Transfer Ribonucleic Acid-induced Hydrolysis of Valyladenylate Bound to Isoleucyl Ribonucleic Acid Synthetase*

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(Received for publication, September 24, 1965)

SUMMARY

When incubated with the appropriate substrates, purified *Escherichia coli* isoleucyl ribonucleic acid synthetase can form a complex with either isoleucyl- or valyladenylate which may be isolated by Sephadex filtration. Whereas the isoleucyladenylate complex reacts with transfer RNA (tRNA) to form isoleucyl-RNA, the valyladenylate complex breaks down in the presence of tRNA. In place of transacylation, the net reaction observed is a tRNA-induced hydrolysis of valyl-AMP. Isoleucine-specific tRNA molecules are the active components. Any alteration of the tRNA which destroys the isoleucine acceptor activity also destroys the ability of the tRNA to induce the hydrolysis of the enzyme-bound valyladenylate.

Aminoacyl ribonucleic acid formation results from two successive reactions catalyzed by a single enzyme (1, 2). The first (Reaction 1) is the formation of an enzyme bound mixed anhydride, the aminoacyl adenylate (3-5), and the second (Reaction 2) is a transacylation in which the aminoacyl group is esterified to the terminal 2'-hydroxyl or 3'-hydroxyl group of tRNA, or both (6–9). In general, a given tRNA species accepts only a unique amino acid (with one exception (10)) and each aminoacyl-RNA synthetase forms aminoacyl-RNAs with a single amino acid.

\[
\text{Enz} + \text{aa} + \text{ATP} \rightarrow \text{Enz} \ldots \text{aa-AMP} + \text{PP}_1 \quad (1)
\]

\[
\text{Enz} \ldots \text{aa-AMP} + \text{tRNA} \rightarrow \text{aa-RNA} + \text{AMP} + \text{Enz} \quad (2)
\]

* Supported in part by research grants from the National Institutes of Health, United States Public Health Service.
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The abbreviations used are: tRNA or tRNA \ldots pCP\text{pA} is used to indicate intact, unreacted tRNA; tRNA_{\text{be}} or tRNA_{\text{val}} refers to those molecules in the mixed population of tRNA which function as acceptors of isoleucine or valine, respectively; tRNA_{\text{be}} or tRNA_{\text{val}} indicates a preparation which has been oxidized by periodate but not specifically treated to promote elimination of the oxidized sugar and base, while tRNA_{\text{IO}} or tRNA_{\text{Be}} or tRNA_{\text{val}} refers to those molecules in the mixed population of tRNA which function as acceptors of isoleucine or valine, respectively; tRNA_{\text{be}} or tRNA_{\text{val}} indicates a preparation which has been oxidized by periodate and then incubated with an amine to eliminate the terminal residue; tRNA_{\text{be}} or tRNA_{\text{val}} is used to indicate the material produced by venom phosphodiesterase treatment of tRNA at 20°C; Ile-RNA is meant to show isoleucyl-RNA, etc.; \text{enz}_{\text{be}} or \text{Ile} (Val)\text{-AMP refers to the isoleucyl-(or valyl)adenylate bound to isoleucyl-RNA synthetase, and enz}_{\text{val}} \ldots \text{Val-AMP means the analogous valyladenylate bound to valyl-RNA synthetase.}

There can, however, be a difference in the specificity of the two reactions catalyzed by an aminoacyl-RNA synthetase. *Escherichia coli* isoleucyl-RNA synthetase converts valine as well as isoleucine to the corresponding aminoacyl adenylate, although only isoleucine is transferred to tRNA (11). A similar situation may exist with *E. coli* Val-RNA synthetase since threonine as well as valine supports the ATP-PP_1 exchange reaction (11); presumably, however (although it has not been tested), threonine is not transferred to tRNA_{\text{val}}.

To learn more about how esterification of the "wrong" amino acid to tRNA is prevented, we have examined the mechanism of exclusion of valine from tRNA_{\text{be}}, by the Ile-RNA synthetase. Earlier (12), we described the isolation of enz_{\text{be}} \ldots \text{Ile-AMP} and enz_{\text{be}} \ldots \text{Val-AMP complexes by Sephadex gel filtration. Both complexes yield ATP in the presence of PP_1 according to Reaction 1. Nevertheless, whereas enz_{\text{be}} \ldots \text{Ile-AMP yields Ile-RNA when tRNA is added, no Val-RNA is formed when the corresponding enz_{\text{be}} \ldots \text{Val-AMP is incubated with tRNA. Actually, the enz_{\text{be}} \ldots \text{Val-AMP complex is destroyed in the presence of tRNA (12).}

This paper describes experiments which further characterize the breakdown of the enz_{\text{be}} \ldots \text{Val-AMP complex in the presence of tRNA. The reaction is induced by and completely dependent on intact tRNA_{\text{be}}, *E. coli* tRNA_{\text{val}} and yeast tRNA_{\text{be}}, which do not accept isoleucine in the reaction catalyzed by *E. coli* Ile-RNA synthetase, and *E. coli* tRNA_{\text{be}} modified so as to be inactive as isoleucine acceptor, are all unable to induce the breakdown of enz_{\text{be}} \ldots \text{Val-AMP. Our experiments indicate that the reaction induced by the appropriate tRNA is hydrolysis of the anhydride linkage of the enzyme-bound valyladenylate and release of AMP and valine.}

**EXPERIMENTAL PROCEDURE**

**Materials**

Enzymes—Ile-RNA synthetase was extracted from *E. coli* B and purified as described in the preceding paper (13). Val-RNA synthetase was prepared according to Bergmann, Berg, and Dieckmann (11). Bacterial alkaline phosphatase (14) was a product of Worthington. It was stored at a concentration of 2 mg per ml as an ammonium sulfate suspension. A unit of phosphatase was defined for purposes of these experiments as 1 unit of phosphatase.
the amount of enzyme required to hydrolyze 1 mmole of 0.01 m AMP at 10°, pH 6.0. Under these conditions, the enzyme had 175 units per mg of protein. Pancreatic RNase was a Worthington product and Tl RNase was purified according to Takahashi (15). 

tRNA—tRNA was isolated by phenol extraction (16) from E. coli B cells, also used as the source of enzyme; tRNAs with different amino acid specificities were obtained by partition chromatography as described by Muench and Berg (17). Yeast tRNA was purchased from General Biochemicals.

To determine whether a unique species of tRNA chain was responsible for enz1le... Val-AMP breakdown and to assess the role of the acceptor end of the tRNA molecule, two types of modification were made in the RNA. Differential inactivation of tRNAs was accomplished by periodate oxidation of the terminal ribose before and after esterification with amino acids (7) and by limited digestion with Crotalus venom phosphodiesterase to remove one or two nucleotides from the ...CCA end of the chain (7).

In Modification a, periodate oxidation was performed as follows. tRNA (300 A260) in 1.0 ml of 0.1 m sodium acetate, pH 5.0, was mixed with a 3- to 10-fold excess of 0.1 m sodium periodate, and after 30 min at 25°, the RNA (RNA ... pCpCp“A”(IOJ)) was precipitated by the addition of 0.1 volume of 5 M NaCl and 2 volumes of cold ethanol. The precipitate was collected by centrifugation, dissolved in 1.0 ml of water, and then made 0.01 m in ethylene glycol to destroy excess periodate. The absorbance at 232 mμ was determined before and after addition of ethylene glycol to establish that an excess of periodate had been present at the end of the reaction. Treatment of RNA ... pCpCp“A”(IOJ) with 0.5 m lysine, pH 8, at 45° for 60 min results in the formation of RNA ... pCpCp (18).

tRNA capable of accepting only isoleucine or valine was prepared as follows. tRNA was esterified enzymatically with either ...C-isoleucine or ...C-valine and the appropriate aminocyl-RNA synthetase under standard assay conditions (19) and then subjected to periodate oxidation followed by incubation with lysine as described above. The lysine treatment also removed the protecting amino acid.

In Modification b, tRNA ... pC was prepared by controlled digestion of tRNA with venom phosphodiesterase at room temperature according to the method of Zubay and Takeani (20). Our preparation had 2% of the original isoleucine acceptor activity, but when incubated with CTP, ATP, and the enzyme which completes the ...CCA-terminal sequence (21), 80% of the original isoleucine acceptor was regenerated. Since the addition of AMP required the presence of CTP, and since approximately equal amounts of CMP and AMP were incorporated, we assume that the predominant tRNA species formed by the phosphodiesterase treatment was RNA ... pC (21).

Substrates—α-32P-ATP and AM32P were purchased from International Chemical and Nuclear Corporation. The ATP32P had a specific activity of approximately 200 mC per mmole at the time of purchase and was usually 70 to 80% pure as judged electrophoretically. 13C-Amino acids were obtained from New England Nuclear.

Methods

Assay for Release of AMP from Enz... aa-AMP Complex—Aminocyclation of tRNA (Reaction 3), breakdown of enz1le... Val AMP (Reaction 4), or in fact any reaction which involves release of AMP from the enzyme-aminocyl adenylate complex, can be followed by the production of P1 from AMP in the presence of alkaline phosphatase (Reaction 5). This is possible because the phosphatase reacts at a negligible rate with the aminocyl adenylate, free or enzyme-bound (cf. Figs. 1, 2, and 10).

Enz1le... Ile-AMP32P + tRNA ⇌ enz + Ile-RNA + AMP32P (3)

Enz1le... Val-AMP32P + H2O ⇌ enz + valine + AMP32P (4)

AMP32P + H2O $\xrightarrow{\text{phosphatase}}$ adenosine + 32P1 (5)

Enz... aa-AMP32P complex formed with α-32P-ATP and isolated by Sephadex gel filtration (12) is stable for a few hours at 0° in the buffer in which it was recovered (0.05 m sodium succinate (pH 6.0), 0.05 m KCl, 0.01 m 2-mercaptoethanol, and 0.001 m EDTA).

Assay mixtures (0.25 ml) consisting of 0.20 ml of enz ... aa-AMP32P containing 0.1 to 0.5 mmole of either the isoleucyl or valyl complex, 0.01 ml of phosphatase (3.5 units), and tRNA were incubated at 10°. Aliquots (50 μl) were removed periodically and added to a suspension containing 0.3 ml of 2 N HCl, 100 mmoles of KH2PO4, and 10 mg of acid-washed Norit. After mixing, the suspension was centrifuged for 2 min at 10,000 rpm. An aliquot of the supernatant fluid was dried on a Whatman glass filter (GF/C, 2.4 cm in diameter), and counted under toluene scintillation solution in a Packard Tri-Carb liquid scintillation spectrometer. Dephosphorylation of free AMP with this level of phosphatase is complete within a few seconds and is not rate-limiting since NH2OH added to either enz... Ile-AMP32P or enz... Val-AMP32P allows the conversion of virtually all of the 32P to 32P1 within the time consumed in removing an aliquot (10 sec) (cf. Figs. 1 and 2).

RESULTS

Formation of Ile-RNA and Release of AMP from Enz... Ile-AMP—When isoleucine is transferred from enz1le... Ile-AMP to tRNA, AMP is concomitantly released and becomes susceptible to phosphatase (Fig. 1). Approximately 60 to 70% of the bound isoleucine is esterified to tRNA and 70 to 80% of the AMP is released as judged by the liberation of P1. With no tRNA added, only 10% of the 32P added as complex yields P1. This 10% "blank" could be due to some free ATP32P not separated from the complex by Sephadex filtration; more probably, it is due to the free AMP32P which results from partial hydrolysis of the complex during its isolation. Enz1le... Ile-AMP does not appear to be hydrolyzed spontaneously during incubation at 10°.

It is not clear why the transfer of isoleucine from enz1le... Ile AMP to tRNA is incomplete. The extent of transfer does not appear to reflect an equilibrium position for the transacylation, since addition of a large excess of tRNA did not increase the extent of transfer. The remaining 20 to 30% of the 32P, however, still appears to be in anhydride linkage, since all of the 32P becomes susceptible to phosphatase after the addition of neutral hydroxylamine (Fig. 1). It is possible that some of the Ile-AMP initially bound to the enzyme dissociates from the protein on addition of tRNA. This is at variance with the quantitative release of AMP from enz1le... Val-AMP in the presence of excess tRNA1le (Fig. 2).
Breakdown of Enz\textsubscript{16}...Val-AMP\textsuperscript{32}P in Presence of tRNA—
As judged by Sephadex gel filtration, both the valyl and AMP moieties of enz\textsubscript{16}...Val-AMP are dissociated from the enzyme on addition of tRNA (12). This reaction is more easily followed by measuring the amount of Pi formed from the AMP released when tRNA is added to enz\textsubscript{16}...Val-AMP (Fig. 2).

Very little Pi is formed in the absence of tRNA, but if excess tRNA is added, there is a rapid production of Pi. At lower concentrations of tRNA, the initial reaction velocity is approximately proportional to the concentration of tRNA (Fig. 3). In most experiments to be presented, less than saturating amounts of tRNA have been employed to reduce the initial velocity of the reaction to a measurable level.

From the reciprocal plots of the initial velocity of AMP release at different concentrations of tRNA, a \( K_m \) of 2.5 \( \times 10^{-7} \) m for tRNA\textsubscript{16} was calculated. This is close to the \( K_m \) for tRNA\textsubscript{16} (1.7 \( \times 10^{-7} \) m) calculated for Ile-RNA formation under standard assay conditions (19), i.e. with catalytic amounts of enzyme and with Ile-RNA synthesis measured as a function of tRNA concentration. The justification for expressing tRNA concentration in terms of tRNA\textsubscript{16} is, as we show next, that breakdown of enz\textsubscript{16}...Val-AMP is induced specifically by these tRNA chains.

Specificity for tRNA\textsubscript{16} in Breakdown of Enz\textsubscript{16}...Val-AMP—
Earlier, experiments with Sephadex filtration (12) revealed that enz\textsubscript{16}...Val-AMP breakdown occurs in the presence of tRNA enriched for tRNA\textsubscript{16}, but not with fractions lacking these chains; tRNA treated with pancreatic RNase did not induce the breakdown. These experiments have been repeated with the use of the phosphatase assay (Fig. 4) and the earlier results have been confirmed. We have also observed that, after T1 RNase digestion of tRNA, the remaining oligonucleotides which still possess the intact ...CCA-terminal sequence do not induce breakdown of enz\textsubscript{16}...Val-AMP.

Inasmuch as the tRNA\textsubscript{16}-enriched fractions from the partition column also contained appreciable amounts of tRNA\textsubscript{16}, it was not possible to decide which of the two species of tRNA catalyzed the breakdown of the enz\textsubscript{16}...Val-AMP complex. This distinction could be made, however, with periodate-treated Ile-RNA or Val-RNA (see "Methods" for their preparation).

Fig. 5 shows clearly that the rapid breakdown of enz\textsubscript{16}...Val-AMP\textsuperscript{32}P is induced by the isoleucyl-protected tRNA but not by the valyl-protected tRNA or by totally unprotected tRNA.
FIG. 4. Specificity of enzile . . Val-AMP breakdown for trNAile. Reaction mixtures contained enzile . . Val-AMP (0.10 μmole, 7100 cpm) and unfractionated tRNA (0.5 A₂₆₀, 0.015 μmole of tRNAile) (●), or a pancreatic RNase digest of tRNA (2.5 A₂₆₀, tRNA incubated for 15 min at room temperature with 2.5 μg of pancreatic RNase) (○), or tyrosine-enriched tRNA (0.5 A₂₆₀, containing 0.045 μmole of tRNAile) (△), or no tRNA (□).

However, enzile . . . Val-AMP complex, formed with purified E. coli Val-RNA synthetase, releases AMP only on addition of the valyl-protected tRNA (Fig. 6), as an expected consequence of the transfer of valine to tRNAile by this enzyme. The breakdown of enzile . . . Val-AMP results, therefore, from a specific interaction between the complex and tRNAile, a reaction related to Ile-RNA synthesis from the enzile . . . Ile-AMP complex.

Specificity of Hydrolytic Reaction for Intact tRNAile—The preceding experiment showed that intact tRNAile but not intact tRNAval or partially degraded tRNAile, induced the breakdown of enzile . . . Val-AMP. We may also conclude that tRNAile . . . pCpCp, which would be present in the unprotected or valyl-protected periodate-oxidized preparations, does not induce the breakdown. The same can be said for RNAile . . . pC. The release of AMP from intact tRNA, trNA . . . pC, or trRNA . . . pCpCp is added to enzile . . . Ile-AMP or enzile . . . Val-AMP complexes is shown in Fig. 7. In all cases, the observed rate of Pᵢ release with a particular RNA preparation is similar with either complex.

The low rate of release of AMP with tRNA . . . pC in each case may be due to a small amount of degraded tRNAile in this preparation (2% of original isoleucyl acceptor activity), but this point requires further investigation.

Yeast tRNA is approximately 5% as active as tRNA from E. coli as an acceptor of isoleucine with the E. coli Ile-RNA synthetase. Similarly, the rate of hydrolysis of the E. coli
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FIG. 8. Failure of tRNA ... CpCp to inhibit breakdown of enzile ... Val-AMP. Reaction mixtures contained enzile ... Val-AMP (0.077 mmoles, 3650 cpm) and no tRNA (O), or tRNA (1.3 A260, 0.026 mmoles of tRNA) (O), or tRNA (1.8 A260) plus tRNA ... pCpCp (1.4 A260) (O), or tRNA (1.3 A260) plus tRNA ... CpCp (1.3 A260) (O).

enzile ... Val-AMP complex in the presence of yeast tRNA was about 5% the rate of breakdown induced by E. coli tRNA.

Absence of Competition between Intact tRNA and Degraded tRNA in Breakdown of Enzile ... Val-AMP—It was of interest to know whether tRNAs which do not induce breakdown of enzile ... Val-AMP fail to do so because they do not interact with the complex. Such tRNA preparations were already known to be inactive as aminoacyl acceptors. Preparations of tRNA ... pC and tRNA ... pCpCp were found to inhibit the formation of Ile-RNA with a $K_r$ of approximately $10^{-6}$ M. By contrast, tRNA terminating in a monoesterified phosphate at the acceptor end (tRNA ... CpCp) did not inhibit Ile-RNA synthesis of enzile ... Val-AMP breakdown (Fig. 8). Whether or not it is the presence of the terminal phosphomonoester group that prevents interaction of the RNA molecule with the enzyme needs further study. Nevertheless, these data indicate that tRNA, which has been modified in the terminal ... CCA sequence and which is capable of interacting with the enzyme (albeit somewhat less effectively than intact tRNA, $K_m = 2 \times 10^{-5}$ M), is still unable to induce enzile ... Val-AMP breakdown.

Requirement of Free 3'-Hydroxyl on Intact tRNA in Breakdown of Enzile ... Val-AMP—The strict correlation between the ability of a tRNA preparation to accept isoleucine and its ability to induce the breakdown of enzile ... Val-AMP suggests a direct involvement of the terminal 2'- or 3'-hydroxyl. Fig. 9 shows that tRNA esterified with isoleucine is markedly less effective in inducing the hydrolysis of enzile ... Val-AMP than is free tRNA. This result is consistent with the interpretation that the terminal hydroxyl group is required. An alternate explanation, however, is that steric hindrance modifies the binding of Ile-RNA to enzile ... Val-AMP, thereby preventing breakdown of the complex.

Enzymatic Nature of Hydrolysis of Enzile ... Val-AMP—The fact that AMP is released when intact tRNA is added to the enzile ... Val-AMP complex indicates that the acyl phosphate anhydride linkage has been broken. However, it is not clear whether hydrolysis of the acyl phosphate bond occurs while Val-AMP is still bound to the enzyme, or in a chemical reaction following the release of Val-AMP from the enzyme.

To distinguish between these two alternatives, Val-AMP, recovered from enzile ... Val-AMP by dissociation and precipitation of the protein with cold trichloroacetic acid (see legend to Fig. 10) was tested for its susceptibility to alkaline phosphatase.

It is clear that the rate of release of $^{32}P$ from free Val-AMP is slow at 10° relative to the rates observed due to the interaction of tRNA with the enzile ... Val-AMP complex (Fig. 10). Addition of tRNA to the Val-AMP complex did not enhance the rate of $^{32}P$ formation. Addition of a stoichiometric amount of en-

FIG. 9. Failure of Ile-RNA to induce the breakdown of enzile ... Val-AMP. Reaction mixtures contained enzile ... Val-AMP (0.11 mmoles, 7500 cpm) and no tRNA (O), or tRNA (5.9 A260, 0.18 mmoles of tRNA) (O), or Ile-RNA (5.8 A260, 0.18 mmoles of Ile-RNA) (O).

FIG. 10. Stability of Val-AMP under the conditions of the phosphatase assay. Val-AMP was prepared by the addition of 0.5 ml of cold 10% trichloroacetic acid to 0.5 ml of enzile ... Val-AMP (1.2 mmoles, 80,000 cpm). After 10 min at 0°, precipitated protein was removed by centrifugation for 10 min at 10,000 rpm leaving free Val-AMP in the supernatant fluid. The supernatant fluid was carefully neutralized to pH 6.0 with KOH. Reaction mixtures contained Val-AMP (0.30 mmoles) and no tRNA (O), or tRNA (2.0 A260, 0.25 mmoles) (O), or 2.5 µg of Ile-RNA synthetase (O). Where indicated, 0.10 M NH$_4$OH was added.

* Unpublished results.
enzyme was likewise without effect. Complete release of $^{32}$P$_1$ was observed, however, after NH$_4$OH addition.

The slow rate of release of $^{32}$P$_1$ may be due to chemical hydrolysis of the free Val-AMP, which is known to be relatively unstable (22). In any case, the rate of $^{32}$P$_1$ formation is insufficient to account for the rate observed when tRNA$_{ile}$ is added to the enzyme Val-AMP complex.

Apparent ATPase Activity of Ile-RNA synthetase in Presence of Valine and tRNA$_{ile}$—Inasmuch as the enzyme Val-AMP complex is unstable in the presence of tRNA$_{ile}$, the interaction of enzyme, valine, ATP, and tRNA should lead to a net production of PP$_1$ from ATP. With $\beta,\gamma-^{32}$P-ATP as substrate, there is a release of $^{32}$P (presumably PP$_1$) as judged by its nonadsorbability to Norit) when valine, tRNA, and enzyme are allowed to react (Fig. 11). Although there is some breakdown of the ATP in the absence of added valine, the rate is significantly higher in the presence of both valine and tRNA. Note also that in the absence of added valine, the rate is significantly higher than in the presence of both valine and tRNA.

First, each aminoacyl-RNA synthetase must distinguish one amino acid from all others and select a unique set of tRNAs as acceptors of that amino acid (2). Subsequent steps in protein assembly rely on the specificity of H-bond interactions between the messenger RNA triplets and different aminoacyl-RNAs to ensure the correct amino acid sequences. Clearly then, the frequency with which two structurally related amino acids appear in place of each other in a protein cannot be less than the frequency with which they are mistaken for each other in aminoacyl-RNA formation.

A particularly interesting case concerns the discrimination between isoleucine and valine. Pauling had estimated (23), on physicochemical grounds, that these two amino acids could be distinguished to the extent of only 1 in 20, yet Loftfield showed (24) that in ovalbumin synthesis, isoleucine and valine are not mistaken for each other at different positions in the peptide chain except perhaps at a frequency of less than 1 in 3000. Thus, one would expect that Ile-RNA synthetase and Val-RNA synthetase distinguish between these two amino acids with great precision.

One might suppose that the Val-RNA synthetase would fail to utilize isoleucine in place of valine because of steric factors, but the reverse distinction, the prevention of valine utilization by Ile-RNA synthetase, must depend on other parameters. Although considerable specificity is achieved at the level of substrate binding ($K_m$ for isoleucine = $5 \times 10^{-4}$ M; $K_m$ for valine = $4 \times 10^{-4}$ M), the precision in the overall synthesis of Ile-RNA is far greater. Bergmann et al. (11) found that, indeed, Ile-RNA synthetase catalyzes the formation of both isoleucyl- and valyladenylates but only isoleucine could be esterified to tRNA. The specificity of the first step depends on the structure of the free amino acid, whereas the specificity of the second reaction depends in some way on the structure of the enz . . . aa-AMP complex. The present work shows that the difference in the reaction of the enz$_{ile}$ . . . Ile-AMP and enz$_{ile}$ . . . Val-AMP complexes with tRNA$_{ile}$ prevents formation of Val-RNA$_{ile}$. No transfer of valine to tRNA$_{ile}$ occurs from enz$_{ile}$ . . . Val-AMP (<0.005%) under conditions in which 60 to 70% of isoleucine in enz$_{ile}$ . . . Ile-AMP is converted to Ile-RNA.

Loftfield and Eigner (25) suggested that tRNA increased the ability of Ile-RNA synthetase to discriminate between isoleucine and valine by changing the relative $K_m$ values for these amino acids and their $V_{max}$ for enz . . . aa-AMP formation. Actually, most of their kinetic data are qualitatively consistent with our knowledge of the fate of the enz . . . aa-AMP complexes in the presence of tRNA. But, even on the assumption that tRNA facilitates discrimination between isoleucine and valine in the first reaction, the RNA-induced breakdown of the enz$_{ile}$ . . . Val-AMP complex provides a positive and probably more efficient mechanism for preventing the formation of an aberrant Val-RNA$_{ile}$ and the consequent replacement of isoleucine by valine in protein.

Since only tRNAs possessing isoleucine acceptor activity induce the breakdown of enz$_{ile}$ . . . Val-AMP it is likely that common structural features in the tRNA are required for the hydrolytic as well as normal aminoacylation reaction. Our results show, however, that the specific interaction between tRNA$_{ile}$ and Ile-RNA synthetase, which must occur during Ile-RNA formation, is essential but not sufficient to induce breakdown of enz$_{ile}$ . . . Val-AMP. For example, removal of the terminal adenylate and one cytidylate residue from tRNA or oxidation of the terminal vicinal hydroxyl group with periodate prevents hydrolysis of enz$_{ile}$ . . . Val-AMP; yet these modifications do not prevent specific interaction with the enzyme since these tRNAs competitively inhibit Ile-RNA formation with intact tRNA.
Similarly, yeast tRNA\textsubscript{ii,j} which presumably can interact with E. coli Ile-RNA synthetase (see Reference 27), does not prevent hydrolysis of enzyme-bound Val-AMP.

One might have proposed that interaction of tRNA with the enzyme effects a conformational change in the protein which in turn promotes hydrolysis of the bound Val-AMP. The fact that several modified tRNAs fail to induce the hydrolysis in spite of their capacity to interact with the enzyme suggests that such a mechanism is not likely. Moreover, tRNA esterified with isoleucine is ineffective in inducing breakdown of the complex, in contrast to the Val-RNA which contains the terminal fragment originating from tRNA\textsubscript{ii,j} (\textldots Cp(UpCp)ApCpCpA (28)) is inactive. It will be of interest to determine whether valine is actually transferred from the Val-AMP to tRNA\textsubscript{ii,j}, and the resulting Val-RNA\textsubscript{ii,j} then hydrolyzed enzymatically, or whether some transition state involved in the transacylation reaction decomposes to yield free valine and tRNA.

At present, little is known about the so called “recognition” between a tRNA and its corresponding aminoacyl-RNA synthetase. Probably, this involves interaction of the protein (as the aminoacyladenylate complex) with a specific region of the polynucleotide chain. However, failure of an RNA to serve as amino acid acceptor or, as in the present case, to induce breakdown of the Val-AMP complex is not necessarily a measure of this “recognition.” Although modifications of a tRNA at its “recognition” site probably result in inactivation of the acceptor activity of that tRNA molecule, inactivation of the acceptor activity of a tRNA need not be diagnostic of an alteration in the “recognition” site. To study the “recognition” problem, methods for measuring the interaction between tRNA and its enzyme are needed, but these methods should not depend on amino acid acceptor activity.

One other activating enzyme has been reported to act as a hydrolase under certain conditions. DeLuca and McElroy (29) have shown that sulphydryl reagents cause the enzymatic hydrolysis of luciferase-bound dehydrodicoluciferyl-adenylate. Dehydrodicoluciferyl-adenylate is an inhibitor of luciferase; the formation of dehydrodicoluciferyl-AMP by luciferase is quite analogous to the formation of Val-AMP by the Ile-RNA synthetase. The mechanism of the luciferase-catalyzed hydrolysis differs, however, from that of the hydrolysis catalyzed by the Ile-RNA synthetase. Whereas hydrolysis of luciferase-bound dehydrodicoluciferyl-adenylate is induced by sulphydryl reagents \textit{e.g.} p-chloromercuribenzoate, arsenite plus 2,3-dimercaptopropanol, and CdCl\textsubscript{2}, hydrolysis of Val-AMP is induced by tRNA\textsubscript{ii,j}, the normal substrate for the enzyme. Hydrolysis of luciferase-bound dehydrodicoluciferyl-adenylate by sulphydryl reagents also requires ATP and magnesium, although the significance of this observation is not clear. However, two enzymes which form enzyme-bound adenylates as the first of two reactions both have increased specificity in the second step, and in each case the adenylate of an “abnormal” substrate may under the appropriate conditions be destroyed by enzymatic hydrolysis.
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