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Kinetic analysis of an *E.coli* phenylalanine-tRNA synthetase mutant

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ABSTRACT

A mutation in the pheS gene, encoding phenylalanyl-tRNA synthetase, in E. coli NP37 confers temperature-sensitivity on the organism. A five-fold increase in $tRNA^{phe}$ levels complements the mutation. Analysis of the kinetic properties of the mutant enzyme indicates that the K_M is 20-fold higher than the wild-type and the dissociation constant of the $tRNA^{phe}$-synthetase complex for the mutant is at least 10-fold higher. These results indicate that the mutation in E. coli NP37 directly affects the $tRNA^{phe}$ binding site on the cognate synthetase.

INTRODUCTION

The initial step in protein biosynthesis is aminoacylation of a specific tRNA with its cognate amino acid. This reaction is catalyzed by a class of enzymes referred to as aminoacyl-tRNA synthetases (1). There is a single, specific aminoacyl-tRNA synthetase for each amino acid. Translational fidelity requires that this reaction transpire with extreme precision since a misacylated tRNA will donate the incorrect amino acid into the nascent peptide chain on the ribosome (2). Given their similarity of function, synthetases exhibit a surprising degree of structural diversity (3). Despite this, Schimmel has proposed that all synthetases contain a "core" which is responsible for catalysis and the additional amino acids are dispensable for this process (4). Considerable research has provided only limited information on the mechanism of the tRNA-synthetase interaction.

E. coli phenylalanyl-tRNA synthetase (pheRS) is an $\alpha_2\beta_2$ enzyme whose individual polypeptides have molecular weights of 39,000 (α) and 94,000 (β) (5). The two subunits are coded for by adjacent genes (pheS and pheT) on the E. coli chromosome (6). An E. coli mutant strain, NP37, has been shown to harbor a tempera-

ture sensitive pheRS; the mutation maps to the α subunit (7). In an earlier report, it was shown that NP37 transformed with a plasmid containing the gene coding for $tRNA^{phe}$ was capable of growth at the non-permissive temperature. The transformed strain exhibited a five-fold overproduction of $tRNA^{phe}$ (8). This observation suggested that the mutant NP37 pheRS has an elevated K_M for its cognate tRNA and that overproduction of $tRNA^{phe}$ is sufficient to provide a functional degree of saturation of pheRS such that normal growth can be restored at the non-permissive temperature. In order to test this hypothesis, a comparison of the kinetic properties of the mutant and wild-type pheRS was undertaken.

MATERIALS AND METHODS

Materials

E. coli NP37 (relA, tonA, pheS5) (9) was obtained from the *E. coli* Genetic Stock Center (CGSC #4913). Frozen *E. coli* MRE 600 cells were obtained from Grain Processing, Muscatine, IA.

Preparation of Phenylalanine-tRNA Synthetase

E. coli cells were ruptured by grinding with alumina or passage through a French press and the post-ribosomal supernatants were prepared as described (10). The pheRS activity was precipitated at 40%-51% saturation of ammonium sulfate, resuspended in 50 mM K-phosphate (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol and applied to a DEAE-cellulose column. pheRS activity was batch eluted with 150 mM K-phosphate (pH 6.5), 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol. The preparation was adjusted to 50% glycerol and stored at -20°C. In one case, the enzyme was purified to near homogeneity by the procedure of Fromant et al. (11). The K_M values for $tRNA^{phe}$ determined with the ammonium sulfate fraction, DEAE-cellulose fraction and pure pheRS were essentially identical. Unless otherwise noted, the pheRS employed in these studies was a preparation purified through the DEAE-cellulose step. The source of wild-type enzyme was either *E. coli* MRE600 or HB101 transformed with pID1 (10).

Aminoacylation of $tRNA^{phe}$ and Determination of Kinetic Parameters

Crude tRNA was prepared from *E. coli* HB101 transformed with pRK3 (which exhibits eight-fold overproduction of $tRNA^{phe}$ (8)) as

described (12). Aminoacylation was performed as described previously (8) in a reaction mixture consisting of 100 mM NaHepes (pH 7.5), 10 mM KCl, 40 mM $MgCl_2$, 1.0 mM dithiothreitol, 5 mM ATP, 10-20 μM [^{3}H]phenylalanine and $tRNA^{phe}$ concentrations ranging from 2.3-230 nM. Reactions were terminated by addition of 5% trichloroacetic acid. The precipitates were collected on glass fiber filters and the radioactivity was measured by liquid scintillation counting. Kinetic parameters were determined from the initial velocities of aminoacylation for at least five $tRNA^{phe}$ concentrations. In some cases, a time-integrated derivation of Michaelis-Menten kinetics (13) was employed to obtain K_M and V_{max} values from a single time point.

Determination of the Dissociation Constant for $tRNA^{phe}$

Pure $tRNA^{phe}$ was 5'-end labelled with γ-[^{32}P]ATP and T4 polynucleotide kinase as described previously (8). The dissociation constant for the binding of $tRNA^{phe}$ to pheRS was determined by nitrocellulose filter binding, essentially as described by Yarus and Berg (14). Varying amounts of ^{32}P-labeled $tRNA^{phe}$ were incubated with partially purified pheRS in 48 mM KH_2PO_4, 2 mM K_2HPO_4, 10 mM $MgCl_2$, 1 mM DTT and 50 μg/ml of bovine serum albumin (BSA) for several seconds at room temperature. The mixture was then slowly filtered through a nitrocellulose filter (Schleicher and Schuell BA85). The filters were washed with additional association buffer (minus DTT and BSA), dried and the bound radioactivity was measured by liquid scintillation counting.

Alternatively, association was measured by gel electrophoresis by a modification of the "gel-retardation" procedure which is widely used for analysis of DNA-protein interactions (15). Various concentrations of $tRNA^{phe}$ and pheRS were incubated in 30 microliters of 200 mM Tris-HCl (pH 8.0), 150 mM KCl, 50 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.05% BSA (w/v). Immediately after addition of tRNA and enzyme, bromophenol blue was added to 0.01% and the entire mixture was subjected to electrophoresis for 3 h on a 7.5% polyacrylamide gel in Tris-borate-EDTA buffer (16). The gel was dried and subjected to autoradiography. The radioactive bands (corresponding to free

$tRNA^{phe}$ and $tRNA^{phe}$-pheRS complex) were excised and quantitated by liquid scintillation counting.

RESULTS

Kinetic Parameters for Wild-Type and Mutant pheRS

K_M with regard to $tRNA^{phe}$ and Vmax were determined for pheRS from wild-type *E. coli* and the NP37 mutant. The values obtained from a large number of independent experiments are presented in table 1. The majority of the determinations were carried out with a partially purified enzyme preparation. This was necessitated by the inability to purify pheRS from NP37 to homogeneity due to its extreme lability in more highly purified preparations (17). Preliminary experiments with wild-type pheRS showed that K_M and Vmax were identical for the pure enzyme and the pheRS activity in the DEAE-cellulose purified fraction employed in these assays.

At 30°C, the permissive temperature for NP37, the K_M with respect to $tRNA^{phe}$ is 20-fold higher for the mutant than for the wild-type enzyme. Vmax, on the other hand, appeared to be essentially identical for both enzyme preparations. This suggests that the lesion in the mutant pheRS is the result of weaker binding of $tRNA^{phe}$ to the enzyme.

TABLE 1
Kinetic Parameters for Wild-type and Mutant pheRS

	Wild-type	Mutant
K_M (nM)	2.1 ± 1.0	43 ± 6.3
Vmax (pmol/min/μg)	0.28 ± .04	0.20 ± 0.1

Aminoacylation experiments were carried out at 30°C employing a pheRS preparation from both the wild-type and mutant which had been purified through the DEAE-cellulose step and K_M and Vmax were determined as described in Materials and Methods. The K_M and Vmax values (± standard deviation) were obtained from four separate determinations. Vmax is expressed relative to the total protein concentration in the partially purified pheRS preparation.

Dissociation Constants for the tRNA-pheRS Complex

To further evaluate the differences in $tRNA^{phe}$ binding to the wild-type and mutant enzymes the dissociation constant (K_d) for the $tRNA^{phe}$-pheRS complex was determined by nitrocellulose filter binding (14). These experiments (and those described below) were carried out with a partially purified pheRS preparation in which pheRS represented 16% of the total protein. The results for the wild-type pheRS are shown in figure 1 and yield a value of 0.5±.08 μM. Similar experiments were carried out with the mutant pheRS but it was not possible to achieve saturation, even with 10-fold higher concentrations of $tRNA^{phe}$.

In an attempt to measure the K_d of the mutant pheRS-$tRNA^{phe}$ complex a "gel-retardation" assay for complex formation was employed. Figure 2 shows the results of such an experiment with wild-type enzyme. The complex migrates more slowly than the free tRNA and is clearly resolved. Measurement of the radioactivity in gel slices enables the direct quantitation of the concentrations of free $tRNA^{phe}$ and $tRNA^{phe}$-pheRS complex. This, in turn, yields a K_d of 0.5±.06 μM for the wild-type complex. This is in excellent agreement with the value obtained by nitrocellulose binding. Unfortunately, the binding of $tRNA^{phe}$ to mutant pheRS

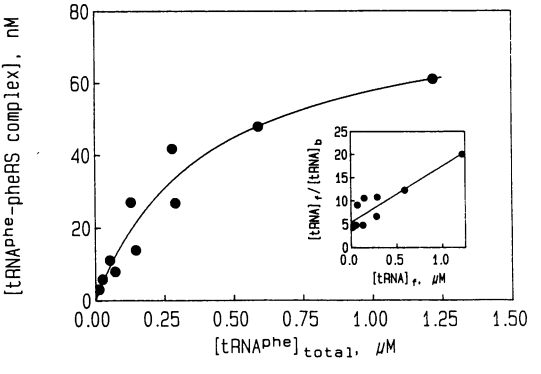

Figure 1. Binding of $tRNA^{phe}$ to wild-type pheRS. The indicated amounts of ^{32}P-labelled $tRNA^{phe}$ were incubated with a fixed amount of pheRS and complex formation was determined by nitrocellulose filter binding as described in Materials and Methods. Inset, the binding data transformed for determination of K_d. The slope of the line is equal to 1/[pheRS] and the y intercept is K_d/[pheRS].

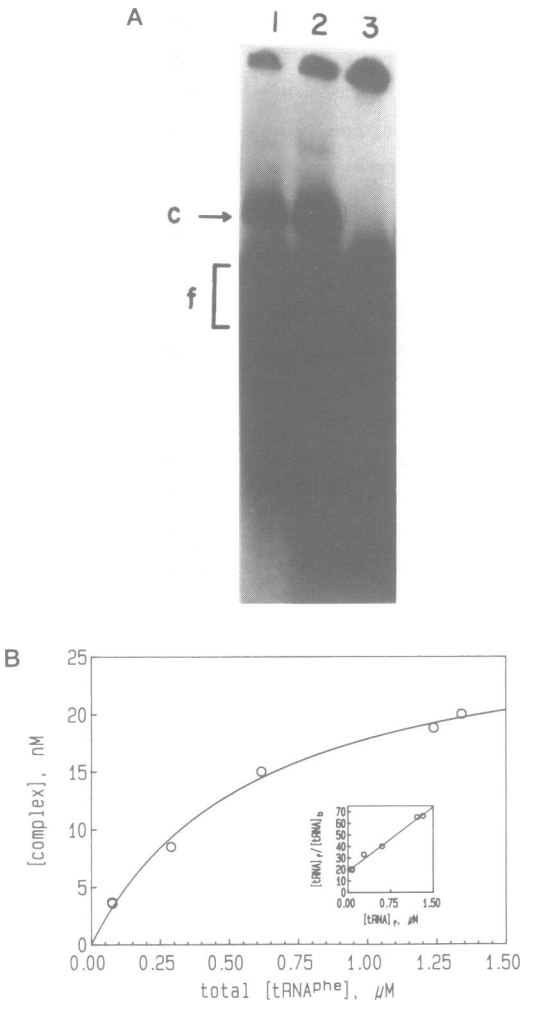

Figure 2. Analysis of wild-type $tRNA^{phe}$-pheRS complex by gel electrophoresis. A) The indicated amounts of ^{32}P-labelled $tRNA^{phe}$ (50,000 cpm/pmole) were equilibrated with a fixed amount of pheRS and the mixtures were subjected to electrophoresis as described in Materials and Methods. Lane 1: 0.31 μM $tRNA^{phe}$; lanes 2 and 3: 0.64 μM $tRNA^{phe}$. Lane 3 contained no pheRS. The band of $tRNA^{phe}$-pheRS complex is indicated as c and the region containing uncomplexed $tRNA^{phe}$ is indicated as f. Figure is an autoradiogram after one hour exposure. B) After autoradiography, the regions of the gel corresponding to free $tRNA^{phe}$ and $tRNA^{phe}$-pheRS complex were excised and the radioactivity was measured by liquid scintillation counting. The data were analyzed as in figure 1.

was too weak to allow measurement of the K_d by this technique, as well. For the wild-type complex, saturation begins between 0.6-1.0μM $tRNA^{phe}$. Addition of up to 12 μM $tRNA^{phe}$ to the NP37 enzyme was insufficient to achieve any degree of saturation. It is, thus, reasonable to conclude that the K_d for the mutant pheRS is at least an order of magnitude higher than that for the wild-type enzyme.

DISCUSSION

To assure a rate of protein synthesis which is adequate to sustain growth, uncharged tRNAs must be aminoacylated at least as rapidly as the charged tRNAs donate their aminoacyl moieties to nascent proteins. The temperature-sensitive *E. coli* strain NP37 apparently fails to meet this condition for phenylalanine at the non-permissive temperature. However, as little as a five-fold oversupply of $tRNA^{phe}$ is sufficient to correct this abnormality (8). The simplest hypothesis to explain this observation is that the velocity of aminoacylation is near limiting for cell growth, that uncharged $tRNA^{phe}$ concentration is not far above K_M and that K_M for the mutant enzyme is greater than that for wild-type. If this were the case, then a five-fold elevation in [$tRNA^{phe}$] would reduce $K_M/[S]$ back down to a level sufficient to restore growth at the elevated temperature.

The results reported here confirm that this is indeed the case. Even at the permissive temperature of 30°C the K_M for $tRNA^{phe}$ of the mutant enzyme is 20-fold greater than that for the wild-type. It is reasonable to assume that at 30°C, when the growth rate of *E. coli* is much slower than at 37°C, the charging rate of $tRNA^{phe}$ is not a growth-limiting process. Thus, although the K_M defect is demonstrable at 30°C, the consequence of the mutation is apparent only at elevated temperatures.

The K_M values for wild-type pheRS obtained in this study were 2.1 nM at 30°C and 4 nM at 37°C. This is somewhat lower than K_M values typically reported for aminoacyl-tRNA synthetases. However, Santi el al. reported K_M values for pheRS of 1.7×10^{-8} - 5.8×10^{-8} M at 37°C (18). Thus the values obtained here are close to those observed earlier given the variation which might be expected due to differences in assay conditions. The key

finding from the current experiments, however, is that under identical conditions the K_M values obtained with the wild-type and mutant pheRS varied significantly.

At first glance, it is somewhat surprising that only a 5-fold increase in [$tRNA^{phe}$] can compensate for the mutation since the K_M of the mutant pheRS is 20-fold higher than that of wild-type. Jakubowski and Goldman (19) reported that there are 1830 molecules of phe-tRNA (charged) and 992 molecules of pheRS in E. coli K38. Since 80% of total tRNA in the cell is charged (20), there are approximately 460 molecules of uncharged $tRNA^{phe}$ in the cell (i.e. 1830/.8 = 2288 total $tRNA^{phe}$; 2288 x .2 = 458). Thus, a 5-fold increase in total $tRNA^{phe}$, which would appear almost entirely in the uncharged tRNA pool, would result in a re-establishment of substrate excess for pheRS and permit the production of sufficient charged $tRNA^{phe}$ to meet cellular requirements.

A pseudorevertant of an E. coli temperature-sensitive glutaminyl-tRNA synthetase mutant has been reported in which the $tRNA^{Gln}$ levels are doubled (21). In that case, the enzyme defect is manifested as irreversible inactivation at the restrictive temperature and complementation is thought to operate by substrate protection of the enzyme. This is apparently not the case for E. coli NP37 since the complementation can be fully explained by the differences in K_M.

The altered K_M for the mutant enzyme appears to be predominantly the result of weaker tRNA binding affinity as determined by a K_d for the $tRNA^{phe}$-pheRS complex which is at least an order of magnitude higher than that for the wild-type. The K_d was determined by both nitrocellulose binding and "gel-retardation" experiments. To our knowledge, this is the first application of the "gel-retardation" assay to the study of tRNA-synthetase interactions. This method has a major advantage over nitrocellulose binding because it can be carried out at pH≈8 rather than pH of 5.5 for nitrocellulose binding (14). Contrary to the generally accepted principle that the synthetase-tRNA interaction is weaker at higher pH (1) the observed values for K_d were identical in both assays. Bartmann et al. have reported a Kd for the $tRNA^{phe}$-pheRS of 0.09-1.2 μM (22) which compares favorably to

the value of 0.5 μM determined in the present study. The fact that the values obtained for K_d were identical in both assays indicates that no ionizable groups with a pKa in the pH range of 6-8 (e.g. histidine) are involved in $tRNA^{phe}$ binding. In this case, as well, the crucial observation is that there is a difference in the Kd of at least an order of magnitude between the wild-type and mutant pheRS.

The results presented here indicate that a mutation in the α subunit of pheRS results in a significant impairment of tRNA binding. The wild-type gene for α-pheRS has been cloned and sequenced (23), but little is known regarding the determinants of the tRNA binding site. Elucidation of the site of mutation in NP37 pheS will provide valuable information with regard to the molecular basis of $tRNA^{phe}$ binding to pheRS.

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