of $tRNA^{Ile}$ (see Figure 1 of the preceding article). A variety of data have indicated that the anticodon comes into close proximity to the enzyme in the synthetase–tRNA complex (Schimmel et al., 1972; Dickson and Schimmel, 1975; Schoemaker and Schimmel, 1976, 1977). Nevertheless, this reverse-sequence, anticodon trinucleotide does not inhibit the synthetase.

Experiments were also done with Tyr-tRNA synthetase. In the case of $tRNA^{Tyr}_2$, the sequence at the 8-9-10 position is U-U-C (see Figure 2 of the preceding article). Addition of U-A-G to an aminoacylation reaction mixture had no effect on the rate of acylation of $tRNA^{Tyr}_2$. This result, together with those described in Table I, suggest that the inhibition of Ile-tRNA synthetase by U-A-G is due to a specific interaction of the trinucleotide with the enzyme.

Mechanism of U-A-G Inhibition. Having reasonable assurance that the inhibition by U-A-G is specific, the next issue is the mechanism by which the oligonucleotide inhibits the

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TABLE I: Inhibition of Ile-tRNA Synthetase by Oligonucleotides.[a]

Oligonucleotide	% inhibition
None	0
U-A-G	48.8
G-A-U	0
A-U-A	0
C-U-G	5.9
U-A	0
U-U	0

[a] The aminoacylation mixture contained in 1 mL: 20 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 7.5 μM [^{14}C]Ile, 0.4 μM $tRNA^{Ile}$, and 2 nM Ile-tRNA synthetase. When an oligonucleotide was added, its approximate concentration was: U-A-G, 70 μM; G-A-U, 76 μM; A-U-A, 100 μM; C-U-G, 100 μM; U-A, 80 μM; U-U, 100 μM. Incubation was at 37 °C and 100-μL aliquots were removed after 5 min (while the aminoacylation is still increasing steadily with time) for assay by the method of acid precipitation (Hoskinson and Khorana, 1965). In some cases the aminoacylation after 5 min in the presence of an oligonucleotide was a little higher than the control; these cases were considered as zero percent inhibition.

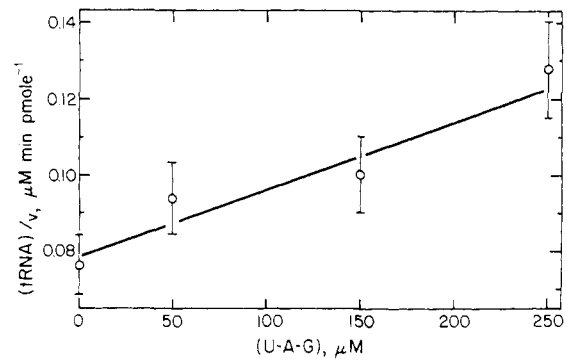

FIGURE 1: Plot of (tRNA)/v vs. (U-A-G). Aminoacylation was carried out at ~pH 7.5, 37 °C, in 20 mM phosphate buffer, 10 mM $MgCl_2$, 5 mM ATP, 7.5 μM [^{14}C]Ile, 5 μM $tRNA^{Ile}$, and catalytic amounts of Ile-tRNA synthetase. Aminoacylation was monitored by the method of acid precipitation (Hoskinson and Khorana, 1965). Initial velocities were based on 2-min time points; in all cases the extent of aminoacylation at 2 min was less than 20% of maximal. The line was obtained by the method of least squares. Error bars correspond to ±10% experimental error.

aminoacylation reaction. Four simple possibilities must be considered. First, it is conceivable that the trinucleotide somehow inhibits the amino acid activation reaction in which isoleucine and ATP are condensed to form isoleucyl adenylate. A second possibility is that the trinucleotide accelerates the deacylation of Ile-$tRNA^{Ile}$ caused by the special deacylase activity of Ile-tRNA synthetase (Schreier and Schimmel, 1972). In this event the net rate of aminoacylation could be substantially reduced due to a large rate of hydrolysis of newly formed Ile-$tRNA^{Ile}$. A third possibility is that the trinucleotide binds to $tRNA^{Ile}$ and thereby interferes with the synthetase-tRNA interaction. For example, binding of complementary oligomers to $tRNA^{Phe}$ has been shown by Barrett et al. (1974) to interfere with aminoacylation by Phe-tRNA synthetase. A final alternative is that the oligonucleotide binds to the enzyme and thereby inhibits its association with $tRNA^{Ile}$.

The four possibilities were each checked. First, it was found that at a concentration of 100 μM (sufficient to produce 50% or so inhibition of aminoacylation (see Table I)) U-A-G has no effect on the ATP-PP_i exchange reaction that is commonly used to measure aminoacyl adenylate formation (see Berg, 1961; Novelli, 1967). This result shows that the trinucleotide does not affect the rate of production of aminoacyl adenylate. Secondly, using [^{14}C]Ile-$tRNA^{Ile}$ and enzyme concentrations and conditions similar to those used in aminoacylation assays in which strong inhibition by U-A-G is observed, it was found that 100 μM U-A-G has no effect on the deacylation of the charged tRNA. (At the concentrations of enzyme used in the aminoacylation assay (a few nM), the enzymatic deacylation reaction (in the presence or absence of U-A-G) is inconsequential compared with the normal solvent mediated ester hydrolysis that occurs at 37 °C.) Thus, under the conditions used, U-A-G does not activate the deacylation reaction so that this reaction also plays no role as far as the inhibition phenomenon is concerned.

Direct binding of U-A-G to $tRNA^{Ile}$ was checked by the microequilibrium dialysis method of Uhlenbeck et al. (1970; see also Schimmel et al. (1972) for earlier studies on hybridization of oligonucleotides to $tRNA^{Ile}$). This method was developed by Uhlenbeck et al. (1970) to study hybridization of tri- and tetranucleotides to unpaired and unshielded regions on tRNA containing complementary sequences to the oligonucleotides. However, in $tRNA^{Ile}$ the sequence complementary to U-A-G—C-U-A— is involved in cloverleaf base-pairing so that no hybridization of U-A-G to $tRNA^{Ile}$ is expected. Indeed, no interaction between U-A-G and $tRNA^{Ile}$ was detected.

Elimination of the first three of the aforementioned possibilities raises the question of whether U-A-G binds directly to the enzyme and thereby blocks binding of $tRNA^{Ile}$. To test for this possibility simple steady-state kinetic experiments were done. Assuming that U-A-G competitively binds to the enzyme with a dissociation constant of K_I, the Michaelis–Menten relationship between the initial velocity v and the tRNA and U-A-G concentration is

$$v = \frac{V_M}{1 + \frac{K_M}{(\text{tRNA})}\left(1 + \frac{(\text{U-A-G})}{K_I}\right)} \quad (1)$$

where K_M is the Michaelis constant for tRNA binding and V_M is the maximal velocity. This equation can be converted into an alternate form to give

$$\frac{(\text{tRNA})}{v} = \frac{K_M + (\text{tRNA})}{V_M} + \frac{K_M}{V_M}\frac{(\text{U-A-G})}{K_I} \quad (2)$$

According to eq 2 a plot of (tRNA)/v vs. (U-A-G) should be linear with a slope of $K_M/(V_M K_I)$ and an intercept of $(K_M + (\text{tRNA})/V_M$, if U-A-G acts by competitive inhibition.

A plot of (tRNA)/v vs. (U-A-G) is shown in Figure 1. The plot covers the range of 0 to 250 μM in U-A-G concentration. The brackets on the points correspond to 10% error limits. Within experimental error, a strictly linear relationship is observed, in accordance with the expectations of simple competitive inhibition.

In order to estimate the dissociation constant K_I, it is necessary to know the parameters K_M and V_M. These were obtained by working under the same conditions with (U-A-G) = 0 and studying the initial velocity of aminoacylation as a function of tRNA concentration. These data were plotted as (tRNA)/v vs. (tRNA) (see eq 2 with (U-A-G) = 0)). From the slope and intercept of this plot values were obtained for K_M and V_M. These yielded values of K_M = 1.8 μM and V_M = 88.5 pmol min^{-1}. Although no direct measurements of Ile-tRNA synthetase-$tRNA^{Ile}$ association at pH 7.5 have been made, the K_M determined in this study is in line with direct measurements of the dissociation constant made at lower pH values, and taking into account the marked decrease in binding that occurs as pH is raised (see Lam and Schimmel, 1975).

Using the values obtained for K_M and V_M, K_I in eq 2 may

be calculated from the slope of Figure 1. This yields a value of 110 μM. Thus, the analysis suggests that the association of the trinucleotide at pH 7.5 is about a factor of 50 weaker than that of the tRNA.

As a check on the kinetic data, it is of interest to note that according to eq 2 the intercept in Figure 1 is given by $(K_M + (tRNA))/V_M$. Using values of K_M and V_M obtained from the plot of $(tRNA)/v$ vs. (tRNA) under conditions where (U-A-G) = 0 (see above), an intercept of 0.077 μM min pmol^{-1} is predicted for the graph in Figure 1. The observed intercept in Figure 1 is 0.078 μM min pmol^{-1}. This close agreement gives further support to the kinetic analysis.

With any kinetic observations, it is only possible to state that the data are consistent with a particular mechanistic scheme. It is always conceivable that a scheme that was not considered is actually operative. However, the various experiments described above eliminate the simple and obvious possibilities that inhibition is achieved by an effect on aminoacyl adenylate formation or on the special deacylase reaction, and also show there is no significant interaction between U-A-G and $tRNA^{Ile}$. Thus, it seems safe to assume that any scheme which postulates an effect mediated by one or more of these simple reactions alone or in combination with other processes can be eliminated, and that the major effect is most likely due to competitive binding of U-A-G to the enzyme.

The number and extent of the experimental investigations with U-A-G were seriously limited by the small amounts of this oligomer that were available. However, preliminary dialysis experiments were also done to check on the binding of U-A-G to Ile-tRNA synthetase at pH 7.5, 4 °C. (The low temperature is used in this approach on the assumption that binding will be greater at lower temperatures and thereby make easier the detection of association (cf. Uhlenbeck et al., 1970)). By this method binding of the oligomer to the enzyme was readily demonstrated. Assuming a 1:1 stoichiometry, a dissociation constant of about 30 μM is estimated from the data. A significant, but weaker interaction was also noted for the oligomers in Table I that do not inhibit the enzyme. This must be due to nonspecific effects. In any event, for U-A-G the dissociation constant estimated from dialysis at 4 °C is within a factor 4 of that obtained from the kinetic experiments at 37 °C.

Tritium Labeling of U-A-G in the Presence and Absence of Ile-tRNA Synthetase. The data obtained above suggest that U-A-G binds to Ile-tRNA synthetase at its tRNA binding site. The question is whether this trinucleotide occupies the same position on the enzyme that the corresponding sequence in the tRNA occupies when the nucleic acid is bound. As shown in the preceding article (Schoemaker and Schimmel, 1977), tritium labeling of U8 in $tRNA^{Ile}$ is greatly accelerated in the presence of Ile-tRNA synthetase, while the labeling of most other uridines is unaffected by bound enzyme. If U-A-G occupies the same site on the enzyme as U8 in $tRNA^{Ile}$, one might expect accelerated H-5 exchange in the trinucleotide similar to that observed in the bound tRNA.

The results of a tritium-labeling experiment with U-A-G are summarized in Table II. In this experiment concentrations of about 100 μM of U-A-G and of Ile-tRNA synthetase were employed. With a dissociation constant of 110 μM, a large fraction of the trinucleotide clearly is not bound. Therefore a correction for the unbound material was made. In the table, the actual data obtained are indicated in parentheses, while the corrected results are listed directly below. In addition, data from the preceding article on labeling of U8 in $tRNA^{Ile}$ are included.

The table shows that H-5 labeling of the uridine in bound U-A-G is greatly enhanced. In the absence of enzyme, U8 in $tRNA^{Ile}$ and the U in U-A-G have closely similar exchange rates. In the presence of enzyme, a 13-fold acceleration is found with U8 of $tRNA^{Ile}$ and an approximately 8-fold effect is observed with U-A-G. The similar effects in the two cases, together with the kinetic data showing that the trinucleotide and tRNA binding are competitive, collectively indicate that U-A-G occupies the same site on the enzyme as the corresponding sequence in bound $tRNA^{Ile}$.

TABLE II: Rate Constants at 37 °C for H-5 Exchange of Uridine in U-A-G and of U8 in $tRNA^{Ile}$ in the Presence and Absence of Ile-tRNA Synthetase.[a]

	Without enzymes (10^{-5} h^{-1})	With enzyme (10^{-5} h^{-1})	Ratio (with/without)
U-A-G	(7.0)[b]	(25.2)[b]	
U-A-G	7.0	56.7	8.1
U8 in $tRNA^{Ile}$	7.1[c]	92.7[c]	13.1[c]

[a] The reaction mixture contained in 500 μL: 20 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$, 100 μM U-A-G, and 100 μM Ile-tRNA synthetase, in tritiated water with a specific activity of 2.5 Ci/mL. Incubation was for 6 h at 37 °C. Data are the average of two experiments. [b] Numbers in parentheses are observed values which were then corrected for the amount of unbound U-A-G to give the numbers in the second row. [c] From Schoemaker and Schimmel (1977); conditions are somewhat different.

Discussion

Over the years there have been attempts to aminoacylate large fragments of transfer tRNAs, as an approach to the synthetase–tRNA recognition question (see reviews by Kisselev and Favorova, 1974; Söll and Schimmel, 1974). Often it has not been possible to find a fragment substantially smaller than the whole molecule which has major aminoacylation activity (Thiebe et al., 1972; Mirzabekov et al., 1971). Thus, previous experiments with fragments have in some respects been discouraging and, therefore, it was somewhat surprising to us to find a small trinucleotide that acts as an effective inhibitor of tRNA binding. The reason for the effectiveness of U-A-G may lie in its small size. For example, one could imagine that a much larger fragment, containing the U-A-G sequence, might not effectively inhibit the enzyme because the trinucleotide sequence would be masked in a structure formed by internal hydrogen bonding within the fragment or by inter-fragment hydrogen bonding.

To our knowledge this is the first example of inhibition of an aminoacyl-tRNA synthetase by a specific trinucleotide derived from a part of the tRNA sequence. Barrett et al. (1974) have synthesized oligonucleotides complementary to the anticodon and 3′-C-C-A terminus of $tRNA^{Phe}$. These oligomers hybridize to $tRNA^{Phe}$ and thereby inhibit aminoacylation, apparently by masking part of the tRNA structure which in turn interferes with synthetase binding. In the present studies, inhibition is caused by direct association of the oligonucleotide with the synthetase.

As mentioned above, the data strongly suggest that U-A-G occupies the same site on the enzyme as the corresponding sequence in bound $tRNA^{Ile}$. But it is questionable that the interaction of this trinucleotide can account for the specificity of the synthetase–$tRNA^{Ile}$ interaction. A number of other *E. coli* tRNAs also contain U-A-G at the 8-9-10 position (Barrell and Clark, 1974). It is not known if these other systems are also inhibited by U-A-G; it is conceivable that other trinucleotide segments play a role in other tRNAs that is analogous to that

of U-A-G in *E. coli* $tRNA^{Ile}$. Certainly, in view of the results of the preceding article (Schoemaker and Schimmel, 1977), which suggest an important role for U8, sequences near and even encompassing this residue are good candidates to be explored in future investigations. And in the case of the $tRNA^{Ile}$ system, it is possible that the U-A-G sequence is part of a hierarchial scheme that requires interaction at several points in the tRNA structure (including, for example, the "discriminator" base—the base at the 4th position from the 3′ terminus, which is believed to play a role in synthetase-tRNA recognition (Crothers et al., 1972)).

In light of the results presented here it is also of interest to recall the studies of Wübbeler et al. (1975) who attempted to aminoacylate heterologous combinations of tRNA fragments that are held together by some complementary base pairing. In particular, using yeast Ala-tRNA synthetase they aminoacylated with *alanine* the 3′-half of *E. coli* $tRNA^{Val}$, when it was associated with the 5′-half of yeast $tRNA^{Ala}$ (containing U-m′G-G at the 8-9-10 position), while no significant aminoacylation with *E. coli* Val-tRNA synthetase was achieved. Conversely, using *E. coli* Val-tRNA synthetase, they aminoacylated with *valine* the 3′-half of yeast $tRNA^{Ala}$ when it was associated with the 5′-half of *E. coli* $tRNA^{Val}$ (containing s^4U-A-G at the 8-9-10 position), while yeast Ala-tRNA synthetase does not significantly aminoacylate this heterologous combination. Also under the condition of these reassociation experiments, the 3′-halves alone are not aminoacylated. These results encourage the idea that information in the 5′-halves of the tRNA fragments has an important influence on specificity. However, caution must be exercised because these same workers found that by using a special scheme the 3′-halves alone could be converted into a form which was aminoacylated specifically with its cognate synthetase, albeit to an erratic and nonreproducible degree.

The demonstration in the present work of a specific interaction of a trinucleotide with a synthetase gives rise to several possibilities for future investigation. Clearly, a most important objective is to pursue similar investigations with other trinucleotides and other synthetases. This will require synthesis of substantial amounts of the trinucleotides, a process which we have initiated. As another possibility, along more speculative lines, it is of interest to consider again the possibility raised in the previous article that the interaction of the enzyme with the U8 position might in some way be necessary for a correct positioning of the 3′ terminus at the amino acid attachment site of the synthetase. The sequence at the 3′ terminus, which clearly must interact in some way with the enzyme, is A-C-C-A. Thus, it is of interest to determine whether this tetranucleotide fragment from the 3′ terminus can be aminoacylated in the presence of U-A-G. Experiments of this type are planned.

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