Interactions of Phenylalanyl Transfer Ribonucleic Acid Synthetase of Neurospora crassa with Valyl Transfer Ribonucleic Acid of Escherichia coli

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SUMMARY

Since Phe-tRNA synthetase of Neurospora crassa reacts with tRNA^Val of Escherichia coli to produce Phe-tRNA^Val, the parameters that effect the reverse reaction were examined. Similarly, the interaction of Val-tRNA synthetase (E. coli) with Phe-tRNA^Val and Val-tRNA^Val (E. coli) was studied.

Phe-tRNA synthetase (N. crassa) can catalyze the deacylation of both Phe-tRNA^Val (E. coli) and Val-tRNA^Val (E. coli), although the mechanism of deacylation is different in the two cases since the presence of AMP and PPi is required only for the former. In contrast, Val-tRNA synthetase (E. coli) can deacylate Val-tRNA^Val but not Phe-tRNA^Val. Val-tRNA synthetase may actually inhibit the nonenzymatic deacylation of Phe-tRNA^Val.

Unfractionated tRNA (N. crassa) markedly inhibits the aminoacylation of tRNA^Val (E. coli) catalyzed by Phe-tRNA synthetase (N. crassa). The inhibition appears to result from a competition between tRNA (N. crassa) and tRNA^Val (E. coli) for binding site (or sites) on Phe-tRNA synthetase.

EXPERIMENTAL PROCEDURE

Materials—Spectroquality (CH₃SO) was obtained from Matheson Coleman and Bell. Cacodylic acid was purchased from Fisher. Reagent grade Trizma base was a product of Sigma. L-[¹⁴C]Phenylalanine (355 and 368 mCi per mmole) and L-[¹⁴C]valine (208.5 mCi per mmole) were purchased from New England Nuclear and L-[¹⁴C]phenylalanine (255 mCi per mmole) from Schwarz. The E. coli tRNA^Val (acceptance of 1100 pmoles per As₁₈₅ units) used was purified by reversed phase chromatography as described by Weiss et al. (5) and was generously provided by them. N. crassa tRNA was prepared according to the method of Barnett (6). A highly purified preparation of Phe-tRNA synthetase C (N. crassa) was prepared according to the procedure of Kull and Jacobson (7) and was a generous gift from Dr. Kull. Val-tRNA synthetase (E. coli) was purified according to the procedure of Calender and Berg (8) and Harvey and Meister (9) as modified by Ritter and Jacobson. Both polyacrylamide gel electrophoresis and cellulose acetate electophoresis indicated that Val-tRNA synthetase represented over 80% of the protein in the enzyme preparation. Other tests showed that the purified enzyme contained little, if any, proteinase, nuclease, ATPase, or tRNA nucleotidyltransferase activity. All other reagents were high purity commercial products and were used without further purification.

Methods—The optimal conditions for the aminoacylation of tRNA^Val (E. coli) by Phe-tRNA synthetase (N. crassa) were studied in a variety of conditions.

It is known that the Phe-tRNA synthetase of Neurospora crassa (L-phenylalanine: tRNA ligase (AMP), EC 6.1.1.4) can aminoacylate tRNA^Val of Escherichia coli to form Phe-tRNA^Val. This unusual heterologous reaction has been studied in some detail (3, 4). In Tris buffer under optimal conditions the final yield of Phe-tRNA^Val is a function of enzyme concentration, and aminoacylation is incomplete even at high enzyme concentrations. When cacodylate buffer is used in place of Tris buffer, the pH optimum for aminoacylation is shifted from >8.1 to 6.3, and various kinetic properties of the reaction are changed. The Michaelis constant for tRNA^Val is 10-fold lower in cacodylate buffer than in Tris buffer, and at low enzyme concentrations over 10 times more Phe-tRNA^Val is formed in cacodylate buffer. In addition, 20% dimethylsulfoxide inhibits formation of Phe-tRNA^Val in cacodylate buffer while it markedly stimulates this aminoacylation in Tris buffer.

In an attempt to gain further insight into the interactions between Phe-tRNA synthetase (N. crassa) and tRNA^Val (E. coli) we have studied the aminoacylation of tRNA^Val (E. coli) in reaction mixtures containing various combinations of Phe-tRNA synthetase (N. crassa), Val-tRNA synthetase (E. coli), and tRNA (N. crassa). The enzymatic and nonenzymatic deacylation of Val-tRNA^Val has also been examined under a variety of conditions.
The optimal conditions for the aminoacylation of tRNAVal by Val-tRNA synthetase (E. coli) in Tris buffer are essentially the same as those for the heterologous Tris system. Under standard aminoacylation conditions, each milliliter of reaction mixture contains 0.5 μmole of ATP, 2.5 μmole of MgCl₂, 2.7 μmole of L-[¹⁴C]phenylalanine or 3.9 μmole of L-[¹⁴C]valine, 50 μg of bovine serum albumin, and 50 μmole of potassium cacodylate (pH 6.3) or 50 μmole of Tris·HCl (pH 8.1). The concentrations of other components are listed separately for each experiment. One unit of Val-tRNA synthetase has been defined as the amount of enzyme that will lead to the formation of 4.2 pmoles of Val-tRNAVal (E. coli) in 5 min in 1 ml of a standard reaction mixture initially containing 160 pmoles of tRNAVal (E. coli). The purified enzyme has a specific activity of approximately 65,000 units per mg of protein; since the molecular weight is known to be 110,000 depending on tRNAVal concentration but decreases with increasing concentrations of tRNAVal, there are about 7 units per pmole of Val-tRNA synthetase (E. coli). A unit of Phe-tRNA synthetase is the amount of enzyme needed to form 3.5 pmoles of Phe-tRNAVal (E. coli) in 1 ml at 20° (4); assuming a molecular weight of 180,000, there are 1.4 units per pmole of Phe-tRNA synthetase (N. crassa). Reactions were run at 21-22° and were initiated by the addition of Phe-tRNA synthetase (N. crassa) or Val-tRNA synthetase (E. coli). Aliquots (0.1 ml) were analyzed for labeled aminoacyl-tRNAs by the filter paper disc technique of Bollum (11). Any deviations from these standard conditions are specified.

A standard Tris·HCl reaction mixture containing 240 pmoles of tRNAVal (E. coli) and 2.5 units of Val-tRNA synthetase per ml was used to prepare [¹⁴C]Val-tRNAVal (E. coli) for isolation. A standard potassium cacodylate reaction mixture containing 240 pmoles of tRNAVal (E. coli) and 46 units of Phe-tRNA synthetase (N. crassa) per ml was used to prepare [¹⁴C]Phe-tRNAVal (E. coli) for isolation. The two reactions were terminated after a 90-min incubation by placing the reaction vessels in an ice bath and adding excess potassium acetate (pH 4.6). The reaction mixtures were placed on separate DEAE-cellulose columns equilibrated at 4° with 0.036 M potassium acetate (pH 4.6), 0.012 M KC1, and 0.01 M MgCl₂ (Buffer A). The columns were extensively washed with 0.3 M KC1 in Buffer A, then the aminoacyl-tRNAs were eluted with 1.0 M KC1 in Buffer A. In each case, peak fractions were combined and dialyzed against tenth strength Buffer A. Analysis showed that over 90% of the tRNAVal had been aminoacylated in both cases. The dialyzed aminoacyl-tRNA solutions were stored at -20°.

Reactions involving the deacylation of the isolated aminoacyl-tRNAs were initiated by the addition of the aminoacyl tRNA released. In each case a zero time point was obtained by analysis of the aminoacyl-tRNA just prior to its addition. Reactions were run at 21-22°, and aliquots (0.1 ml) were analyzed by the filter paper disc technique.

**RESULTS**

Fig. 1 indicates that in Tris buffer the presence of Phe-tRNA synthetase (N. crassa) results in an apparent inhibition of Val-tRNA synthetase (E. coli); the extent of Val-tRNAVal formation is decreased progressively, but not linearly, by increasing the concentration of Phe-tRNA synthetase. Although the data are not shown, we found that at a given Phe-tRNA synthetase concentration, the percentage reduction of Val-tRNAVal formation is independent of tRNAVal concentration but decreases with increasing Val-tRNA synthetase concentration. Preincubation of tRNAVal with Phe-tRNA synthetase prior to the addition of Val-tRNA synthetase has no effect on the decreased extent of product formation. Higher concentrations of (CH₃)₂SO markedly increase the inhibitory action of the N. crassa enzyme (Fig. 2). In the absence of Phe-tRNA synthetase (N. crassa), similar concentrations of (CH₃)₂SO have no effect on the final yield of Val-tRNAVal (E. coli), although they do lead to a decrease in the initial rate of aminoacylation (Fig. 3).

Phe-tRNA synthetase can rapidly deacylate Val-tRNAVal in standard reaction mixtures where Val-tRNA synthetase is actively catalyzing Val-tRNAVal formation (Fig. 4). If Val-tRNAVal synthesis is allowed to proceed in the absence of Phe-tRNA synthetase until most of the tRNAVal has been aminoacylated, the addition of Phe-tRNA synthetase leads to a very...
FIG. 3. Effect of (CH₃)₂SO on the aminoacylation of tRNA₉₅₁ (Escherichia coli) by Val-tRNA synthetase (E. coli) in Tris buffer in the absence of Phe-tRNA synthetase (Neurospora crassa). A, each reaction mixture contained 117 pmoles of tRNA₉₅₁ (E. coli), 12.5 units of Val-tRNA synthetase (E. coli), and 3.9 nmoles of [¹⁴C]valine per ml. B, each reaction mixture contained 117 pmoles of tRNA₉₅₁ (E. coli), 3.9 nmoles of [¹⁴C]valine, and approximately 1 unit of Val-tRNA synthetase (E. coli) per ml. See "Methods" for other assay conditions.

FIG. 4. The deacylation of Val-tRNA₉₅₁ (Escherichia coli) by Phe-tRNA synthetase (Neurospora crassa) in standard Tris reaction mixtures (see "Methods") where tRNA₉₅₁ is actively being aminoacylated by Val-tRNA synthetase (E. coli). Each reaction mixture contained 0.5 mM Tris.HCl (pH 8.1) or 50 mM cacodylate (pH 6.3). Phe-tRNA synthetase concentration (units per ml) is indicated on each curve. Isolated aminoacyl-tRNAs were added to start the reactions. See "Methods" for other assay conditions.

FIG. 5. Deacylation of Val-tRNA₉₅₁ (Escherichia coli) (---) and Phe-tRNA₉₅₁ (E. coli) (-----) in Tris and cacodylate buffers, both nonenzymatic and catalyzed by Phe-tRNA synthetase (Neurospora crassa). Each reaction mixture contained 0.5 mM AMP, 0.01 mM pyrophosphate, 7.5 mM MgCl₂, and 50 mM Tris-HCl (pH 6.3) or 50 mM cacodylate (pH 6.3). Phe-tRNA synthetase concentration (units per ml) is indicated on each curve. Isolated aminoacyl-tRNAs were added to start the reactions. See "Methods" for other assay conditions.

TABLE I

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rapid decrease in Val-tRNA₉₅₁ concentration. Addition of excess [¹⁴C]valine along with the Phe-tRNA synthetase leads to the nearly complete disappearance of all [¹⁴C]Val-tRNA within 20 min. The [³H]Val-tRNA has a half-life of 184 min if excess [¹⁴C]valine is added alone. The presence of excess [³H]Valine throughout the reaction effectively eliminates the incorporation of [³H]Valine into acid-precipitable material.

Fig. 5 indicates that Phe-tRNA synthetase (N. crassa) catalyzes the deacylation of both Phe-tRNA₉₅₁ and Val-tRNA₉₅₁ in both Tris and cacodylate buffers in reaction mixtures containing AMP and PP_i but no ATP. The enzymatic deacylation of Phe-tRNA₉₅₁ is much more rapid in cacodylate buffer than in Tris buffer, while the deacylation of Val-tRNA₉₅₁ is rapid in both buffers. The data in Table I show that the deacylation of Val-tRNA₉₅₁ catalyzed by Phe-tRNA synthetase is independent of AMP and PP_i concentrations. In contrast, the same enzyme will not catalyze the deacylation of Phe-tRNA₉₅₁ in the absence
of AMP and PPi. None of the decays catalyzed by Phe-tRNA synthetase are pseudo-first order. In the absence of enzymes decay is pseudo-first order and the half-life of Phe-tRNAVal is 52 min in Tris-HCl (pH 8.1) and 189 min in potassium cacodylate (pH 6.3). The half-life of Val-tRNAVal is 128 and 206 min in Tris and cacodylate buffers, respectively, when no enzymes are present.

While Val-tRNA synthetase can catalyze the decay of Val-tRNAVal, it cannot catalyze the decay of Phe-tRNAVal (Fig. 6). In fact, the decay of Phe-tRNAVal may actually be slower in the presence of Val-tRNA than in its absence. The enzymatic decay of Val-tRNAVal is much more rapid in in cacodylate buffer than in Tris buffer. In other experiments we showed that Val-tRNA synthetase cannot catalyze the decay of Val-tRNAVal in the absence of AMP and PPi.

The half-life of Phe-tRNAVal under conditions optimal for amination can be determined by adding excess [14C]phenylalanine to reaction mixtures after amination has reached equilibrium (Fig. 7). Under such conditions the half-life of Phe-tRNAVal is approximately 1000 min in cacodylate buffer and 62 min in Tris buffer. Thus, in cacodylate buffer the half-life of Phe-tRNAVal is much longer under amination conditions than under the conditions used for nonenzymatic decay (compare Figs. 5 and 7). The addition of both AMP and excess [14C]phenylalanine to reaction mixtures after net aminoacylation has ceased leads to a significant decrease in the half-life of Phe-tRNAVal (as compared with the addition of [14C]phenylalanine alone) in cacodylate buffer but has no effect on decay in Tris buffer. Although not shown, the addition of both PPi and excess [14C]phenylalanine has little, if any, effect on the half-life of Phe-tRNAVal in either buffer.

Phe-tRNA synthetase (N. crassa) does not interact well with tRNAVal (E. coli) in Tris buffer in the presence of homologous tRNA (Fig. 8). Under standard conditions approximately 37 pmol of Phe-tRNAVal (E. coli) are formed in 1 ml of reaction mixture containing 59 pmol of tRNAVal and 16 units of Phe-tRNA synthetase (N. crassa) (Fig. 8A). The substitution of 2.5 A260 units of tRNA (N. crassa) for the tRNAVal results in the formation of 40 pmol of Phe-tRNA (Fig. 8B). If both tRNAVal (E. coli) and tRNA (N. crassa) are present in a reaction mixture, the final yield of Phe-tRNA is less than 44 pmol per ml (Fig. 8C), considerably lower than the 77 pmol predicted on the assumption that the final yield obtained in the presence of both tRNAs would be the sum of the final yield obtained when each tRNA was alone in the reaction mixture. If a reaction mixture containing only tRNAVal (E. coli) is allowed to react until net aminoacylation has ceased, the subsequent addition of tRNAVal (E. coli) or tRNA (N. crassa) results in a relatively rapid and substantial increase in Phe-tRNA concentration (Fig. 8C). In the case where tRNA (N. crassa) is added, Phe-tRNA concentration reaches a maximum shortly after the addition and then drops off rather rapidly. If a reaction mixture containing only tRNA (N. crassa) is allowed to react until net aminoacylation has ceased, the subsequent addition of tRNAVal (E. coli) leads to little, if any, increase in Phe-tRNA concentration (Fig. 8B). In contrast, the subsequent addition of tRNA (N. crassa) results in a marked increase in Phe-tRNA concentration, so that the final concentration of Phe-tRNA is doubled when the amount of tRNA (N. crassa) is doubled.

**DISCUSSION**

This study started with the observation that Phe-tRNA synthetase (N. crassa) can apparently inhibit Val-tRNA formation catalyzed by Val-tRNA synthetase (E. coli). Fig. 4 indicates that this inhibition is due to a rapid decay of Val-tRNAVal by the N. crassa enzyme. Net Val-tRNAVal synthesis stops when the rate of Val-tRNAVal formation (catalyzed by Val-tRNA synthetase (E. coli)) is equal to the rate of Val-tRNAVal decay (as catalyzed by Phe-tRNA synthetase (N. crassa)).
The greater the Phe-tRNA synthetase concentration the greater the rate of deacylation and the lower the steady state concentration of Val-tRNAVal. At a given Phe-tRNA synthetase concentration an increase in Val-tRNA synthetase concentration leads to an increase in the steady state concentration of Val-tRNAVal.

Dimethylosulfoxide is known to stimulate the formation of Phe-tRNAVal (E. coli) in Tris buffer (4) catalyzed by Phe-tRNA synthetase. Fig. 3 indicates that (CH₃)₂SO only slightly inhibits the rate of Val-tRNAVal formation but does not affect the extent of product formation. For these reasons, (CH₃)₂SO would be expected to decrease the steady state concentration of Val-tRNAVal under the conditions described in Fig. 1. Fig. 2 indicates that this is indeed the case. The (CH₃)₂SO presumably inhibits the rate of aminoacylation by Val-tRNA synthetase, while it stimulates the rate of deacylation by Phe-tRNA synthetase.

Fig. 5 shows that the Phe-tRNA synthetase (N. crassa) can deacylate both Val-tRNAVal (E. coli) and Phe-tRNAVal (E. coli) under deacylation conditions that include the presence of AMP and PP₁ but not of ATP. However, the deacylation of Val-tRNAVal is independent of AMP and PP₁ concentrations, while the deacylation of Phe-tRNAVal is dependent on AMP and PP₁ (see "Results"). These latter facts suggest that the mechanism of Val-tRNAVal deacylation is different from the mechanism of Phe-tRNAVal deacylation. The deacylation of Phe-tRNAVal presumably results from a reversal of the normal aminoacylation reaction. Such a reversal would require AMP and PP₁ and would be expected to proceed more rapidly in cacodylate buffer than in Tris buffer. In contrast, the deacylation of Val-tRNAVal, which is independent of AMP and PP₁, may represent a simple hydrolysis reaction. Our hypothetical explanation assumes that the Phe-tRNA synthetase interacts with the RNA portion of Val-tRNAVal and causes the ester bond to hydrolyze. The valine that is released cannot bind to the amino acid site on the enzyme and is released from the complex.

In contrast to the Phe-tRNA synthetase (N. crassa), the Val-tRNA synthetase (E. coli) cannot deacylate Phe-tRNAVal (Fig. 0), although it does catalyze the deacylation of Val-tRNAVal normally, as expected. The deacylation of Val-tRNAVal is dependent on PP₁ and AMP, indicating that the deacylation results from a reversal of the acylation reaction. An indication that Val-tRNA synthetase does interact with Phe-tRNAVal is seen in the fact that the half-life of the latter is slightly longer in the presence of Val-tRNA synthetase than in its absence. This is true in both Tris and cacodylate buffers. Since the enzyme concentration is only ~0.7 pmole per ml, as compared to the Phe-tRNAVal concentration of 1.8 pmoles per ml, further studies may show that greater protection of Phe-tRNAVal by the enzyme can be achieved and that the conditions that critically affect this interaction can be explored. The mechanism by which such interactions stabilize the aminoacyl ester bond to chemical hydrolysis presumably involves the positioning of the ester bond in a protected site in the tertiary structure of the tRNA or enzyme. In summary, the abilities of the two enzymes to interact with an abnormal substrate are to be contrasted on the basis of the finding that Phe-tRNA synthetase catalyzes the deacylation of Val-tRNAVal, whereas Val-tRNA synthetase does not catalyze the deacylation of Phe-tRNAVal but may inhibit its chemical deacylation.

The stability of Phe-tRNAVal is also markedly affected by other conditions. In cacodylate buffer the half-life of Phe-tRNAVal is longer under aminoacylation conditions (approximately 1000 min (Fig. 7)) than under the conditions used for nonenzymatic deacylation (180 min (Fig. 5)). This may indicate that the Phe-tRNAVal is partially protected from deacylation under aminoacylation conditions, possibly through interactions with Phe-tRNA synthetase. Similar results were reported for the Ile-tRNA synthetase (E. coli), in that the hydrolytic deacylation of Ile-tRNAVal was inhibited by ATP, by tRNAValΔ₁, and probably by isoleucyl adenylate (12). If the Phe-tRNA synthetase of N. crassa is also inhibited by the corresponding components, it is possible to imagine that the Phe-tRNAVal is associated with the enzyme in an inhibited state and that the enzyme shields the ester bond in some way.

It has been suggested previously that the low final yields of Phe-tRNAVal obtained in Tris buffer may be due to the reversible formation of an inactive enzyme complex (4). This proposal is supported by the observation that AMP cannot stimulate the enzymatic deacylation of Phe-tRNAVal in a Tris-buffered amino-
acylation mixture after net aminoacylation has ceased (Fig. 7). In contrast, AMP markedly decreases the half-life of Phe-tRNA\(^{Val}\) in cacodylate buffer under similar conditions. This ability of AMP to decrease the half-life of Phe-tRNA\(^{Val}\) in cacodylate indicates that the enzyme is active, whereas the failure of AMP to cause a similar effect in Tris buffer suggests an inactive enzyme.

Another demonstration that a complex forms between Phe-tRNA synthetase and tRNA is seen in Fig. 8. The amount of Phe-tRNA formed when both tRNA\(^{Vsr}\) and tRNA\(^{Phe}\) are present is only slightly greater than when tRNA\(^{Phe}\) alone is present (Fig. 8, B and C). For these experiments unfractionated tRNA (N. crassa) was used, but we assume that only the tRNA\(^{Phe}\) reacts with the enzyme. The addition of tRNA\(^{Phe}\) shows that the enzyme reacts readily with either tRNA\(^{Vsr}\) or tRNA\(^{Phe}\). Thus the enzyme may not always act as simple catalysts (16), and one may be studying a complex steady state reaction instead of a simple equilibrium. In general, one must be very cautious in interpreting the results of crude heterologous reactions.

REFERENCES

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