JOURNAL OF BACTERIOLOGY, Sept. 1971, p. 828-832

Vol. 107, No. 3
Printed in U.S.A.

Biochemical Characterization of a Mutant Isoleucyl-Transfer Ribonucleic Acid Synthetase from *Escherichia coli* K-12

GERDA TREIBER AND MAURIZIO IACCARINO

International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy

Received for publication 26 March 1971

Isoleucyl-transfer ribonucleic acid (tRNA) synthetase [L-isoleucine: soluble RNA ligase (adenosine monophosphate), EC 6.1.1.5; IRS] was partially purified from *Escherichia coli* K-12 and from an *ileS* mutant that appears to be altered in IRS. The half-life of wild-type IRS, incubated at 60.5 C, is 69 min, whereas that of mutant IRS is 8 min. Mutant IRS shows about a 100-fold lower affinity than wild-type IRS for isoleucine, DL-valine, thiaisoleucine, and O-methyl-DL-threonine, both in the pyrophosphate exchange assay and in the assay of isoleucyl-tRNA formation. The affinity of the mutant enzyme for adenosine triphosphate in the assay of isoleucyl-tRNA formation is 15-fold lower than that of the wild-type enzyme. The affinity of mutant IRS for tRNA is not changed as compared with wild-type IRS. These data show that mutant IRS has an altered structure and clearly confirm that *ileS* is the structural gene for IRS.

A new marker (*ileS*) on the genetic map of *Escherichia coli* K-12 has been described, and it appears to be the structural gene for isoleucyl-transfer ribonucleic acid (tRNA) synthetase (L-isoleucine: soluble RNA ligase (adenosine monophosphate, EC 6.1.1.5; IRS) (reference 7). Although the described *ileS* mutant requires isoleucine for growth, it appears both from biochemical and genetic experiments that it contains normal isoleucine biosynthetic enzymes; the only difference that has been found in extracts of the mutant as compared to wild-type extracts is a striking increase (more than 100-fold) in the K_m for isoleucine of IRS. Since the K_m defect and the isoleucine auxotrophy of the mutant appear to be located at the same locus, it has been concluded that they are both consequences of the same mutation. IRS activity measured in crude extracts of the *ileS* mutant is low, mainly because of the modification of the K_m for isoleucine, whereas the apparent V_{max} is 20 to 25% of the wild type. Although this difference in V_{max} might be due to differences in extraction or stability of the mutant enzyme, it could also be caused by other alterations of enzyme activity which result in the reduction of the apparent V_{max} or by a decreased rate of transcription of the mutated gene (regulation of IRS synthesis has been described; reference 14).

In this investigation, we report experiments on partially purified preparations of IRS extracted from the mutant and wild-type strains, which give more evidence that the *ileS* gene is the structural gene for IRS.

MATERIALS AND METHODS

Reagents. ^{14}C-L-isoleucine and ^{32}P-sodium pyrophosphate (^{32}PPi) were purchased from New England Nuclear Corp., Boston, Mass.; adenosine triphosphate (ATP) was from Sigma Chemical Co., St. Louis, Mo. *E. coli* B tRNA (Schwarz BioResearch, Orangeburg, N.Y.) was used for most assays of aminoacyl-tRNA formation, except in some experiments where *E. coli* K-12 tRNA (also Schwarz BioResearch) has been used (in several instances equal results were obtained by using either tRNA species). Thiaisoleucine-hydrochloride (2-amino-3-methylthiobutyrate) and O-methyl-DL-threonine were obtained from Reef Laboratory, Santa Paula, Calif.; DL-valine and DL-leucine from Nutritional Biochemicals Corp., Cleveland, Ohio; diethylaminoethyl (DEAE) cellulose from Whatman Co., England; hydroxyl-apatite (Bio Gel HTP) from Bio-Rad Laboratories, Richmond, Calif.

The DL forms of valine and leucine were chosen since they are prepared synthetically and should, therefore, be less contaminated by other natural amino acids. It is assumed, however, that only the L isomer interacts with IRS, and the K_m or K_i values given are for the concentration of the L isomer only.

Strains, media, and growth conditions. Wild-type enzyme was extracted from PB154, an arginine- and tryptophan-requiring derivative of *E. coli* K-12, and the mutant enzyme was extracted from M11, a derivative of PB154, which has an additional requirement for isoleucine as a consequence of the mutation in IRS. Both

strains were previously described (7). Bacteria were grown aerobically at 37 C in minimal medium (13) supplemented with 100 μg of L-arginine per ml, 25 μg of L-tryptophan per ml, and, in the case of M11, 50 μg of L-isoleucine per ml. The bacteria were harvested from cultures in exponential growth phase and stored at −20 C until ready for use.

Enzyme assays. IRS activity has been assayed in two different ways. (i) The isoleucine-dependent exchange of ^{32}PPi with ATP was measured by adsorption of ATP on Norit (4); (ii) the rate of isoleucyl-tRNA (ile-tRNA) formation was followed by measuring the conversion of ^{14}C-isoleucine to the acid-insoluble ^{14}C-ile-tRNA (9). The same assay was used for valyl-tRNA synthetase [L-valine: soluble RNA ligase (AMP), EC 6.1.1.9].

Proteins were measured by the method of Groves (5).

Enzyme purification. Frozen PB154 cells were suspended in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 8.0), containing 0.01 M $MgCl_2$ and 10% glycerol (3.5 ml of final volume per each gram), and thawed. Portions (5 ml) of this suspension were sonically oscillated for 2 min with a sonic oscillator (Measuring & Scientific Equipment, Ltd.), and the suspension was centrifuged at 198,000 × *g* (50,000 rev/min in a Spinco Beckman no. 50 rotor) for 2 hr. The supernatant fluid (1,330 mg of proteins) was adsorbed on to a DEAE cellulose column (2.5 cm diameter by 47 cm), previously equilibrated with 0.02 M potassium phosphate (*p*H 7.5) containing 0.05 M 2-mercaptoethanol. The enzyme was eluted with a linear gradient made of 2,000 ml of 0.02 M potassium phosphate, (*p*H 7.5) and 2,000 ml of 0.25 M potassium phosphate (*p*H 6.5). Both solutions contained 0.05 M 2-mercaptoethanol (3). The peaks of IRS and valyl-tRNA synthetase were localized by assaying the rate of ile-tRNA formation and valyl-tRNA formation. The combined fractions of the IRS peak were concentrated and further purified by adsorption onto a second DEAE cellulose column (2 cm diameter by 17 cm), equilibrated with 0.02 M potassium phosphate (*p*H 7.5) containing 0.05 M 2-mercaptoethanol and eluted with 0.25 M potassium phosphate containing 0.05 M 2-mercaptoethanol. The fractions containing the IRS activity were combined and dialyzed against 0.02 M potassium phosphate (*p*H 7.5) containing 0.01 M 2-mercaptoethanol. The fractions were adsorbed onto a hydroxylapatite column (2 cm diameter by 26 cm) which was first washed with 0.02 M potassium phosphate (*p*H 7.5) containing 0.01 M 2-mercaptoethanol and then with 100 ml of 0.05 M potassium phosphate (*p*H 7.5) containing 0.01 M 2-mercaptoethanol. The enzyme was eluted with a linear gradient made of 400 ml of 0.05 M potassium phosphate (*p*H 7.5) and 400 ml of 0.3 M potassium phosphate (*p*H 7.5), both containing 0.01 M 2-mercaptoethanol (1). Fractions containing IRS activity were dialyzed against 1 mM potassium phosphate (*p*H 6.8), containing 20 mM 2-mercaptoethanol, concentrated by covering the dialysis tube with dry Sephadex G-200, and again dialyzed against the same solution. This procedure gives a preparation with a specific activity of 750 nmoles of ile-tRNA formed in 10 min at 37 C per mg of protein, with a 40-fold purification and a 40% yield.

Several preparations of IRS from M11 cells have been obtained by the same method; the purification obtained was about 30-fold with a 20 to 25% yield.

Valyl-tRNA synthetase in the PB154 IRS-purified preparation used in the experiments described in this paper was less than 0.035% of the extract, whereas for the M11 IRS preparations it was less than 0.002%.

RESULTS AND DISCUSSION

Figure 1 shows the inactivation at 60.5 C of IRS purified from the wild-type strain and from M11. Although the half-life of the wild-type enzyme is 69 min, that of the mutant enzyme in the same conditions is 8 min.

It has been reported (7) that when the rate of ile-tRNA formation is assayed as a function of isoleucine concentration in extracts of wild type and M11 strains, the K_m value for isoleucine in the mutant enzyme is greatly increased. We measured this parameter for both the purified wild type and the mutant enzyme, in both reaction (1) and (2).

$$\text{isoleucine} + \text{ATP} + \text{IRS} \rightleftharpoons \text{IRS (isoleucyl-AMP)} + \text{PPi} \quad (1)$$

$$\text{isoleucine} + \text{ATP} + \text{tRNA} \xrightleftharpoons{\text{IRS}} \text{ile-tRNA} + \text{AMP} + \text{PPi} \quad (2)$$

Figure 2 shows double reciprocal plots of en-

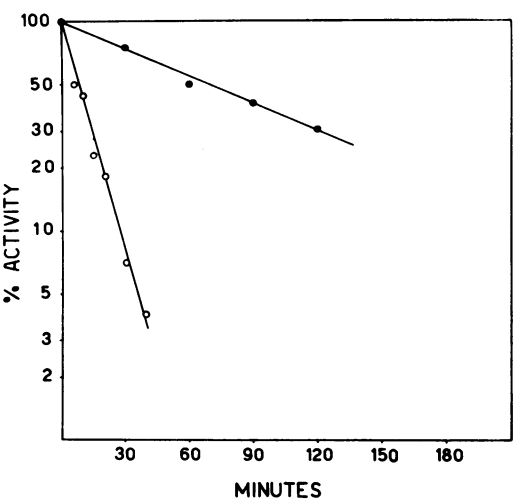

FIG. 1. *Rate of inactivation of wild-type (●) and mutant (○) IRS at 60.5 C. The enzymes (5.3 and 4 mg/ml, respectively) in 1 mM potassium phosphate (pH 6.8), containing 20 mM 2-mercaptoethanol and 50% glycerol were incubated at 60.5 C for the indicated times in the absence of any substrate; they were then placed in an ice bath before assaying the rate of ile-tRNA formation. The enzyme preparations used in this experiment were purified only through the first DEAE cellulose column. The specific activity of PB154 IRS was 22 units/mg and that of M11 IRS was 1.5 units/mg, when measured with the standard isoleucine concentration.*

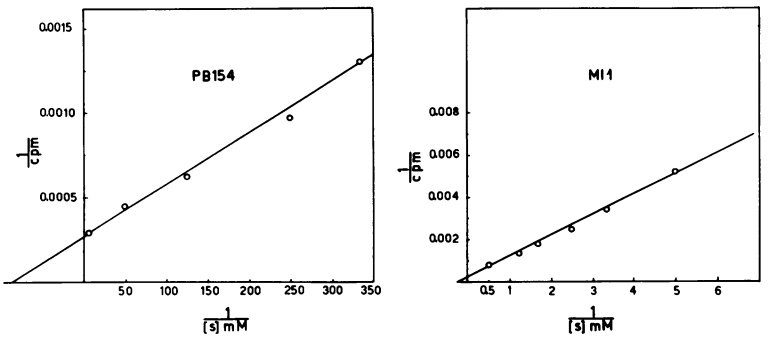

FIG. 2. *Double reciprocal plots of counts per minute as a function of isoleucine concentration for PB154 and MI1 enzymes in the pyrophosphate exchange assay (reaction 1). K_m values are given in Table 1.*

zyme activity in reaction (1) versus substrate concentration, as measured with wild-type IRS and mutant IRS; Table 1 gives K_m values calculated from these plots and from similar plots where valine, thiaisoleucine, and O-methyl-threonine were used as substrates.

Figure 3 shows double reciprocal plots, analogous to those of Fig. 2, of enzyme activity in reaction (2) versus concentration of substrate or substrate plus valine, as measured with wild-type IRS and mutant IRS. Table 2 gives K_m values for isoleucine and K_i values for valine, thiaisoleucine, and O-methyl-threonine. It is evident from the data in Tables 1 and 2 that the affinity of all these substances for the IRS is more than 100-fold lower in the preparation obtained from the mutant cells, both in reaction 1 (Fig. 2 and Table 1) and in reaction 2 (Fig. 3 and Table 2). The K_m of DL-leucine in reaction 1 was also measured and found to be 0.16 mM (concentration of the L-isomer) in the wild-type IRS. This value is much higher in MI1 IRS since we obtained no measurable activity with 50 mM DL-leucine (final concentration). Table 1 shows that isoleucine, valine, and two isoleucine analogues (thiaisoleucine and O-methyl-threonine) can all form the IRS (aminoacyl-AMP) complex, as judged from their ability to substitute isoleucine in the pyrophosphate exchange assay. Formation of the IRS (valyl-AMP) complex by *E. coli* B IRS (2, 3) and by *E. coli* K-12 IRS (8) has been reported. That thiaisoleucine is a substrate in reaction (1) was suggested by Szentirmai and Umbarger (11), who reported evidence for thiaisoleucyl-tRNA formation. O-methyl-threonine has been reported to act as a substrate for IRS in *E. coli* 15 extracts (10). In preliminary experiments, we also found evidence for thiaisoleucyl-tRNA and O-methyl-threonyl-tRNA formation

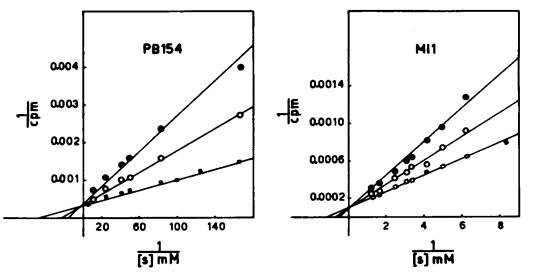

FIG. 3. *Double reciprocal plots of counts per minute as a function of isoleucine concentration (□) for PB154 and MI1 enzymes in the assay of ile-tRNA formation (reaction 2); ○, same, in the presence of 1.5 mM DL-valine (PB154) or 40 mM DL-valine (MI1); ●, same, in the presence of 3 mM DL-valine (PB154) or 80 mM DL-valine (MI1). K_m and K_i values are given in Table 2.*

TABLE 1. *Affinity of isoleucine and some isoleucine analogues for IRS in the pyrophosphate exchange assay*

Substance tested	K_m (mM)	
	Wild-type IRS	Mutant IRS
L-Isoleucine	0.012	3.3
DL-Valine[a]	0.39	77
Thiaisoleucine	0.16	20
O-methyl-DL-threonine[a]	0.25	55

[a] Concentration of the L-isomer.

TABLE 2. *Affinity of isoleucine and some isoleucine analogues for IRS in the assay of isoleucine-transfer ribonucleic acid formation*

Substance tested	K_i (mM)	
	Wild-type IRS	Mutant IRS
L-Isoleucine	0.02[a]	1.05[a]
DL-Valine	0.7	40
Thiaisoleucine	0.36	23.5
O-methyl-DL-threonine	0.4	60

[a] K_m value.
[b] Concentration of the L-isomer.

with *E. coli* K-12 tRNA and the IRS purified from PB154. Inhibition of ile-tRNA formation by valine and thiaisoleucine has been reported for *E. coli* K-12 IRS (8, 12).

Figure 4 is a reciprocal plot of enzyme activity (ile-tRNA formation) as a function of ATP concentration, which shows that the affinity of the mutant enzyme for ATP is about 15-fold lower than that of the wild-type enzyme. Thus, the K_m is 0.085 mM ATP for the wild-type enzyme and 1.3 mM for the mutant enzyme. This experiment has been performed with the isoleucine concentration of the standard assay (0.2 mM), which is a saturating concentration for the wild-type enzyme but gives only 20% of the V_{max} with the mutant enzyme (Fig. 3). One possible inference which could be drawn from this experiment, namely that the value of the ATP K_m is dependent on the saturation of the isoleucine site, is ruled out by an experiment in which the ATP K_m for the wild-type enzyme was measured at a concentration of isoleucine (1 μM) which gives 3% of V_{max}. The ATP K_m in these conditions is 0.024 mM, i.e., even lower than the value found for MI1 (Fig. 4). The mutant enzyme is, therefore, altered not only in the affinity for isoleucine, but also in that for ATP. Since the IRS activity measured at the ATP concentration (1 mM) used in the standard assay of ile-tRNA formation is 40% of V_{max} (Fig. 4), the apparent V_{max} measured in crude extracts (7) should be increased accordingly.

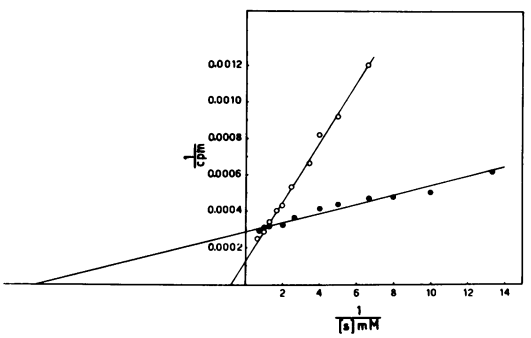

FIG. 4. *Double reciprocal plot of counts per minute as a function of ATP concentration for PB154 (●) and MI1 (○) enzymes in the assay of ile-tRNA formation (reaction 2). K_m values are given in the text.*

Figure 5 shows a reciprocal plot of rate of ile-tRNA formation as a function of tRNA concentration. The K_m value is 1.5 μM for both enzymes.

In conclusion, the difference between the wild-type and mutant IRS in the K_m for isoleucine previously identified in crude extracts (7) has been confirmed by the experiments reported here on the purified preparations. It is, moreover, shown (Fig. 4) that the ATP site is also altered in the mutant enzyme. The mutation has apparently caused no alteration at the tRNA site, thus causing a dissociation of this site from the one for isoleucine and ATP. This effect appears to be analogous to what was found with IRS purified from *E. coli* B (6), where the chemical modification of the most reactive sulphydryl group affects the turnover number by affecting the isoleucine-ATP site, with no alteration at the tRNA site.

The experiments reported clearly show that the mutant IRS has an altered structure and, therefore, confirm that *ileS* is the structural gene for IRS.

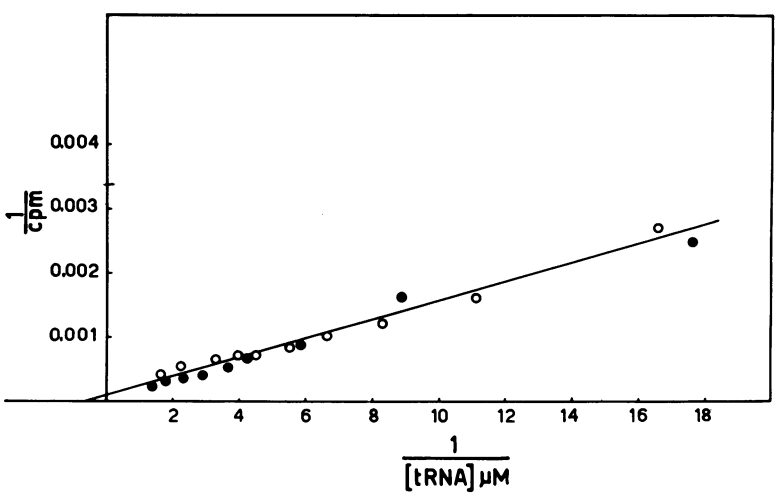

FIG. 5. *Plot of the reciprocal of counts per minute as a function of the reciprocal of the concentration of tRNA for PB154 (●) and MI1 (○) enzymes in the assay of ile-tRNA formation (reaction 2). K_m values are given in the text. tRNA from E. coli K-12 was used, but an equal result was obtained with E. coli B tRNA.*

ACKNOWLEDGMENTS

This work was supported by a research grant from the Jane Coffin Childs Memorial Fund for Medical Research.

LITERATURE CITED

1. Baldwin, A. N., and P. Berg. 1966. Purification and properties of isoleucyl ribonucleic acid synthetase from *Escherichia coli*. J. Biol. Chem. **241**:831-838.
2. Baldwin, A. N., and P. Berg. 1966. Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase. J. Biol. Chem. **241**: 839-845.
3. Bergman, F. H., P. Berg, and M. Dieckmann. 1961. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. II. The preparation of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid synthetases from *Escherichia coli*. J. Biol. Chem. **236**:1735-1740.
4. Calendar, R., and P. Berg. 1966. Tyrosyl tRNA synthetase from *Escherichia coli* B, p. 384-399. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper & Row, New York.
5. Groves, W. E., F. C. Davis, Jr., and B. Sells. 1968. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal. Biochem. **22**:195-210.
6. Iaccarino, M., and P. Berg. 1969. Requirement of sulphydryl groups for the catalytic and tRNA recognition functions of isoleucyl-tRNA synthetase. J. Mol. Biol. **42**:151-169.
7. Iaccarino, M., and P. Berg. 1971. Isoleucine auxotrophy as a consequence of a mutationally altered isoleucyl-tRNA synthetase. J. Bacteriol. **105**:527-537.
8. Kondo, M., and C. R. Woese. 1969. Specificity of aminoacyl transfer ribonucleic acid synthetases from *Escherichia coli* K12. Biochemistry **8**:4177-4182.
9. Muench, K. H., and P. Berg. 1966. Preparation of aminoacyl ribonucleic acid synthetases from *Escherichia coli*, p. 375-383. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper & Row, New York.
10. Smulson, M. E., M. Rabinowitz, and T. R. Breitman. 1967. O-methylthreonine inhibition of growth and of threonine deaminase in *Escherichia coli*. J. Bacteriol. **94**: 1890-1895.
11. Szentirmai, A., and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XIV. Effect of thiaisoleucine. J. Bacteriol. **95**:1666-1671.
12. Szentirmai, A., M. Szentirmai, and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XV. Biochemical properties of mutants resistant to thiaisoleucine. J. Bacteriol. **95**:1672-1679.
13. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. **218**:97-106.
14. Williams, L. S., and F. C. Neidhardt. 1969. Synthesis and inactivation of aminoacyl-transfer RNA synthetases during growth of *Escherichia coli*. J. Mol. Biol. **43**:529-550.