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Mutants of *Escherichia coli* K-12 with an Altered Glutamyl-Transfer Ribonucleic Acid Synthetase

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Three streptomycin-suppressible lethal mutants of *Escherichia coli* K-12 have been shown to possess structurally altered glutamyl-transfer ribonucleic acid (tRNA) synthetases. Each mutant synthetase displays a K_m value for glutamate which is 10-fold higher than the parental value, and the mutations reside in two widely separate loci on the genetic map. Mixing of the mutant extracts in pairs gave no indication of in vitro complementation. All three enzymes charge the minor $tRNA^{glu}$ fraction identically, but one (EM 120) charges the major fraction at a twofold lower rate than do the other two (EM 102 and EM 111). Possible explanations for the existence of the two synthetase loci are presented.

The established role of aminoacyl-transfer ribonucleic acid (tRNA) synthetases in protein synthesis, their involvement in regulatory processes, and their use in studying the basic phenomenon of nucleic acid "recognition" by specific proteins have all been reviewed recently (2, 3, 13, 20, 21, 23, 27, 29, 30–32, 35). Much of the insight into the structure and function of these enzymes has come from the study of genetically modified synthetases. Temperature-sensitive mutants have been found with alterations in the synthetases for valine (2, 36), phenylalanine (21), and alanine (10). Analogue-resistant synthetase mutants have been found for phenylalanine (3), arginine (9), isoleucine (32), and histidine (27). Mutants with synthetases displaying elevated amino acid K_m values have appeared among auxotrophs requiring histidine (20), tryptophan (1), tyrosine (30), glycine (4), or methionine (8).

A previous communication (19) related the isolation of three mutants with decreased ability to charge tRNA with glutamic acid. In this paper we verify that these streptomycin (Sm)-suppressible lethal mutants have altered glutamyl-tRNA synthetases and discuss similarities and differences among them.

[1] Predoctoral trainee of the U.S. Public Health Service. The material in this paper is taken in part from a dissertation presented to the Graduate School of Yale University by E. J. Murgola in partial fulfillment of the requirements for the Ph.D. degree.

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MATERIALS AND METHODS

The derivation of the mutants, the media employed, the methods used for mapping and growth studies, and the preparation of S100 extracts were all previously described (19).

Diethylaminoethyl (DEAE)-cellulose extracts. Cells grown in medium D with Sm to late-logarithmic phase were harvested by centrifugation, washed three times with medium D, and suspended in fresh medium D with no Sm to a cell density of approximately 2×10^7/ml. The cells were incubated further for 4 or 5 hr (Sm-deprivation), and harvested. All further operations were performed at 4 C. After suspension in buffer S [10% glycerol; 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-chloride, *p*H 7.9; 0.01 M $MgCl_2$; 0.02 M β-mercaptoethanol], the cells were passed through a French pressure cell at a pressure of 8,000 psi. The lysed cell suspension was centrifuged in a Beckman no. 40 rotor at 36,000 rev/min for 2.5 hr. The clear supernatant solution was withdrawn to a level even with the uppermost portion of the pellet. The extract was applied to a DEAE-cellulose column (2.2 by 11 cm) previously equilibrated with buffer S. The column was washed with buffer S until the absorbance at 280 nm fell to the base level, and then eluted with buffer S containing 0.25 M NH_4Cl. The peak resulting from the change of buffer was assayed for glutamyl-tRNA synthetase activity, and the activity-containing fractions (50 ml) were pooled and concentrated fourfold by dialysis against buffer S containing 30% polyethylene glycol (PEG), molecular weight 6,000. Concentration was followed by overnight dialysis against buffer S (no PEG), and the extract was stored in 50% glycerol at −17 C.

Aminoacyl-tRNA synthetase assays. All 20 amino-

acyl-tRNA synthetases were assayed in the "charging reaction" which measures the overall reaction of activating an amino acid and attaching it to its cognate transfer RNA. By use of radioactive amino acids, the rate of the forward reaction was determined by measuring the amount of label incorporated into a cold acid-insoluble material. The standard reaction mixture contained, per 0.1 ml, the following constituents in the final concentrations indicated: Tris acetate, 0.1 M, pH 7.8; magnesium acetate, 0.02 M; cytidine 5'-triphosphate, 0.0005 M; adenosine 5'-triphosphate (ATP), 0.01 M; phosphoenolpyruvate (PEP), 0.01 M; ^{14}C-L-amino acid (7.5 μc/μmole), 5×10^{-5} M; β-mercaptoethanol, 0.05 M; PEP kinase, 1 μg/ml; tRNA (unfractionated, *Escherichia coli* K-12), 1 mg/ml; enzyme extract protein, 30 to 120 μg/ml (S100 extracts were used, except where noted otherwise). For studies on glutamyl-tRNA synthetase, the tRNA concentration used was 4 mg/ml and the Mg/ATP ratio was changed to 15:1 (25) by using magnesium acetate at 0.03 M and ATP at 0.002 M. The assays were done at 30 C and the reaction was terminated by placing samples on filter-paper discs (17) and immersing the discs in cold 10% trichloroacetic acid for 0.5 hr. The discs were washed three times (15 min each) with cold 5% trichloroacetic acid (containing nonradioactive glutamic acid in the glutamyl-tRNA synthetase assays), once with ether-ethanol (3:1), and once with ether. After being dried with an infrared lamp, the discs were counted in a Beckman LS-250 liquid scintillation counter, with a scintillation fluid containing 15.2 g of 2,5-diphenyl oxazole (PPO), and 0.38 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP) in 3.75 liters of toluene. A blank containing bovine serum albumin in place of enzyme extract was incubated with each set of assay tubes. Activity values are expressed as counts per minute obtained above the blank value (usually around 50 counts/min). In kinetic experiments, the activities are expressed as counts per minute above a value obtained with a zero-time sample.

Preparation of tRNA. Unfractionated tRNA (from *E. coli* K-12) was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. To increase the acceptor capacity of certain species of tRNA (14), the RNA solution was heated at 60 C for 5 min in the presence of 0.01 M magnesium acetate (and Tris acetate, pH 7.8), and then cooled to room temperature before being frozen in small portions. The fractionated tRNA used consisted of two fractions of $tRNA^{glu}$. The fractions were obtained as two separate peaks, a major ("fraction I") and a minor ("fraction II"), in benzoylated DEAE-cellulose column chromatography (6) by J. Bartz and D. Söll, and samples of each were generously provided by them.

RESULTS

Screening. As described in the accompanying communication (19), a number of mutants was isolated which exhibit a progressive decline in the rate of protein synthesis following the removal of Sm from the medium. Ten of these mutants were chosen for analysis of aminoacyl-tRNA synthetase activities. The S100 extract of EM-0 (the parent strain) was first checked for linearity of the charging reaction with enzyme concentration and time, for each amino acid. Extracts of the 10 mutants, prepared from Sm-starved cells, were then assayed for the activities of all 20 aminoacyl-tRNA synthetases at 30 C, using the predetermined appropriate reaction times and enzyme concentration. Table 1 shows the results for 10 amino acids. Activities less than twofold lower than the parent were not considered significant, but doubtful values were rechecked kinetically (time course of the reaction) and by using different extract concentrations. Three of the 10 mutants (EM 102, EM 111, and EM 120) appeared defective in their ability to charge tRNA with glutamic acid, with no apparent defect in the other 19 activities. The rest of the mutants examined showed no difference from the parent with regard to any of the synthetase activities. Mixing experi-

TABLE 1. *Screening for mutants with altered aminoacyl-tRNA synthetases*[a]

Strain	glu	leu	ϕala	ser	isol	ala	val	apn	cys	gln
Parent	320	500	760	500	1,150	220	1,500	300	450	1,540
EM 100	400				1,950	500	2,300	450	505	1,773
EM 101	250	600	900	1,100	1,200	450	1,750	350	440	1,460
EM 102	130	600	1,100	1,000	1,300	290	2,200	450	430	1,190
EM 103	290	900	800	1,000	1,800	350	2,100	460	525	1,460
EM 107	250	850	700	950	1,500	165	2,200	400	456	1,190
EM 108	450	950	900	1,000	1,750	400	2,200	650	460	1,475
EM 109	320	950	1,000	1,050	1,600	300	2,300	500	447	1,175
EM 111	100	900	550	1,500	2,000	400	2,000	620	397	1,629
EM 117	290	1,100	950	950	1,750	380	1,500	500	460	1,525
EM 120	120	600	550	550	950	300	950	300	402	900

[a] Results are expressed as counts per min per 0.1 ml of reaction at 30 C. Each amino acid was used at a concentration of 5×10^{-5} M and a specific radioactivity of 7.5 μc/μmole. For the assay of each amino acid, the same predetermined (linear range) amount of extract protein was used for parent and mutants.

ments, in which the parent extract was mixed with each of the mutant (102, 111, 120) extracts in different ratios, gave no indication of the presence in the mutant extracts of inhibitory or glutamate-degrading activity.

Kinetics of charging with glutamate. The data in Figure 1 verify kinetically the defective charging ability of the three mutants in question. EM 107 was included since, in the screening, it gave a value that was next lowest after 102, 111, and 120. Its charging ability was no different from that of the parent. Two revertants of EM 111 (spontaneous revertants which do not require Sm for growth on complete medium) were included to indicate that the altered phenotype (Sm-dependence on complete medium) of the mutant corresponds to the altered charging capacity with glutamate. Further, to strengthen the conclusion that the mutant effect is specific for glutamate, the parent, mutant 111, and the two revertants were examined for their kinetics of charging with proline. The enzymes of all four strains behaved exactly the same. Also, addition of Sm to the glutamate assay mixture (final concentration of 200 μg/ml) showed no effect on either the parent or the mutant 111. This result was expected from studies with a conditionally Sm-dependent ornithine transcarbamylase mutant (7).

Heat inactivation. In an attempt to show a structural difference between parent and mutants, the rate of inactivation at 50 C of EM 111 was compared with that of the parent. The results are shown in Fig. 2. The mutant is more stable than the parent, but what is surprising is that the parental enzyme is labile at all at this temperature. The glutamyl-tRNA synthetases of *E. coli* B (11) and *E. coli* W (25) have been shown to be stable even at 55 C. An unrelated K-12 strain, S26Rle, has a stable enzyme, so apparently strain EM-0 acquired an alteration in that enzyme before being used as parent for the Sm-suppressible lethal mutants. This is quite probable since it was derived from AB 1621 after nitrosoguanidine mutagenesis. Also included in Fig. 2 is strain 111R1, a revertant of EM 111. The failure of the heat stability to return to that of the parent indicates that the lethal difference between parent and mutant is not directly related to the difference in heat stability.

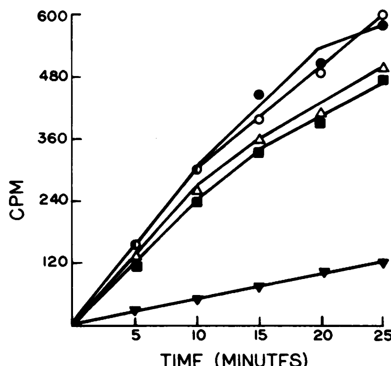

FIG. 1. *Kinetics of glutamate charging. Symbols:* ●, *111R1;* ○, *111R2;* △, *EM-0;* ■, *EM 107;* ▼, *composite of EM 102, EM 111, and EM 120.*

K_m determinations. In a preliminary experiment with a 10-fold higher glutamate concentration than usual, the 102, 111, and 120 mutant extracts yielded glutamyl-tRNA synthetase activities which were either parental or less than twofold lower than the parental. This suggested that the mutant extracts do not simply contain less enzyme but rather that they contain enzymes with lower affinity for glutamate. Consequently, the apparent K_m values of each mutant (and parent) enzyme for glutamate were determined. The results are shown in Table 2. The mutant values can be seen to be approximately 10-fold higher than the parental value. Revertants of each mutant, 102R1, 111R1, and 120R1, have the parental value. An extract made of EM 111 grown without Sm deprivation also has (approximately) the parental value. The behavior of the revertants and of undeprived EM 111 verifies that the Sm-suppressible mutation corresponds to the altered glutamyl-tRNA synthetase. As further support of the specificity of the alteration in these three mutants, two others in the collection were examined, EM 100 and EM 101. These two have K_m values almost 2.5-fold higher than that of the parent. It was reasoned, however, that if this difference is unrelated to the Sm-suppressible lethal mutation, revertants of EM 100 and EM 101 should display the same K_m value as those two mutants; and indeed this is the case. When the values of three other mutants were determined, they all had approximately the parental value, as was also true of an unrelated strain, S26Rle. It can be concluded, therefore, that mutants 102, 111, and 120 possess structurally altered glutamyl-tRNA synthetases.

Further studies. The finding of three glutamyl-tRNA synthetase mutants was unexpected, but it was even more surprising to discover that the mutations map in at least two different places. The mutation in EM 111 is 5% cotransducible with *xyl* and has been shown by conjugation to be located between *mtl* and *gltC*. EM 102 has not yet been shown to be cotransducible with *xyl*, but by conjugation with the Hfrs AB 1868 (0–12) and AB 2271 (0–13) it is known to map very close to *xyl*. EM 120, on the other hand, maps near *his*. The wild-type allele of this mutant locus is brought in by Hfr KL 96 (0–11) during the first

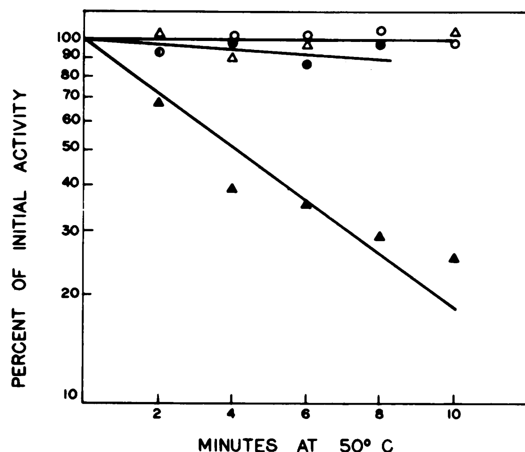

FIG. 2. *Rate of inactivation of glutamyl-tRNA synthetase activity at 50 C. The extracts were incubated for the indicated times at 50 C, pH 7.8, in the absence of any substrates, and then placed on ice. Each sample was then assayed for synthetase activity for 20 min at 30 C. Symbols:* ▲, *EM-0;* ○, *111R1;* ●, *EM 111;* △, *S26R1e. The same amount of enzyme extract protein was used in each case (12 µg per 0.1 ml of reaction).*

few minutes of transfer. Also, recombinants are formed in matings with the F′ strain KLF 3 (for description of Hfr and F′ strains, see Fig. 2 in reference 19). Hence the mutation in EM 120 is located between minute 38 and minute 41 on the *E. coli* genetic map (33). Since the designation *gltS* has already been used for the structural gene of glutamate permease (18), the loci specified by the 111 (and 102 presumably) and 120 mutations shall be referred to by the designations *gltE* and *gltM*, respectively.

For use in further experiments, DEAE-cellulose column preparations were made of extracts of the parent and three mutant strains (see Materials and Methods). After examining the parent extract for kinetics of charging at different enzyme concentrations, and for the amount of charging as a function of tRNA concentration, the K_m values for glutamic acid were determined for the four extracts. The 10-fold difference between parent and mutants was verified, but the values in each case were threefold higher (see Table 3, bracketed numbers). This result may reflect the loss, with enzyme purification, of a factor involved in substrate binding, as has been suggested for a different system (28). It would be of interest to find out if further purification of the mutants yields enzymes displaying even higher K_m values, and if the mutants differ among themselves in this regard.

To investigate the possibility that the two genetic loci might specify two different glutamyl-tRNA synthetases, each of which specifically charges a different subspecies of $tRNA^{glu}$, extracts of the three mutants and the parent were examined for the kinetics of charging of each of two separated $tRNA^{glu}$ fractions (see Materials and Methods). The results are shown in Table 3. All three mutant enzymes charged both fractions at lower rates than the parent. With the minor fraction, there was no apparent difference between the mutants, whereas, with the major fraction, the enzyme of EM 120 charged at a rate approximately two times lower than that of the enzyme of either EM 102 or EM 111. This result indicates a difference between 120 and the other two mutants, but the significance of this in terms of the lethality of each mutation is unclear.

The rationale for the following experiment was the possibility that the two genetic loci might specify nonidentical subunits of one enzyme (nonidentical since a lethal mutation was obtained at each locus). If this were true, then one might be able to obtain in vitro complementation by mixing the mutant extracts in pairs and looking for an increase in activity over that expected from the component extracts. To increase the chances of subunit formation (12), the glycerol concentration of each extract was decreased to less than 2% by dilution with enzyme dilution buffer (0.1% bovine serum albumin; Tris acetate, 0.01 M, *p*H

TABLE 2. *Apparent K_m values for glutamic acid*[a]

Strain[b]	M (× 10⁵)	Strain	M (× 10⁵)
Parent [2]	(6) 6.5	EM 100	13
EM 102 [20]	(3) 55	EM 101	15
EM 111 [20]	(4) 59	100R1	14
EM 120 [20]	(3) 64	101R1	15
102R1	6.2	EM 103	6.2
111R1	6.0	EM 109	6.2
120R1	6.5	EM 115	6.5
EM 111 + Sm-200[c]	7.5	S26R1e	5.9

[a] The K_m values were estimated from a double reciprocal plot of velocities and substrate concentrations. Each point on the plot was obtained as the reciprocal of an initial velocity determined by taking at least five time points up to 12 min of reaction at 30 C for each glutamate concentration. When average K_m values are given, the number of determinations is indicated in parentheses.

[b] Determinations were done on S100 extracts of each strain listed. The bracketed numbers next to the first four strains are the K_m values (M × 10^4) determined for extracts prepared by DEAE-cellulose column chromatography.

[c] EM 111 was grown without streptomycin (Sm) deprivation before making the cell extract.

TABLE 3. *Rates[a] of aminoacylation[b] of fractionated tRNA^{glu}*

Strain	tRNA^{glu} fraction I[c]	tRNA^{glu} fraction II[c]
EM-0	119	34
EM 102	58	22
EM 111	47	22
EM 120	24	21

[a] Rates are expressed as picomoles ($\times 10^2$) of ^{14}C-glutamate attached per minute per microgram of tRNA.

[b] Kinetics of aminoacylation were examined at 30 C, with DEAE-cellulose extracts at a final concentration of 200 μg/ml. For the reaction with fraction I, the glutamate concentration was 4.7×10^{-5} M; for fraction II, 6.3×10^{-5} M.

[c] See Materials and Methods.

7.8; and magnesium acetate, 0.01 M); after equal volumes of the diluted extracts were mixed in pairs, the mixtures were allowed to incubate for 1 hr on ice (12). To allow for reassociation of heterologous subunits, each mixture was incubated at 30 C for 10 min before being assayed for enzyme activity (at 30 C, for 10 min). No increased activity (i.e., complementation) was detected. Prior to this experiment, two similar attempts to obtain complementation were made, without the 1-hr incubation on ice: once with the DEAE-cellulose extracts and once with the S100 extracts. The same negative result was obtained.

DISCUSSION

Many questions still remain regarding the three mutants described in this paper. The main one is: Does *E. coli* have two enzymes for charging tRNA with glutamate or only one enzyme with nonidentical subunits? If each gene codes for a subunit, the subunits would have to be nonidentical since a mutation in either gene can be lethal. A number of aminoacyl-tRNA synthetases have been shown to consist of subunits (12) and in one case, bovine pancreas tryptophanyl-tRNA synthetase, nonidentical subunits have been demonstrated (24). A subunit structure has been shown for glutamyl-tRNA synthetase in *Micrococcus cryophilus* (16), and the possibility of this in *E. coli* is not ruled out by the failure to achieve complementation with extracts of EM 102, 111, and 120.

If one assumes the existence of two synthetases in *E. coli*, each isozyme would have to have an indispensable function in the cell. Two obvious possibilities are the following. First, each enzyme could specifically charge one of two subspecies of tRNA^{glu}. Second, one enzyme might charge one tRNA^{glu} fraction which responds to either glutamic acid codon for general protein synthesis, while the second enzyme charges another tRNA^{glu} which is used for some limited but essential function as, for example, attachment of glutamate to phosphatidyl glycerol (15), or to tRNA^{gln}, that is, tRNA which responds to the glutamine codons (34). Both hypothesis, however, make it difficult to explain why S100 extracts of mutants from *both* genetic loci, when examined for the charging of *unfractionated* tRNA, exhibit 10-fold higher K_m values for glutamate, unless one assumes that each mutant has become greatly derepressed for the synthesis of the altered isozyme and concomitantly repressed for the unaltered one. Furthermore, most of the present (*E. coli*) data are consistent with the existence of only one aminoacyl-tRNA synthetase for each amino acid (13, 23). Preliminary evidence was presented (37) for the existence of several leucyl-tRNA synthetases in *E. coli* K-12, but a more recent study (26) indicates that the phenomenon is due to formation of aggregates of a single enzyme.

A third possible explanation of the functions of the *gltE* and *gltM* genes, other than two enzymes or nonidentical subunits, would be one enzyme made up of identical subunits, specified by one gene, and a modifying factor, specified by the other gene. The modifier might, during the course of subunit synthesis, perform on them an alteration necessary for their proper association or interaction, or both, with each other or with the enzyme substrates. This interpretation is more consistent with the results of the charging of the two tRNA^{glu} fractions than is the two-enzyme hypothesis. In the latter case, one would expect that one enzyme would, for example, charge fraction I well and fraction II poorly, while the second enzyme would show the converse. Instead, the experiment indicated that all three mutants charge fraction II identically but that EM 120 charges fraction I twice as slowly as do the other two mutants. The hypothesis of one gene coding for a modifying factor is consistent with this result and also with the glutamate K_m differences seen between parent and mutants. The modification of one synthetase has been shown (22), and data suggesting the possibility of bacterial synthetase modifiers now exist (see reference 28, and discussion in the Results section of K_m determinations on DEAE-cellulose extracts). The resolution of this problem of the two genetic loci must await further studies with fractionated tRNA and purified enzymes.

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