Trypsin-Modified Isoleucyl Transfer Ribonucleic Acid Synthetase with Partial Retention of Activity†

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ABSTRACT: In a search for an active fragment of isoleucyl-tRNA synthetase pure enzyme from *Escherichia coli* B was subjected to limited trypsin digestion, which produced two fragments with molecular weights of 69,000 and 39,000. The cleavage site generating the fragments is extraordinarily sensitive to trypsin, being about 2500 times more sensitive than the other potentially susceptible sites in the isoleucyl-tRNA synthetase molecule. The two fragments remain tightly but noncovalently bound, and the treated enzyme is indistinguishable from the native enzyme by gel filtration on Sephadex G-100, by chromatography on DEAE-cellulose and hydroxylapatite, and by polyacrylamide disc gel electrophoresis in the absence of denaturing agent. The trypsin-modified isoleucyl-tRNA synthetase has no isoleucine-dependent ATP-PP_i exchange activity and cannot charge isoleucine to tRNA. Although the modified enzyme is inactive in the AMP- and-PP_i-dependent removal of isoleucine from Ile-tRNA, the enzyme still recognizes and specifically hydrolyses Ile-tRNA.

Isoleucyl-tRNA synthetase of *Escherichia coli* B is a representative of the group of a few aminoacyl-tRNA synthetases consisting of a single polypeptide chain (Arndt and Berg, 1970). Most of the other synthetases have subunits (for review, see Kisselev and Favorova, 1974). Isoleucyl-tRNA synthetase was first purified and studied by Berg and his colleagues (Berg *et al.*, 1961; Norris and Berg, 1964; Baldwin and Berg, 1966a,b). Subsequently, many studies have been carried out on this enzyme, including kinetic analyses (Arndt and Berg, 1970; Cole and Schimmel, 1970; Holler and Calvin, 1972; and Holler *et al.*, 1973), substrate binding (Yarus and Berg, 1967; Iaccarino and Berg, 1969; Arndt and Berg, 1970; Eldred and Schimmel, 1972), substrate specificity (Yarus and Mertes, 1973), fluorescence properties (Penzer *et al.*, 1971), active site topology (Holler *et al.*, 1973), and, recently, sequences of thiol peptides (Kula, 1973). Of particular interest is the observation that each enzyme molecule binds one molecule each of its substrates: isoleucine, ATP, and $tRNA^{Ile}$ (Norris and Berg, 1964; Yarus and Berg, 1967), whereas the synthetases with subunits have more than one binding site for their substrates (for references, see Kisselev and Favorova, 1974). The absence of subunits and the single substrate binding site makes this enzyme a good candidate for the study of polypeptide–polynucleotide interactions. The high molecular weight of this enzyme (112,000, Baldwin and Berg, 1966b) makes the structural analysis, which is essential for the above study, difficult. Therefore it would be useful to produce a modified enzyme smaller in size yet still wholly or partially active.

In searching for such a modified isoleucyl-tRNA synthetase, a series of trypsin digestions were performed. It should be pointed out that similar work has been reported for other synthetases (Cassio and Waller, 1971; Rouget and Chapeville, 1971). For example, Cassio and Waller (1971) have shown that after trypsin digestion, methionyl-tRNA synthetase from *E. coli* releases a small peptide with the remaining major polypeptide still active. Recently Koch *et al.* (1974) have shown that trypsin digestion of valyl-tRNA synthetase from *Bacillus thermophilus* produces two structurally similar fragments. In this paper I shall describe the results of limited trypsin digestion of isoleucyl-tRNA synthetase in which the native molecule can be cleaved at a single, extraordinarily sensitive site to yield two fragments with ⅓ and ⅔ of the original molecular weight. These two fragments are, however, held tightly together and can only be separated in the presence of a denaturing agent. This modified enzyme still retains the ability to hydrolyze Ile-tRNA. A preliminary report of this work has been presented (Lee, 1974). After this manuscript was completed, similar work by Piszkiewicz and Goitein (1974) was published. The present communication partially confirms their results and extends their observations.

Experimental Section

Materials

E. coli B grown in a minimal medium and harvested at ¾ log phase was purchased from Grain Processing Corp., Muscatine, Iowa. Bovine pancreatic trypsin, L-(1-tosyl-2-phenyl)ethyl chloromethyl ketone treated, 220–300 units/mg, soybean trypsin inhibitor, and bovine pancreatic α-chymotrypsin, three times crystallized, 68 units/mg, were purchased from Worthington Biochemical Co. [^{32}P]PP_i and [^{3}H]- and [^{14}C]-L-amino acids were from New England Nuclear. Acrylamide and bisacrylamide were from Canalco. Sodium dodecyl sulfate (SDS),[1] dansyl chloride, and dansyl amino acids were obtained from Pierce Chemical Co. Unfractionated *E. coli* B tRNA was from Schwarz/Mann. Iodoacetic acid was purchased from Aldrich Chemical Co. and was recrystallized in chloroform. Unlabeled amino acids were from Calbiochem. α-Aminobutyric acid, β-aminobutyric acid, γ-aminoisobutyric acid, GTP, and dATP were from Sigma Chemical Co. Hydroxylapatite was

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[1] Abbreviation used is: SDS, sodium dodecyl sulfate.

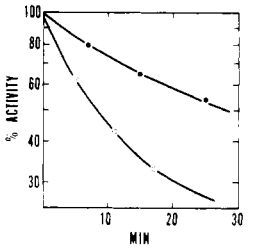

FIGURE 1: Stability of trypsin. Trypsin was prepared at a concentration of 1 mg/ml in 1 mM HCl. Immediately before use the trypsin was diluted to 15 μg/ml with 10 mM potassium phosphate buffer (pH 7.5) with (○) or without (●) 10 mM 2-mercaptoethanol. The trypsin solution was then incubated at 37° for the time indicated. At each time interval, samples of 30 and 60 μl were withdrawn and added to 0.85 ml of 0.1 M Tris-HCl buffer (pH 7.5) with 20 mM $CaCl_2$. Then 0.1 ml of 10 mM *p*-toluenesulfonyl-L-arginine methyl ester was added, and the increase in absorbance at 247 nm was recorded every 30 sec for up to 5 min. The linear portion was taken for the computation of the activity.

prepared according to Main *et al.* (1959). Cellulose thin layer sheets (0.1 mm), Polygram cell 300, were purchased from Brinkmann Instruments, Inc., Westbury, N.Y. Cheng Chin polyamide layer sheets were obtained from Gallard-Schlesinger.

Isoleucyl-tRNA synthetase was purified simultaneously with tryptophanyl-tRNA synthetase from the same cell extracts. The procedures used for isoleucyl-tRNA synthetase were essentially the same as described by Norris and Berg (1964) with some modifications. The details for simultaneous purification of isoleucyl- and tryptophanyl-tRNA synthetase will be published elsewhere.

The markers used for the determination of molecular weights by SDS-acrylamide gel electrophoresis and Sephadex gel filtration are obtained from Worthington Biochemical Co. except for whale myoglobin, a gift of Dr. B. Cameron, and tryptophanyl-tRNA synthetase, a gift of Dr. G. Kuehl.

Methods

Assay of Isoleucyl-tRNA Synthetase. L-Isoleucine-dependent ATP-PP_i exchange reaction was done as described by Baldwin and Berg (1966b). The charging of tRNA with [^{14}C]isoleucine was performed according to the method of Muench and Berg (1966). AMP- and PP_i-dependent or independent deacylation of [^{14}C]Ile-tRNA was carried out as described by Schreier and Schimmel (1972) except that the tRNA used had a specific activity of 100 pmol of $tRNA^{Ile}$/A_{260} unit. Protein was determined by the method of Lowry *et al.* (1951).

Polyacrylamide Disc Gel Electrophoresis. Analytical disc gel electrophoresis at pH 8.5 was performed as described by Davis (1964). SDS-acrylamide gel electrophoresis was performed according to the method described by Weber and Osborn (1969). The acrylamide concentration was 10%, and the bisacrylamide concentrations was varied from 0.15 to 0.6% as specified in each experiment.

N-Terminal Residue Determination. N-Terminal residues of peptides were dansylated with dansyl chloride by the method of Gros and Labouesse (1969), and the dansyl amino acids were then identified on 5 × 5 cm polyamide layer sheets developed by solvent systems as described by Hartley (1970).

Trypsin Digestion. Concentrated isoleucyl-tRNA synthetase was first dialyzed against 10 mM potassium phosphate buffer (pH 7.5) with 10 mM 2-mercaptoethanol. The concentration of the enzyme was then adjusted to 0.5–2.0 mg/ml by addition of the above buffer. Trypsin, 1 mg/ml, was prepared and stored for up to 7 days in 1 mM HCl. Trypsin was diluted with the above buffer immediately before use. In a typical digestion, the 0.25-ml reaction mixture contained 10 mM potassium phosphate buffer (pH 7.5), 10 mM 2-mercaptoethanol, 250 μg of isoleucyl-tRNA synthetase, and 2.5 μg of trypsin. The ratio of synthetase/trypsin (w/w) as well as the duration of digestion varied as indicated in each experiment. The presence of 2-mercaptoethanol in the reaction mixture has two purposes: (1) to prevent denaturation of isoleucyl-tRNA synthetase (this enzyme loses more than 80% of its activity when the mercaptoethanol is removed); and (2) to inactivate trypsin after a certain time of incubation. As can be seen in Figure 1, trypsin has a half-life of about 8 min in the digestion mixture. An amount of soybean trypsin inhibitor equimolar to the trypsin was added at the times indicated to inhibit the remaining trypsin activity. The digestion products were then examined by gel electrophoresis in the presence or absence of SDS as described above.

In one experiment (see under Results, trypsin digestion), trypsin-modified isoleucyl-tRNA synthetase was carboxymethylated before gel electrophoresis: trypsin-modified isoleucyl-tRNA synthetase (0.3 ml, 1 mg/ml) was first passed through a Sephadex G-25 column, 0.9 × 16.5 cm, equilibrated with 10 mM potassium phosphate (pH 6.9)–10 mM 2-mercaptoethanol. To the breakthrough material (0.50 ml), 0.20 ml of 1 M Tris-HCl (pH 8.5) was added. Then 0.10 ml of iodoacetic acid (50 mg/ml in 1 M Tris-HCl (pH 8.5)) was added and the mixture incubated at 37° for 2 hr. After addition of 600 mg of urea, another 0.10 ml of the iodoacetic acid was introduced and the mixture incubated again for another 1 hr at 37°. The unreacted reagent was removed by dialysis against 10 mM sodium phosphate buffer (pH 6.9). Before gel electrophoresis the treated isoleucyl-tRNA synthetase was incubated with SDS as described by Weber and Osborn (1969) except that no 2-mercaptoethanol was present.

Results and Discussion

Trypsin Digestion. When the native isoleucyl-tRNA synthetase was subjected to trypsin digestion, isoleucyl-tRNA synthetase was cleaved into two pieces, large fragment, Fr. L, and small fragment, Fr. S. (Figure 2). As can be seen in Figure 2 the limited digestion was essentially complete in 1 min at 37°. Incubation up to 20 min produced no further digestion of the major fragments. The molecular weights of the fragments were estimated by SDS polyacrylamide gel electrophoresis with different amounts of cross-linking in the gel (Weber and Osborn, 1969). It can be seen from Figure 3 that the large fragment has a molecular weight of 69,000, whereas that of the small fragment is 39,000. In the communication just reported by Piszkiewicz and Goitein (1974), with slightly different experimental conditions, isoleucyl-tRNA synthetase was cleaved into two pieces with molecular weights of 76,000 and 41,000, agreeing within experimental error with the present observations. The sum of the molecular weights of the large and small fragments is 108,000, a figure slightly lower than that reported for the molecular weight of the native enzyme, 112,000 (Arndt and Berg, 1970). Allowing an error of 5–10% for the determination of the molecular weight by the gel electrophoresis method (Weber and Osborn, 1969) the above discrepancy is acceptable. Nonetheless, attempts were made to find a

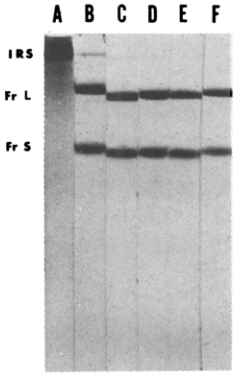

FIGURE 2: Kinetics of trypsin digestion of isoleucyl-tRNA synthetase. Isoleucyl-tRNA synthetase was prepared and digested with trypsin as described in Methods. The isoleucyl-tRNA synthetase concentration was 1.0 mg/ml, 9×10^{-6} M, and the trypsin concentration was 10 μg/ml, 0.4×10^{-6} M. The digestion was carried out at 37° for 0 (A), 0.5 (B), 1 (C), 5 (D), 10 (E), and 20 min (F). At each interval, a sample of 20 μl was withdrawn and added to 0.2 μg of soybean trypsin inhibitor. The digestion products were then analyzed by polyacrylamide gel electrophoresis in the presence of SDS with the normal amount of cross-linking (0.3% bisacrylamide) (Weber and Osborn, 1969). The origin is at the top.

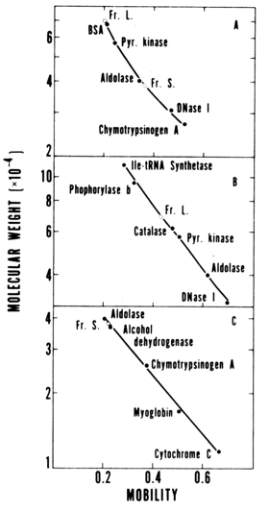

FIGURE 3: Molecular weights of the large and small fragments. Isoleucyl-tRNA synthetase was digested with trypsin as described in Figure 2. Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Weber and Osborn (1969) with 0.3% (A), 0.6% (B), and 0.15% (C) bisacrylamide. The standards used (●) and the large (Fr. L) and small fragments (Fr. S) (○) are indicated.

missing fragment(s), if any, with a molecular weight of 5000 or less by digesting isoleucyl-tRNA synthetase with a wide range of trypsin concentrations for 10 sec to 2 min. The digestion products were then analyzed on a 10 or 15% acrylamide gel with twice the normal amount of cross-linking (0.6% bisacrylamide) in the gel (Weber and Osborn, 1969). In no case was the above fragment detected, although the β-chain insulin, used as a marker, with a molecular weight of 3500, was clearly seen. To see whether even smaller peptide fragments were released, the isoleucyl-tRNA synthetase after limited trypsin digestion was passed through a Sephadex G-25 column, which retards peptides with molecular weights of less than 5000. A broad fluorescence peak corresponding to a molecular weight of about 2000 to 3500 was noted. The fraction with highest fluorescence was chromatographed on a cellulose thin-layer plate with butanol–acetic acid–water–pyridine (150:30:20:100 in volume) as a developing solvent. There were at least six peptides identifiable. Dansylation of the material from the same fraction showed that at least five N-terminal residues were present (glycine, alanine, serine, lysine, and glutamine or glutamic acid), although the yields were low (less than 15%). No attempt was made to determine the stoichiometry of these peptides relative to the large and small fragments. The origin of these peptides remains to be elucidated.

To see whether there is a conversion of the large fragment to the small fragment upon digestion of isoleucyl-tRNA synthetase with high concentrations of trypsin, isoleucyl-tRNA synthetase was treated with increasing amounts of trypsin as shown in Figure 4. Both the large and small fragments appeared simultaneously. When the molar ratio of trypsin/isoleucyl-tRNA synthetase reached 5, both fragments began to disintegrate. It is, therefore, unlikely that the small fragment is derived from the large fragment. It is worth noting that the trypsin-sensitive site of cleavage between the two major fragments is about 2500-fold more sensitive than the other trypsin cleavage sites, because the major fragments are readily formed with a trypsin/isoleucyl-tRNA synthetase ratio of 0.002, whereas the other sites are at least partially resistant to digestion with a trypsin/isoleucyl-tRNA synthetase ratio 2500 times greater (Figure 4). Bovine pancreatic chymotrypsin, under conditions similar to those in Figure 4, did not reveal such an extraordinarily sensitive site. Isoleucyl-tRNA synthetase remained resistant to digestion with chymotrypsin/isoleucyl-tRNA synthetase molar ratio of 1:240, began to show multiple bands at the ratio of 1:80, and was digested into small pieces not detectable with 10% acrylamide SDS gels when the ratio reached 1:24.

Attempts to Separate the Large and Small Fragments. Attempts were made to separate the large fragment from the small fragment under mild conditions to preserve the fragments for assay of residual activities. However, the two fragments remained tightly associated in the absence of a denaturing agent. When trypsin-modified isoleucyl-tRNA synthetase was cochromatographed with a catalytic amount of native isoleucyl-tRNA synthetase on a Sephadex G-100

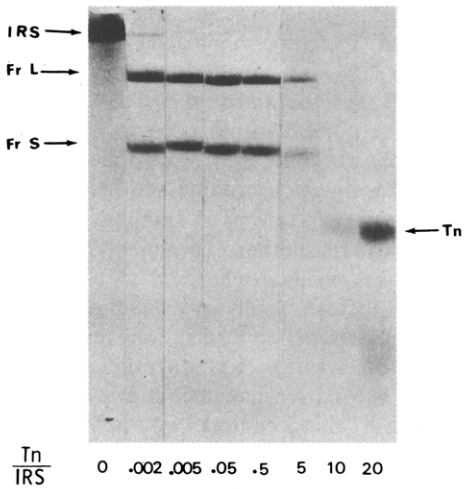

FIGURE 4: Effects of increasing trypsin concentration on digestion of isoleucyl-tRNA synthetase. Isoleucyl-tRNA synthetase, 1.0 mg/ml, was digested with increasing amounts of trypsin as indicated (trypsin/isoleucyl-tRNA synthetase in molar ratio). Incubation was at 37° for 5 min. The reaction was terminated and products were electrophoresed in SDS gels as described in Methods. Each gel contained 15 μg of isoleucyl-tRNA synthetase.

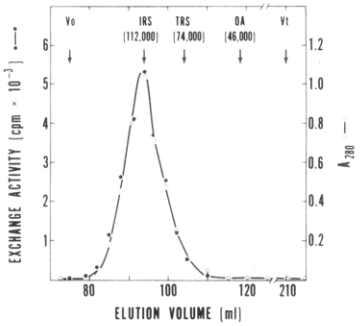

FIGURE 5: Cochromatography of native and trypsin-modified isoleucyl-tRNA synthetase. A Sephadex G-100 (20–120-μ beads) column, 1.6 × 110 cm, was equilibrated with 100 mM potassium phosphate buffer (pH 7.5) with 10 mM 2-mercaptoethanol; 5 mg of isoleucyl-tRNA synthetase in 5 ml was digested with trypsin as described in Figure 2 with a ratio isoleucyl-tRNA synthetase/trypsin of 100:1. The digestion was at 37° for 5 min and was terminated by addition of 55 μg of trypsin inhibitor. An aliquot of the digestion products was analyzed by SDS polyacrylamide gel electrophoresis, which showed the presence of the two fragments and no undigested isoleucyl-tRNA synthetase. As soon as the digestion was finished, a catalytic amount of native isoleucyl-tRNA synthetase (200 μg) was added, and the mixture was cochromatographed on the Sephadex column with the same buffer as developer. The flow rate was 8 ml/hr. The isoleucyl-tRNA synthetase activity was assayed as described in Methods. The recovery of the isoleucyl-tRNA synthetase activity was 93%. On a separate run, markers of tryptophanyl-tRNA synthetase (molecular weight 74,000, Joseph and Muench, 1971) and egg albumin (molecular weight 46,000, Weber and Osborn, 1969) were cochromatographed. The former was detected by the tryptophan-dependent ATP–PP_i exchange reaction and the latter by optical absorption at 280 nm. The recovery of tryptophanyl-tRNA synthetase was 88%. Vo, TRS, OA, and Vt indicate the elution volume determined by Dextran Blue, tryptophanyl-tRNA synthetase, egg albumin, and [^{14}C]glycine, respectively.

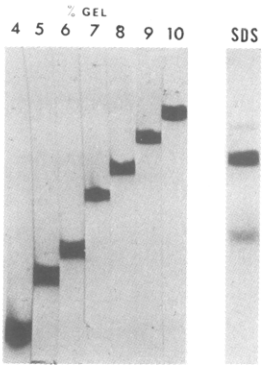

FIGURE 6: Disc gel electrophoresis of the trypsin-modified isoleucyl-tRNA synthetase with varying concentrations of acrylamide. The gels containing 4–10% of acrylamide were prepared as described by Hedrick and Smith (1968). Isoleucyl-tRNA synthetase was first digested with trypsin for 5 min as described in Figure 2; 20 μg of protein was applied to each gel and electrophoresed, in the absence of SDS, as described by Hedrick and Smith (1968). The gel on the right shows the same material electrophoresed in an SDS gel as described by Weber and Osborn (1969).

column, a single A_{280} peak, which arose from the digested material, was superimposed on the ATP–PP_i exchange activity peak of the undigested enzyme (Figure 5). (The digested enzyme has lost its exchange activity, see below.) In contrast to this observation, the recent report by Piszkiewicz and Goitein (1974) showed that trypsin-modified enzyme chromatographed slightly behind the native enzyme. This discrepancy can be adequately explained by the fact that in their gel filtrations native and modified enzymes were chromatographed separately rather than together, as in my experiment. Variation in phosphate buffer concentration from 25 to 200 mM did not alter the elution profile. The same results were obtained when DEAE-cellulose and hydroxylapatite chromatographies were performed. Furthermore, on polyacrylamide gel electrophoresis in the absence of SDS the digestion product showed only one band with acrylamide concentrations in the gels ranging from 4 to 10% (Figure 6). In another experiment in which the digested product was coelectrophoresed with the native isoleucyl-tRNA synthetase, again only one band was detected throughout the 4–10% gel concentrations tested. Therefore, the trypsin-treated isoleucyl-tRNA synthetase was indistinguishable from the native enzyme in size and in surface charge as judged by this technique. Piszkiewicz and Goitein (1974) reported that after 1 hr of trypsin digestion, the modified enzyme has a different electrophoretic mobility than the native enzyme. In the present studies, the trypsin digestion was done in 5 min, and the electrophoresis and coelectrophoresis were performed immediately afterwards. I have noticed that if the electrophoresis is not done within 30 min after digestion, the modified enzyme may show a different electrophoretic mobility, presumably because the modified enzyme is not stable in the reaction mixture and undergoes some conformational change.

To see if the large and small fragments were held together by a disulfide linkage, the isoleucyl-tRNA synthetase was digested in the usual manner with trypsin (Methods). After digestion, the 2-mercaptoethanol originally presented in the reaction mixture, as well as the accessible thiol(s) in the isoleucyl-tRNA synthetase, were reacted with iodoacetic acid. Urea was then added to expose the buried thiols and disulfide bond(s), if any. The latter should have remained intact, as the 2-mercaptoethanol had already reacted with iodoacetic acid. More iodoacetic acid was then added to carboxymethylate the thiols exposed by urea. The carboxymethylated enzyme was isolated by gel filtration, treated with SDS, and electrophoresed in the ordinary manner except without mercaptoethanol. The large and small fragments could be clearly separated, a fact indicating that they are not held together by a disulfide linkage.

Activity of the Modified Isoleucyl-tRNA Synthetase. Isoleucyl-tRNA synthetase catalyzes at least four reactions: (1) L-isoleucine-dependent ATP–PP_i exchange (Berg *et al.*, 1961), (2) aminoacylation of tRNA (Berg *et al.*, 1961), (3) hydrolysis of Ile-tRNA in the absence of AMP and PP_i (Schreier and Schimmel, 1972), and (4) discharge of Ile-tRNA in the presence of AMP and PP_i (Berg *et al.*, 1961; Schreier and Schimmel, 1972). The modified enzyme was examined for these activities. As can be seen from Figure 7A, the modified enzyme has no exchange activity. Increases in the substrate concentrations of up to 50-fold in any combination did not significantly improve the exchange activity by the modified enzyme, a fact mitigating against the possibility that failure of the modified enzyme to interact with substrate(s) was due to an increase of their K_m's. Neither does modified isoleucyl-tRNA synthetase aminoacylate tRNA, and again a tenfold increase of substrate concentrations does not improve the charging activity. These observations confirm the results reported by Piszkiewicz and Goitein (1974).

Spermine stimulates aminoacylation of tRNA (Takeda *et al.*, 1972). For isoleucyl-tRNA synthetase, the presence of 0.1 mM spermine tetrahydrochloride enhanced the charging activity of the native enzyme by 50%, but did not stimulate the charging activity of the modified enzyme.

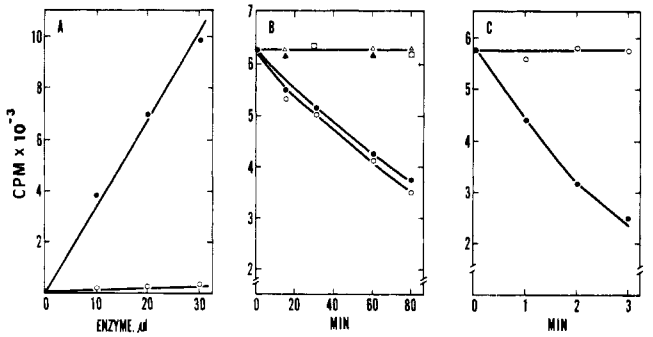

FIGURE 7: Activity of trypsin-modified isoleucyl-tRNA synthetase. In all panels, identical amounts of native and modified isoleucyl-tRNA synthetase were used. (A) Isoleucine-dependent ATP-PP_i exchange reaction. The isoleucyl-tRNA synthetase was first incubated without (●) or with (○) trypsin at 37° for 5 min; the reaction was terminated with trypsin inhibitor as described in Figure 2. The enzymes were then diluted to 10 µg/ml with the same buffer; 10, 20, and 30 µl was withdrawn and assayed for exchange activity as described in Methods. (B) AMP-PP_i independent deacylation (hydrolysis) of [^{14}C]Ile-tRNA. The isoleucyl-tRNA synthetase was treated with trypsin as above; 50 µg of isoleucyl-tRNA synthetase (native, ●; trypsin treated, ○) was then incubated in 0.5 ml of deacylation mixture as described by Schreier and Schimmel (1972). The molar ratio of [^{14}C]Ile-tRNA/isoleucyl-tRNA synthetase used in the deacylation mixture was 4.0. At each time interval, 100 µl of sample was withdrawn, precipitated, washed, and counted as described by Muench and Berg (1966). (△) The deacylation of Ile-tRNA in the absence of any enzyme; (□ and ▲) the deacylation of Ser-tRNA and Tyr-tRNA, respectively, by the modified isoleucyl-tRNA synthetase; (C) AMP-PP_i dependent deacylation of [^{14}C]Ile-tRNA. The modified isoleucyl-tRNA was prepared as above; 10 µg each of native (●) or trypsin treated (○) isoleucyl-tRNA synthetase was incubated in a 0.5-ml incubation mixture as described by Schreier and Schimmel (1972). At each time interval a 100-µl sample was withdrawn, precipitated, dried, and counted as above. In all cases, the trypsin digestion products were confirmed by analysis on SDS gel electrophoresis to ensure proper digestion.

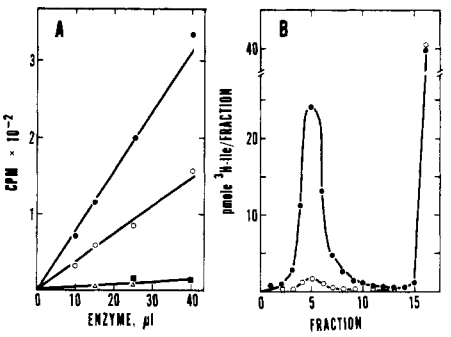

FIGURE 8: Formation of enzyme-substrate complexes. (A) Formation of [^{14}C]Ile-tRNA-isoleucyl-tRNA synthetase complex. Isoleucyl-tRNA synthetase at a concentration of 1.5 mg/ml was treated with trypsin as described in Figure 2 with an isoleucyl-tRNA synthetase/trypsin ratio of 200:1; 10, 15, 25, and 40 µl of native (●) or modified (○) isoleucyl-tRNA synthetase was incubated at pH 5.5 in the reaction mixture described by Yarus and Berg (1969). The [^{14}C]Ile-$tRNA^{Ile}$ concentration in the mixture was 4.8×10^{-6} M (specific activity 2.3 Ci/mol). The complex formed was trapped on a nitrocellulose filter, washed, dried, and counted as described by Yarus and Berg (1969). (△ and ■) Control experiments for the binding of trypsin-treated isoleucyl-tRNA synthetase and [^{14}C]Ser-tRNA or [^{14}C]Tyr-tRNA, respectively. (B) Formation of Ile-AMP-isoleucyl-tRNA synthetase complex. Trypsin digestion was as described in Figure 2. About 11 µg (0.1 nmol) of native (●) or trypsin-treated isoleucyl-tRNA synthetase (○) was then incubated in the reaction mixture described by Eldred and Schimmel (1972) except the reaction mixture (0.1 ml) contained 10 nmol of [^{3}H]isoleucine (specific activity 15 µCi/nmol) and 100 nmol of ATP. After incubating at 37° for 5 min, each incubation mixture was immediately passed through a Sephadex G-50 column, 1.2 × 29 cm, equilibrated with 10 mM sodium cacodylate buffer (pH 6.0) with 0.4 mM EDTA and 50 mM KCl. The eluate was collected at a flow rate of 30 ml/hr with 0.52-ml fractions numbered after discarding the first 8 ml. Aliquots (0.10 ml) were counted in 10 ml of Bray's solution (Bray, 1960).

However, the hydrolytic activity against Ile-tRNA, in the absence of AMP and PP_i, was still preserved in the modified enzyme (Figure 7B). This activity was specific as the modified enzyme failed to hydrolyze Ser-tRNA or Tyr-tRNA. In sharp contrast to the above result, the modified isoleucyl-tRNA synthetase did not deacylate Ile-tRNA in the presence of AMP and PP_i (Figure 7C). This last reaction was done at a lower Mg^{2+} concentration (Schreier and Schimmel, 1972), and the time of reaction was so much shorter (Figure 7C) that the AMP-PP_i independent hydrolysis could not play any significant role. The ability of the modified isoleucyl-tRNA synthetase to specifically hydrolyze Ile-tRNA shows that the modified enzyme is still able to recognize $tRNA^{Ile}$. That recognition is further demonstrated by the formation of a specific complex between modified isoleucyl-tRNA synthetase and tRNA adsorbed to nitrocellulose filters by the method of Yarus and Berg (1967) as shown in Figure 8A. The recognition of tRNA by the modified enzyme and its hydrolytic activity were not reported in the recent publication of Piszkiewicz and Goitein (1974).

In spite of the absence of the isoleucine-dependent ATP-PP_i exchange reaction, the possibility of formation of the Ile-AMP-enzyme complex by modified isoleucyl-tRNA synthetase remained. However, as shown in Figure 8B when identical amounts of native and modified isoleucyl-tRNA synthetase were tested for the ability to form a complex with isoleucine and ATP by gel filtration, only the native enzyme formed the complex. The inability of modified isoleucyl-tRNA synthetase to reverse the charging reaction in spite of the recognition of Ile-tRNA, and the inability to activate isoleucine indicate that the modification affects at least the ATP-AMP binding site of the enzyme.

To see if the modified isoleucyl-tRNA synthetase has altered its substrate specificity, the exchange reactions in the presence of each of the following substrates analogs were examined: 2–20 mM of L-valine, L-leucine, L-norleucine, L-norvaline, DL-methylisoleucine, α-aminobutyric acid, β-aminobutryic acid, and γ-aminoisobutyric acid. None of the substrate analogs was able to support the exchange reaction by the modified enzyme. Neither did GTP or dATP, 0.5–10 mM, support any exchange reaction or the formation of Ile-tRNA. The modified isoleucyl-tRNA synthetase did not synthesize valyl-$tRNA^{Val}$ or deacylate it.

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Ribosome Biosynthesis in *Escherichia coli.* Concerning the Limiting Step†

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ABSTRACT: By studying the kinetics of labeling of newly formed ribosomes in *Escherichia coli* cells growing at 37° on different media it has been found that the average time required to convert a single 26S precursor particle (p30S) into a 30S subunit increases with the culture doubling time. This process is immediately blocked by the addition of chloramphenicol. The maturation of 17S precursor RNA (p16S) into 16S RNA occurs after 30S ribosomes have been formed. *In vitro* reconstitution experiments from free rRNA and free ribosomal proteins have shown that p16S RNA is incorporated into 30S particles with the same efficiency as 16S RNA. p30S are converted into 30S particles by the simple addition of ribosomal proteins, instantaneously and in the cold. These results are interpreted as an indication that the rate limiting step in 30S ribosome formation which leads to the accumulation of p30S particles is not a spontaneous conformational rearrangement of the same particles, or the maturation of rRNA. Ribosome formation is probably controlled by the availability of ribosomal proteins.

The assembly of a bacterial ribosome is a relatively slow process. It takes 1–2 min to synthesize an entire chain of ribosomal RNA (rRNA) and less than 1 min to synthesize a single ribosomal protein. But to assemble the macromolecules into a complete particle takes a significant fraction of a cell generation time (McCarthy *et al.,* 1962; Mangiarotti *et al.,* 1968; Michaels, 1971). In bacteria growing in a poor medium this can amount to 15–20 min.

The reason why the process is so slow is not clear. Free ribosomal proteins are present in the cell (Schleif, 1969; Gierer and Gierer, 1968; Gupta and Singh, 1972; Gausing, 1974; Marvaldi *et al.,* 1974) and they can probably interact with RNA chains while these are still being synthesized (Mangiarotti *et al.,* 1968). The addition of proteins, however, occurs in a discontinuous way (Sells and Davis, 1970; Marvaldi *et al.,* 1972; Nierhaus *et al.,* 1973), leading to the formation of incomplete particles sedimenting at 21–27 S (p30S), at 32 S ($p_1$50S), and at 43 S ($p_2$50S) (McCarthy *et al.,* 1962; Mangiarotti *et al.,* 1968; Osawa, 1968; Hayes and Hayes, 1971). The conversion of these particles into complete ribosomal subunits is the rate-limiting step in

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