Ribosomes of streptomycin-resistant strains (Str-R) translate genetic messages with fewer errors than ribosomes of streptomycin-sensitive (Str-S) strains. I have examined whether the increased level of tRNA discrimination by Str-R ribosomes, relative to Str-S ribosomes, occurs in an initial discrimination step before GTP hydrolysis during enzymatic tRNA binding, or in a second discrimination, or proofreading, step following GTP hydrolysis. Str-S or Str-R ribosomes, programmed with polyuridylate (poly(U)) and carrying N-acetylphenylalanyl-tRNA in the P-site, were allowed to react with elongation factor Tu, GTP, and Leu-tRNA\textsuperscript{1\textsubscript{SA}} (codon CUU) or Ile-tRNA (codon AUU). In the presence of poly(U), these noneognate tRNAs stimulated the hydrolysis of GTP at a much greater rate than the rate of amino acid incorporation into dipeptides, confirming a previous report that rejection of noneognate tRNAs by ribosomes occurs following GTP hydrolysis. tRNA discrimination by Str-R ribosomes is greater than that of Str-S ribosomes in the first discrimination step, as evidenced by lower rates of GTP hydrolysis, and in the second discrimination step, as evidenced by higher GTP/dipeptide ratios for noneognate tRNAs. The addition of streptomycin essentially abolished the second discrimination step, resulting in GTP/dipeptide ratios of approximately 1 for Str-S ribosomes. In addition, streptomycin appeared to increase the rate of GTP hydrolysis for noneognate tRNAs. Because these results parallel the known effects of Str-R mutations and of streptomycin on the fidelity of protein synthesis in vivo, tRNA discrimination probably takes place in two steps in vivo, one before and one after GTP hydrolysis. Ribosomal protein S12 influences tRNA selection at both discrimination steps.

Ribosomal proteins appear to be involved in determining the fidelity of protein synthesis. This has been demonstrated in studies of Escherichia coli using certain aminoglycoside antibiotics and mutants in which the structures of ribosomal proteins are altered (1, 2). For example, streptomycin causes a dramatic increase in the error frequency of protein synthesis (for a review, see Ref. 1). In addition, it is known that ribosomes of streptomycin-resistant strains translate mRNA with much greater accuracy than wild type, streptomycin-sensitive ribosomes both in vivo (3-5) and in vitro (6). This restriction of misreading and the Str-R\textsuperscript{1} phenotype are due to alterations in protein S12 of the 30 S subunit (7).

In order to explain the high fidelity of translation, Hopfield proposed that the involvement of GTP hydrolysis in tRNA binding allows the selectivity of codon-anticodon interactions to be imposed in two (or more) steps (8). In the specific model proposed by Hopfield (see Fig. 1), discrimination between cognate and noneognate aminoacyl-tRNA first takes place in Reaction 1. Any incorrect AA-tRNA, which escaped from the first discrimination step, remaining bound long enough to undergo the second reaction driven by GTP hydrolysis (Reaction 2), will reach another state of AA-tRNA-ribosome complex, and will then be subjected to the second discrimination step, that is, most of the noneognate AA-tRNAs will be dissociated (Reaction 3), while most of cognate AA-tRNAs will participate in peptide bond formation (Reaction 4). Thus, the degree of discrimination between cognate and noneognate AA-tRNAs will be increased. Hopfield generalized this kind of mechanism which involves a series of irreversible steps with side reactions to reject incorrect substrates and termed it "kinetic proofreading" (8). Thompson and Stone (9) recently reported that in the reactions with poly(U) and noneognate AA-tRNA, and Leu- and Ile-tRNA, GTP hydrolysis is much greater than that expected from peptide bond formation, demonstrating the rejection step (Reaction 3) predicted by Hopfield (8).

I have studied mistranslation of poly(U) and asked whether the alteration of ribosome structures caused by Str-R mutations influences discrimination before or after the hydrolysis of GTP. The results presented in this paper demonstrate that the mutation increases the degree of discrimination in both steps. I have also found that the addition of streptomycin to the in vitro system practically abolishes the second discrimination reaction. These experiments demonstrate correlation in the fidelity of translation between in vitro and in vivo systems and imply that both discrimination steps occur in vitro. The significance of the results to fidelity of translation and previously proposed models is discussed.

**EXPERIMENTAL PROCEDURES**

**Preparation of Ribosomes and EF-Tu**—Ribosomes were prepared from E. coli K12 strains PR-C600 (Str-S) and PR-SM3 (Str-R). These two strains are isogenic with the only known difference at the strA locus. PR-C600 was constructed by introducing the ara\textsuperscript{E} to str\textsuperscript{A} region from C600 to PR13 (an ara\textsuperscript{E} strain; see Ref. 10) by P transduction. PR-SM3 was similarly constructed by introducing the ara\textsuperscript{E} to Str\textsuperscript{A} region from Sm3 to PR13. SM3 is a spontaneous Str-R

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* This work has been supported by Grants GM 20427 from the National Institutes of Health and PCM 7818490 from the National Science Foundation, administered by Masayasu Nomura. This is part 2348 from the Laboratory of Genetics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Training Grant GM 07133 from the National Institutes of Health.

‡ Supported by Training Grant GM 07133 from the National Institutes of Health.
Ribosomal Discrimination of Aminoacyl-tRNA

RESULTS AND DISCUSSION

I used poly(U) as mRNA and partially purified Leu-tRNA as noncognate tRNA, and measured both GTP hydrolysis and peptide bond synthesis caused by misreading of the UUU codon. The minor leucine accepting tRNA, tRNA\(^{Lue}\), recognizes the CUU and CUC leucine codons and misreads the UUU codon with high frequency (9, 23). Two kinds of ribosomes were compared, one from Str-S wild type E. coli and the other from a restrictive Str-R mutant strain.

Ribosomes programmed with poly(U) and carrying AcPhe-tRNA in the P-site were reacted with the ternary complex of EF-Tu, \([\gamma-32P]GTP\), and \(^{3}H\)-labeled leucyl-tRNA. The amounts of GTP hydrolyzed and dipeptide synthesis were assayed (Fig. 2, A and B). To compare results obtained with Str-S ribosomes with those obtained with Str-R ribosomes, observed values were corrected for the activity differences of the two ribosome-poly(U)-AcPhe-tRNA preparations, as determined by AcPhePhe synthesis (see "Experimental Procedures"). It can be seen that the addition of poly(U) and Leu-tRNA\(^{Lue}\) stimulated hydrolysis of GTP to larger extents than formation of dipeptide (AcPheLeu); molar ratios of GTP hydrolyzed to peptide bond formed were approximately 5 to 1 with Str-S ribosomes.

Str-R ribosomes exhibit less stimulation of GTP hydrolysis than Str-S ribosomes, but GTP cleavage is not restricted by the Str-R mutation as severely as is dipeptide formation. This stimulation of GTP hydrolysis by poly(U) plus Leu-tRNA\(^{Lue}\), although weak with Str-R ribosomes, is evidently due to Leu-tRNA\(^{Lue}\) since tRNA charged with tyrosine, valine, glycine, or lysine shows no stimulation of GTP hydrolysis by poly(U) with Str-R ribosomes (data not shown). Str-R ribosomes synthesized dipeptides at about 4.5% the rate of Str-S ribosomes in this experiment and hydrolyzed GTP at 18% the rate of Str-S ribosomes. For the calculation of the latter value, the amount of GTP hydrolyzed in the absence of poly(U) is subtracted from the experimental values. The amount of GTP hydrolyzed relative to dipeptide produced is then 4-fold higher with the Str-R ribosomes than with Str-S ribosomes.

These results are summarized for three such experiments with tRNA\(^{Lue}\) as in Table I. As can be seen, the ratio of GTP to dipeptide is 3- to 4-fold higher with Str-R ribosomes. Thus, the rejection of noncognate tRNA, i.e. Leu-tRNA, in the second discrimination step is much more efficient with Str-R ribosomes than with Str-S ribosomes. The rate of GTP hydrolysis is also found to be 2.5- to 6-fold lower with Str-R ribosomes in these experiments, indicating that the efficiency of discrimination in the first step is also increased.

The product of peptide bond formation was analyzed by paper electrophoresis as previously described (12). About 80% of the radioactive product migrated with the expected mobility of AcPheLeu, although the additional peaks with lower mobility, probably corresponding to tri- and tetrapeptides, were detected. The reaction product, referred to as "dipeptide" in this paper, provides a measurement of peptide bond formation.

Fig. 1. Scheme for AA-tRNA selection in protein synthesis proposed by Hopfield (8), including a second discrimination, or proofreading, step. Rib denotes ribosome. See introduction.
Similar results were found when Ile-tRNA was used for dipeptide synthesis (Fig. 3). Although the amount of poly(U)-dependent GTP hydrolysis and dipeptide synthesis were more difficult to determine with Str-R ribosomes, the amount of GTP hydrolyzed relative to dipeptide produced was significantly higher with Str-R than with Str-S ribosomes in all experiments performed (Table II, see below).

Streptomycin was added to parallel reactions in the experiments with leucine (Fig. 2) and isoleucine (Fig. 3). It was observed that streptomycin greatly stimulates the rate of dipeptide synthesis with these noncognate AA-tRNAs and that the dipeptide synthesis rates then approached the rate of GTP hydrolysis (Table II). In fact, with Str-S ribosomes, molar ratios of the GTP hydrolyzed to dipeptide synthesized became approximately 1 for leucine and isoleucine in all experiments. With Str-R ribosomes, this ratio was about 1 for leucine and 1.7 to 2.8 for isoleucine. Although the effect of abolishing proofreading is more striking, streptomycin usually increases the rate of GTP hydrolysis with noncognate tRNA misreading poly(U) (Figs. 2 and 3). The only exception is the combination of Leu-tRNA<sub>2</sub> and Str-S ribosomes, which

![Fig. 2. Reaction of the ternary complex of Leu-tRNA<sub>2</sub> with poly(U)-programmed ribosomes. The complex of [<sup>14</sup>C]Leu-tRNA<sub>2</sub>, EF-Tu, [gamma-<sup>32</sup>P]GTP and an AcPhe-tRNA<sub>2</sub> complex were reacted as described under “Experimental Procedures.” Streptomycin (500 µM) was also included in the reaction when indicated (C and D). Specific activities of [gamma-<sup>32</sup>P]GTP were 1900 cpm/pmol and 3045 cpm/pmol, respectively. The amount of [gamma-<sup>32</sup>P]PO<sub>4</sub>, present prior to mixing with ribosomes, 0.33 to 0.38 pmol, has been subtracted. The ratio of the activity of Str-R ribosomes to that of Str-S ribosomes was 1.4, and this value has been used to correct the observed values for Str-R ribosomes. Open symbols, Str-S ribosomes; filled symbols, Str-R ribosomes; solid curves, A, A, with poly(U); dashed curves, O, O, without poly(U). Note that the vertical scale in A is expanded 5-fold relative to B, C, and D for visualization of dipeptide curves in the absence of streptomycin.

![Fig. 3. Reaction of the ternary complex of Ile-tRNA with poly(U)-programmed ribosomes. Reactions were carried out as described under “Experimental Procedures” except that reactions were terminated by adding aliquots (160 µl) to an equal volume of 0.1 N HCl. Equal portions (150 µl) of the quenched reactions were assayed for dipeptide formed or phosphate released. The amount of [gamma-<sup>32</sup>P]PO<sub>4</sub>, present prior to incubation, 0.035 to 0.050 pmol in these reactions, has been subtracted. The activity of Str-R ribosomes relative to Str-S ribosomes was 0.95. This value has been used for correction. Specific activities of [gamma][H]Ile and [gamma-<sup>32</sup>P]GTP were 3900 cpm/pmol and 3900 cpm/pmol, respectively. Open symbols, Str-S ribosomes; filled symbols, Str-R ribosomes; solid curves, A, A, with poly(U); dashed curves, O, O, without poly(U). Note that the vertical scale in A is expanded 5-fold relative to B, C, and D.

**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>GTP (Str-R)/GTP (Str-S)</th>
<th>Dipeptide (Str-R)/dipeptide (Str-S)</th>
<th>GTP/dipeptide (Str-R)</th>
<th>GTP/dipeptide (Str-S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.21</td>
<td>0.065</td>
<td>4.8</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>0.043</td>
<td>4.5</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>0.43</td>
<td>0.11</td>
<td>12</td>
<td>45</td>
</tr>
</tbody>
</table>
TABLE II

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>Number of experiments</th>
<th>Str-S</th>
<th>Str-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcPheLeu</td>
<td>3</td>
<td>7.1</td>
<td>96</td>
</tr>
<tr>
<td>AcPheIle</td>
<td>3</td>
<td>8.3</td>
<td>56</td>
</tr>
<tr>
<td>AcPhePhe*</td>
<td>1</td>
<td>1.16</td>
<td>107</td>
</tr>
</tbody>
</table>

Values given are averages obtained from the indicated number of experiments. Calculations were made as described for Table I. In each experiment, two to four measurements were made after 1.5 min to 15 min of incubation. There was no systematic variation of ratios as reactions proceeded. Values varied by less than 30% from the mean in each experiment, although variation between experiments was sometimes greater (see Table I).

Although the synthesis of AcPhePhe was 80% complete after 15 s of incubation, additional measurements were made after 2 and 5 min. For measurements of GTP hydrolysis, the values obtained in the absence of poly(U) were subtracted from experimental values. The poly(U)-independent GTP hydrolysis was slow but became significant after longer incubation times needed for the reactions with noncognate Leu-tRNA and Ile-tRNA (cf. Figs. 2 and 3). The fact that GTP, to dipeptide ratios, calculated in the Phc reaction, remained approximately 1, supports the validity of subtraction of the background values from experimental ones in calculating the amounts of GTP specifically hydrolyzed according to Reaction 1, indicated in Fig. 1.

Ribosomal Discrimination of Aminoacyl-tRNA

Values given are averages obtained from the indicated number of experiments. Calculations were made as described for Table I. In each experiment, two to four measurements were made after 1.5 min to 15 min of incubation. There was no systematic variation of ratios as reactions proceeded. Values varied by less than 30% from the mean in each experiment, although variation between experiments was sometimes greater (see Table I).

The extent that peptide bond formation may result from nonenzymatic binding of tRNA in these experiments was also investigated. The omission of GTP reduced the rate of dipeptide synthesis more than 10-fold in experiments similar to those of Figs. 2 and 3 but with EF-Tu and Leu-tRNA_{Aeu} added uncomplexed (data not shown). The addition of nonhydrolyzable analogues of GTP, GMP-PNP or GMP-PCP, yielded the same reduced rates. Thus, as expected under the experimental conditions used (6 mM Mg^{2+}), the dipeptide synthesis observed is essentially dependent on enzymatic tRNA binding utilizing GTP.

I have demonstrated that ribosomes of strA mutants, known to translate with reduced misreading in vivo (1, 4–6), show higher efficiency in rejecting noncognate tRNAs in both the first and second discrimination steps than the wild type ribosomes. Similarly, streptomycin, which is known to cause misreading in vivo, both decreases initial discrimination and reduces GTP/dipeptide ratios, essentially abolishing the second discrimination step for tRNAs especially prone to misreading. These results support the hypothesis that a proofreading step following an initial discrimination step, occurs in vivo.

Ninio (26) proposed that restriction of misreading by Str-R ribosomes could result from enhanced kinetic discrimination due to a decrease in the rate of a single forward reaction, which could correspond to Reaction 2 or 4 in Fig. 1. It is conceivable that tRNA binding is altered with Str-R ribosomes such that the rates of both Reactions 2 and 4 are decreased, as would be required to increase discrimination both before and after GTP hydrolysis. A simpler explanation, in accordance with kinetic models (8, 26), is that a decreased stability of tRNA bound to Str-R ribosomes would increase discrimination at both stages by affecting dissociation in Reactions 1 and 2. This alteration of tRNA binding would have more pronounced effects on noncognate tRNAs that bind weakly to the codon at the A-site than on tRNAs with strong anticodon-codon binding and, in this way, increase discrimination. Alternatively, ribosome structures may distinguish between correct and incorrect base pairings by imposing steric (27) or allosteric (28) restrictions on codon-anticodon interactions rather than by kinetic mechanisms of discrimination.

Since GTP cleavage is essentially irreversible in vitro, we may consider tRNA discrimination to occur in two separate steps, terming the second discrimination step “proofreading.” However, there is no evidence that the discrimination has a kinetic basis, as proposed by Hopfield (see introduction and Ref. 8) utilizing energy of GTP hydrolysis to drive a proofreading reaction. Enzymatic tRNA binding, utilizing GTP, may simply increase the rate of an interaction of tRNA, mRNA, and ribosomes that alone determines the degree of discrimination.

In summary, these experiments provide additional evidence that rejection of noncognate tRNAs, following GTP hydrolysis in enzymatic tRNA binding, occurs in vitro. In addition, the results obtained with Str-R ribosomes as well as the observed effect of streptomycin are consistent with previous observations made in vivo and imply that tRNA selection by ribosomes in vivo involves discrimination both before and after GTP hydrolysis.

Acknowledgments—I thank Masayasu Nomura, in whose laboratory this investigation was performed, for advice and encouragement, and Julian Davies for critically reading the manuscript.

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J L Yates


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