Isoleucyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli*. Effect of Limited Cleavage by Trypsin on Activity and Structure†

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ABSTRACT: Isoleucyl transfer ribonucleic acid synthetase when treated with trypsin rapidly and simultaneously lost its abilities to catalyze the ATP–[^{32}P]pyrophosphate exchange reaction and to esterify isoleucine to transfer ribonucleic acid. Transfer ribonucleic acid had no detectable effect on the rate of inactivation, and, therefore, probably binds at a site which is distinct from and does not interact with the site of trypsin cleavage. Saturating concentrations of isoleucine, ATP, and isoleucyl-AMP formed *in situ* protected the activity substantially, but inactivation by trypsin still occurred at a significant rate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the trypsin-modified synthetase showed the formation of two stable polypeptide fragments with molecular weights of 76,000 and 41,000. These fragments could not be separated by polyacrylamide gel electrophoresis. Their chromatographic behavior on Sephadex G-150 and their behavior in sedimentation equilibrium suggested that an equilibrium was set up between the two fragments and their associated form. The fragments were separated by column chromatography on agarose in 6 M guanidine hydrochloride as eluent. A single cysteinyl residue of the enzyme has been modified by *N*-ethylmaleimide with a resultant markedly reduced catalytic ability (Iaccarino, M., and Berg, P. (1969), *J. Mol. Biol. 42*, 151). This residue was found in the 76,000 fragment.

The aminoacyl-tRNA synthetases are a group of enzymes responsible for esterifying the 20 amino acids to their cognate tRNAs as the first step in protein synthesis (Novelli, 1967). While the catalytic properties of these enzymes have been studied extensively (Novelli, 1967; Mehler and Chakraburtty, 1971; Mehler, 1970), relatively little is known about their structures. We have begun a study of the structure of one of these enzymes, isoleucyl-tRNA synthetase (EC 6.1.1.5) of *Escherichia coli*. The native enzyme is composed of a single polypeptide chain having a molecular weight of 114,000 and approximately 1000 amino acid residues (Arndt and Berg, 1970). To simplify the problem of determining the amino acid sequence of this protein we explored the possibility of cleaving it into a limited number of fragments which might be attacked more easily than the intact polypeptide chain.

Baldwin and Berg (1966a) reported that exposure of this synthetase to trypsin resulted in a loss of its ability to catalyze the exchange of [^{32}P]pyrophosphate into ATP, and the presence of ATP and Mg^{2+} plus saturating amounts of isoleucine partially protected this catalytic activity. However, they did not conduct a detailed investigation of this reaction. In this communication we report our findings on the inactivation of isoleucyl-tRNA synthetase by trypsin, the effects of substrates on this inactivation reaction, and some findings on the structure of the modified synthetase.

Experimental Section

Materials. E. coli B tRNA and [^{14}C]isoleucine were from Schwarz/Mann; [^{32}P]pyrophosphate was from ICN; trypsin was from Worthington Biochemicals; ATP and soybean trypsin inhibitor were from Calbiochemicals. Isoleucyl-tRNA synthetase was prepared from *E. coli* B (obtained from Grain Processing, Muscatine, Iowa) by the method of Baldwin and Berg (1966a) as modified by Eldred and Schimmel (1972). The enzyme was concentrated by reverse dialysis against polyvinylpyrrolidone, then stored in the freezer at a concentration of 1 mg/ml in 60% 0.02 M phosphate buffer containing 0.01 M mercaptoethanol at pH 7.5 and 40% glycerol. Aliquots of this stock solution were diluted with the appropriate buffer when used.

Assays of Catalytic Activity. The ATP–[^{32}P]pyrophosphate exchange reaction was done by the method of Baldwin and Berg (1966b). The esterification of [^{14}C]isoleucine to tRNA was measured in 0.05 M Tris-HCl at 30° for a period of 10 min with all other conditions and procedures as described by Muench and Berg (1966).

Hydrolysis by Trypsin. Isoleucyl-tRNA synthetase (0.1–0.2 mg/ml in 0.1 M phosphate buffer (pH 8.10)) was incubated with trypsin (2.0 μg/ml) at 30°. At timed intervals 0.2-ml aliquots were taken, and 1.0 μg of soybean trypsin inhibitor was added to them to stop tryptic hydrolysis. The quenched enzyme solutions were then diluted 100-fold with 0.02 M phosphate buffer which contained 0.01 M mercaptoethanol at pH 7.5, and assayed by either of the methods described above. For those reactions in which the products were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see below) 0.1 M NH_4HCO_3 at pH 8.10 was used to buffer the reaction since the potassium phosphate buffer formed a precipitate with the electrophoresis buffer.

Electrophoresis of protein on buffered polyacrylamide gels was performed as described by Davis (1964).

The molecular weights of protein and protein fragments were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Weber and Osborn (1969); the molecular weights of the standard proteins are those given by these authors.

Gel Filtration. Chromatography of native and trypsin treated isoleucyl-tRNA synthetase was done on a column (0.9

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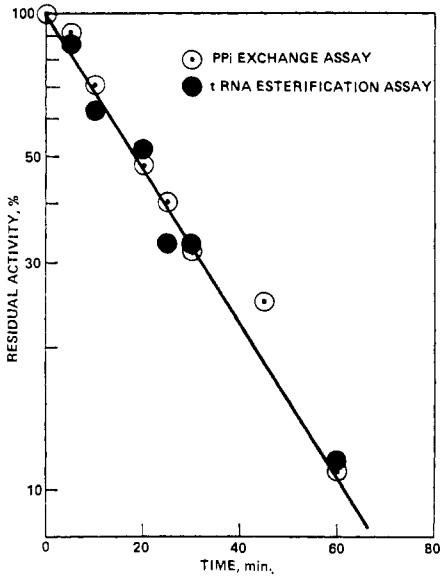

FIGURE 1: The effect of trypsin on the activities of isoleucyl-tRNA synthetase as a function of time: (○) ATP–[^{32}P]pyrophosphate exchange assay; (●) tRNA esterification assay.

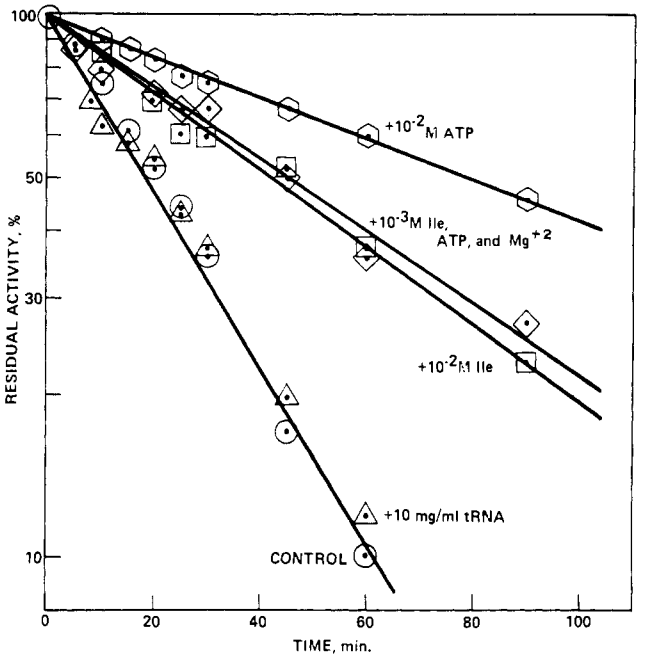

FIGURE 2: Time course of the inactivation of isoleucyl-tRNA synthetase by trypsin in the presence of substrates.

× 93 cm) of Sephadex G-150 in 0.02 M phosphate buffer (pH 7.5). The molecular weights of standard proteins were given by Weber and Osborn (1969). They were located in the eluted fractions by their ultraviolet absorption at 280 nm. Native and trypsin modified synthetase had anomalously high base lines, presumably due to the presence of polyvinylpyrrolidone picked up during concentration. Therefore, these proteins were located in eluted fractions by the turbidimetric method of Rosenbloom *et al.* (1968).

Sedimentation Experiments. The sedimentation equilibrium experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with interference optics and an RTIC temperature control unit at 30,000 rpm and at 20°. A six-channel Yphantis cell was used in the AN-D rotor. After centrifugation for 48-hr fringe patterns were recorded on Kodak II-g spectrographic plates. Measurements of fringe displacement (f) *vs.* radial distance (r) were made with a Gaertner comparator. Molecular weight was calculated by the method of Yphantis (1964). The partial specific volume ($\bar{v}$ = 0.744) of isoleucyl-tRNA synthetase was calculated by Baldwin and Berg (1966a). The term d ln f/dr^2 was obtained as 2.303 times the slope of the log f *vs.* r^2 plot.

Separation of Polypeptide Fragments. Synthetase which had been treated with trypsin for 1 hr was reacted with iodoacetic acid in 6 M guanidine-HCl to carboxymethylate its cysteinyl residues (Piszkiewicz *et al.* 1971). After the reaction was stopped by addition of mercaptoethanol, the product mixture was applied directly to a column (0.9 × 88 cm) of agarose (Bio-Gel A 5m) with 6 M guanidine-HCl as eluent (Fish *et al.*, 1969).

Labeling with [^{14}*C*]-*N-Ethylmaleimide.* The synthetase (0.1 mg/ml) in 0.1 M phosphate buffer (pH 7.0) was reacted with [^{14}C]-*N*-ethylmaleimide (10^{-3} M, 0.5 Ci/mol) for 30 min at 30° according to the method of Iaccarino and Berg (1969). After removal of excess reagent by dialysis, the amount of radioactive label incorporated was measured by counting the product in Aquascint II liquid scintillation fluid (from ICN). Aliquots (0.2 ml) of samples eluted from agarose (1.0 ml) were evaporated to dryness in scintillation vials, suspended in Omnifluor scintillation fluid (New England Nuclear), and then counted. No corrections were made for quenching by guanidine-HCl.

Amino Acid Analyses. Samples were hydrolyzed in evacuated glass tubes at 110° for 24 or 72 hr with 6 N HCl containing one drop of 1% phenol in water and 5 μl of mercaptoacetic acid. Analyses were performed on a Beckman Model 121 amino acid analyzer.

Results

The time course of the reaction of isoleucyl-tRNA synthetase (0.1 mg/ml) with trypsin is shown in Figure 1. This reaction resulted in a rapid loss of the ability of the synthetase to catalyze the exchange of [^{32}P]pyrophosphate into ATP by an apparently first-order process; this experiment confirmed a similar observation by Baldwin and Berg (1966a). Exposure of the synthetase to trypsin also resulted in a loss of ability to catalyze the formation of isoleucyl-tRNA by an apparently first-order process (Figure 1). The rates of the loss of catalytic activity as measured by these two methods appeared identical. At reaction times greater than 120 min less than 3% of initial synthetase activity could be detected by either assay procedure. Therefore, loss of catalytic activities were, within experimental limits, complete. In the absence of trypsin under the conditions employed, the synthetase retained over 95% of its activity after 120 min.

The time course of the inactivation of isoleucyl-tRNA synthetase by trypsin in the presence of substrates was also studied. Residual synthetase activity was determined by the tRNA esterification assay in all cases. In the control experiment which excluded substrate (Figure 2), the rate of loss of catalytic activity had an apparent first-order rate constant of k_{obsd} = 3.7 × 10^{-2} min^{-1}. When ATP (10^{-2} M) was included at a concentration which should have saturated the synthetase (K_d = 5 × 10^{-4} M) (Holler and Calvin, 1972), a decrease of the rate of inactivation was observed (Figure 2). ATP afforded substantial protection against inactivation, but loss of activity still took place at a significant rate (k_{obsd} = 8.7 × 10^{-3} min^{-1}). Isoleucine (10^{-2} M) at a concentration which would saturate the synthetase (K_d = 5.8 × 10^{-6} M) (Holler and Calvin, 1972) also protected it (Figure 2); however, the rate of inactivation in the presence of isoleucine was substantial (k_{obsd} = 1.6 ×

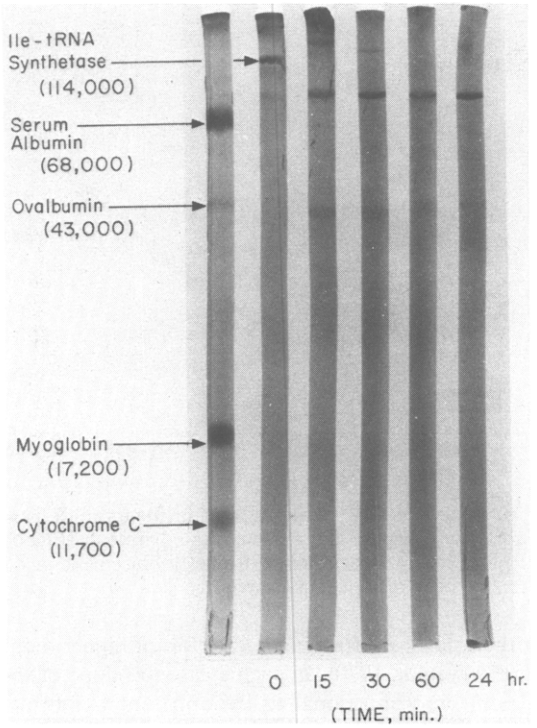

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of standard proteins (gel at left), and isoleucyl-tRNA synthetase after varying times of exposure to trypsin (five gels at right).

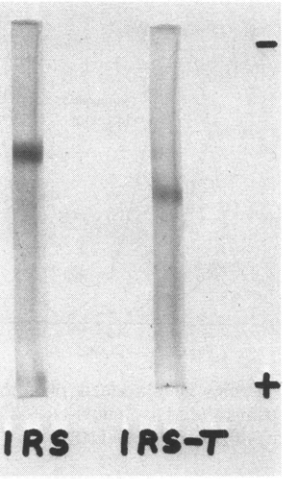

FIGURE 4: Electrophoresis of native isoleucyl-tRNA synthetase (IRS) and trypsin-treated isoleucyl-tRNA synthetase (IRS-T) on polyacrylamide gels.

10^{-2} min^{-1}). The rate of inactivation of isoleucyl-tRNA synthetase by trypsin in the presence of unfractionated tRNA (10 mg/ml) was approximately that of the control which excluded substrate (Figure 2). If one assumes that isoleucyl-tRNA is 5% of the unfractionated material, and that tRNAs have molecular weights of 25,000 to 30,000 (RajBhandary and Stuart, 1966), one can calculate that 10 mg/ml represents an isoleucyl-tRNA concentration of approximately 2×10^{-5} M. Since the dissociation constant for the synthetase-tRNA complex is 5.9×10^{-7} M (Yarus and Berg, 1969), it is clear that no protection was afforded to the synthetase even at a concentration of tRNA (10 mg/ml) at which the enzyme is completely saturated by this substrate.

The effect of isoleucyl-AMP on the rate of inactivation of isoleucyl-tRNA synthetase by trypsin was also tested. To accomplish this, the synthetase was incubated with 10^{-3} M isoleucine, 10^{-3} M ATP, and 10^{-3} M Mg^{2+} for 10 min at pH 8.10 and 30° prior to the addition of trypsin. Similar conditions have been used to saturate this enzyme with isoleucyl-AMP by *in situ* synthesis (Yarus and Berg, 1970). While synthetase activity was partially protected under these conditions (Figure 2), a significant rate of inactivation was observed after addition of trypsin ($k_{obsd} = 1.5 \times 10^{-2}$ min^{-1}). Baldwin and Berg (1966a) observed that the ability of the synthetase to catalyze the [^{32}P]pyrophosphate exchange reaction was substantially, but not completely protected from tryptic inactivation by the addition of ATP, Mg^{2+}, and saturating amounts of isoleucine. Thus, isoleucyl-AMP formed *in situ* only partially protected isoleucyl-tRNA synthetase from inactivation by trypsin.

Isoleucyl-tRNA synthetase is composed of a single polypeptide chain having a molecular weight of 114,000 (Arndt and Berg, 1970). The rate of production of polypeptide fragments from this protein by treatment with trypsin and the sizes of these fragments were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The synthetase was incubated with trypsin for varying lengths of time and the reactions were terminated by addition of soybean trypsin inhibitor. Samples were then subjected to electrophoresis. The band corresponding to the slow moving, uncleaved protein decreased in intensity with time of incubation with trypsin and had disappeared after 1 hr (Figure 3). As the band corresponding to the uncleaved protein disappeared, two bands which had migrated farther down the gel appeared. The relative intensities of these two bands (Figure 3) appeared not to change measurably for up to 24 hr. Thus, the two major fragments formed from isoleucyl-tRNA synthetase by limited tryptic cleavage were resistant to further tryptic hydrolysis.

The polypeptide fragments formed by tryptic cleavage of the synthetase in the presence of 10^{-2} M isoleucine, 10^{-2} M ATP, 10 mg/ml of tRNA, and isoleucyl-AMP formed *in situ* were also examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These fragments were indistinguishable from those formed in the absence of added substrate.

The molecular weights of the fragments were estimated from their electrophoretic mobilities in the sodium dodecyl sulfate–polyacrylamide gels. The electrophoretic mobilities of five standard proteins (Figure 3) when plotted *vs.* the logarithms of their molecular weights gave a linear relationship as expected. The electrophoretic mobilities of the two fragments obtained by limited tryptic cleavage of isoleucyl-tRNA synthetase when plotted on this line (figure not shown) indicated molecular weights of 76,000 and 41,000.

In order to understand how limited tryptic hydrolysis effected a loss of isoleucyl-tRNA synthetase activity, we found it desirable to determine if the polypeptide fragments dissociated after cleavage. Our first approach to answering this question was to examine the relative electrophoretic mobilities on polyacrylamide gels of the native enzyme and enzyme which had been treated with trypsin for 1 hr. Two such samples run simultaneously on separate gels are shown in Figure 4. The modified enzyme (IRS-T) was found as one band which migrated substantially farther toward the anode than the native enzyme (IRS). This result suggested that the fragments of the modified enzyme (IRS-T) remained associated. Furthermore, cleavage of a peptide bond would be expected to introduce a negative charge into the protein at the pH of the gel (pH 8.9) since the new α-carboxyl group would be in the dissociated, anionic form, and the new α-amino group would be unprotonated (Edsall, 1943). Therefore, the greater migration of the

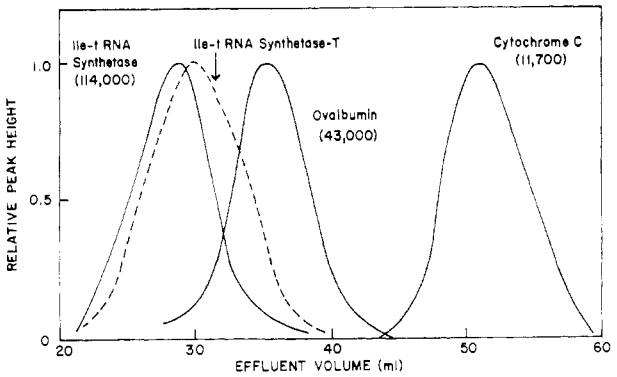

FIGURE 5: Elution profiles of standard proteins, isoleucyl-tRNA synthetase, and trypsin-treated isoleucyl-tRNA synthetase from a column (0.9 × 93 cm) of Sephadex G-150.

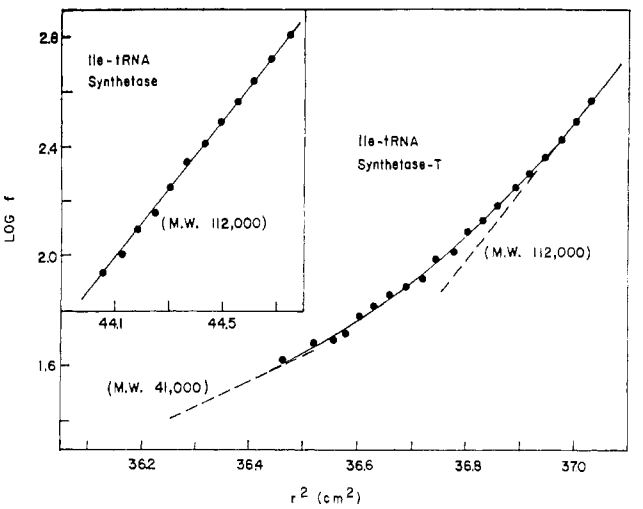

FIGURE 6: Sedimentation equilibrium centrifugation of isoleucyl-tRNA synthetase (inset) and trypsin-treated isoleucyl-tRNA synthetase; plots of the logarithms of fringe displacement in microns ($\log f$) *vs.* the radial distance squared (r^2).

modified protein (IRS-T) toward the anode could be explained, at least in part, as a result of the introduction of this additional negative charge into the protein.

As a further test for possible dissociation, enzyme which had been treated with trypsin for 1 hr was chromatographed on Sephadex G-150. The rates of elution of 1.0-mg samples of isoleucyl-tRNA synthetase, ovalbumin, and cytochrome *c* were determined to calibrate the column. Their order of elution and elution volumes, which are shown in Figure 5, were as expected on the basis of their molecular weights (all peak heights have been normalized to simplify comparisons of elution volumes). Isoleucyl-tRNA synthetase which had been treated with trypsin emerged as a single, broad peak with an elution volume slightly greater than that of the native enzyme (Figure 5). Qualitatively, this behavior of the trypsin-cleaved synthetase resembled that of a reversibly associating system on molecular sieve chromatography (Ackers and Thompson, 1965). Extensive studies have been conducted (Zimmerman and Ackers, 1971) on the behavior of the reversible association of identical components on molecular sieves; their rates of elution and their elution profiles were found to be dependent upon a complex set of factors which included the dissociation constant. However, due to the complexities involved, evaluation of the dissociation constant with any degree of confidence was deemed unlikely (Zimmerman and Ackers, 1971). We are unaware of any similar studies of associating, nonidentical components. Thus, the observed elution of trypsin-cleaved isoleucyl-tRNA synthetase from Sephadex G-150 (Figure 5) is consistent with a dissociation equilibrium of the two fragments which lies in the direction of the associated form.

Sedimentation equilibrium centrifugation was also done to determine if the fragments of trypsin treated isoleucyl-tRNA synthetase dissociated. Native and trypsin treated enzyme (both at 0.2 mg/ml) which had been exhaustively dialyzed against buffer (0.2 M phosphate containing 0.01 M mercaptoethanol at pH 7.5) were run simultaneously in a six-channel Yphantis cell. For the native synthetase in the control experiment, a plot of the log of the fringe displacement (f) *vs.* the radial distance squared (r^2) yielded a straight line (inset, Figure 6). A molecular weight of 112,000 was calculated by using the partial specific volume ($\bar{v}$ = 0.744) for this protein (Baldwin and Berg, 1966a). This molecular weight agrees well with the value of 114,000 ± 2000 determined for this protein by a variety of methods (Arndt and Berg, 1970). For the trypsin treated protein, a plot of the log of the fringe displacement *vs.* the radial distance squared gave a curved line (Figure 6). An upward curved plot such as this is generally interpreted as indicating either that the solute is heterogeneous or that the solutes are forming aggregates of higher molecular weight (Chervenka, 1970). In such a case the slope of the plot at any point is proportional to the apparent weight average molecular weight of the mixture at the corresponding position of the centrifuge cell. A tangent to the top of the curve (derived from the bottom of the cell) had a slope approximately equal to that given by the native protein and representing a species having a molecular weight of 112,000 (assuming $\bar{v}$ = 0.744 (Baldwin and Berg, 1966a)). The bottom portion of the curve approached a line having a slope one would expect for the polypeptide fragment having a molecular weight of 41,000 (assuming $\bar{v}$ = 0.744 (Baldwin and Berg, 1966a) as for the intact protein). The contents of the cell could contain at most three components: the 41,000 fragment, the 76,000 fragment, and the two associated into the 112,000 form. The smaller fragment and the associated form were clearly present along with forms having intermediate weight average molecular weights. Thus, an association equilibrium of the two fragments to give the associated form is indicated. Since a separation of these fragments could not be affected by electrophoresis on polyacrylamide gel (Figure 4) or chromatography on Sephadex G-150 (Figure 5) this equilibrium must lie in the direction of the associated form. It would be desirable to calculate an equilibrium constant; however, ultracentrifuge studies of associating systems conducted to date have concentrated on the association equilibria of identical subunits (Roark and Yphantis, 1969). Since it is not within the scope of this study to develop new ultracentrifuge methods, no attempt has been made to develop a treatment for determination of the equilibrium constant for the association of these nonidentical subunits.

If this method of tryptic cleavage of isoleucyl-tRNA synthetase is to be of use in analysis of the structure of this enzyme, the two product fragments must be separated in milligram quantities. We have accomplished such a separation by column chromatography on agarose with 6 M guanidine-HCl as eluent by the method of Fish *et al.* (1969). In the elution profile (Figure 7A) polypeptide material was found in two cleanly separated peaks. On this molecular sieve column the first peak to emerge (IRS-T-I) would be expected to be the larger (76,000) fragment; the second peak (IRS-T-II) would be expected to be the smaller (41,000) fragment. This identification was confirmed by electrophoresis on sodium dodecyl-

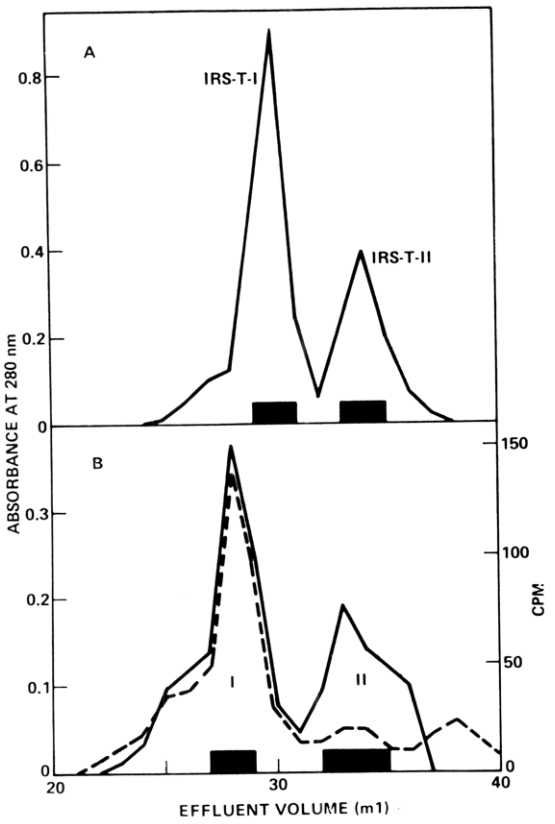

FIGURE 7: (A) Elution profile of trypsin treated isoleucyl-tRNA synthetase from a column (0.9 × 88 cm) of agarose with 6 M guanidine-HCl as eluent. (B) Elution profile of trypsin-treated [^{14}C]-*N*-ethylmaleimide-labeled isoleucyl-tRNA synthetase from the same column: (—), absorbance at 280 nm; (- - -), counts per minute; (solid bars) pooled fractions.

sulfate–polyacrylamide gels (Figure 8) after desalting on Sephadex G-15.

The amino acid compositions of isoleucyl-tRNA synthetase

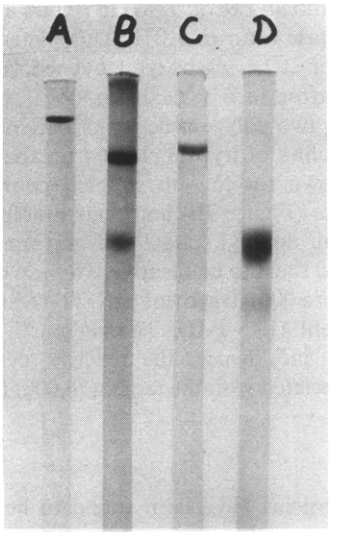

FIGURE 8: Sodium dodecyl sulfate–polyacrylamide gels: (A) isoleucyl-tRNA synthetase; (B) trypsin-cleaved isoleucyl-tRNA synthetase; (C) IRS-T-I of Figure 8A; (D) IRS-T-II of Figure 8A.

and the two separated fragments have been presented in Table I. The sum of the compositions of the fragments is, within the limits of the method, equal to the composition of the total protein. It may also be noted that fragment I (76,000) is significantly enriched in proline and the basic residues histidine and arginine, while fragment II (41,000) is enriched in the nonpolar residues alanine and tyrosine.

Iaccarino and Berg (1969) have found that one cysteinyl residue of the synthetase reacts rapidly with *N*-ethylmaleimide. Modification of this group markedly reduced the ability of the enzyme to catalyze the ATP–[^{32}P]pyrophosphate exchange reaction and to esterify isoleucine to tRNA, however, the modified enzyme was still capable of forming the synthetase–isoleucyl-AMP complex. We have reacted isoleucyl-tRNA synthetase with [^{14}C]-*N*-ethylmaleimide by the reported

TABLE I: Amino Acid Composition of Isoleucyl-tRNA Synthetase and Fragments Formed by Trypsin Cleavage.

Amino Acid	Fragment I (Mol Wt 76,000)	Fragment II (Mol Wt 41,000)	Fragment I + Fragment II	Isoleucyl-tRNA Synthetase *a*	Isoleucyl-tRNA Synthetase *b*
Aspartic acid	72.6	33.0	105.6	99.0	100.7
Threonine	34.8	21.9	56.7	48.2	54.9
Serine	30.9	14.3	45.2	46.4	55.5
Glutamic acid	59.9	39.5	99.4	99.3	83.0
Proline	30.9	9.9	40.8	47.2	43.8
Cysteine	11.5[c]	4.8[c]	16.3	15.0	7.0
Glycine	61.8	32.9	94.7	82.0	82.0
Alanine	58.0	49.4	107.4	100.0	106.9
Valine	49.3[d]	27.4[d]	76.7	76.7	81.3[d]
Methionine	11.6	5.5	17.1	24.5	20.7
Isoleucine	30.9[d]	12.4[d]	43.3	48.7	43.0[d]
Leucine	50.3	26.3	76.6	80.3	83.4
Tyrosine	18.4	14.3	32.7	32.7	33.4
Phenylalanine	19.3	11.0	30.3	32.1	30.3
Histidine	18.4	4.4	22.8	24.8	23.5
Lsyine	39.6	23.0	62.6	65.3	67.0
Arginine	36.8	10.3	47.1	48.7	43.8
Tryptophan				24.5	

[a] Composition from Baldwin and Berg (1966a). [b] Composition from this study. [c] Determined as *S*-carboxymethylcysteine. [d] Determined after hydrolysis for 72 hr.

method (Iaccarino and Berg, 1969) to obtain a product with a label to synthetase ratio of 1.0:1. This material was treated with trypsin for 2 hr, carboxymethylated, then chromatographed on agarose in 6 M guanidine-HCl. As with the unlabeled protein, two polypeptide peaks were obtained (Figure 7B) (elution profiles of trypsin cleaved unlabeled and labeled protein are shown together to simplify comparison). Their elution positions (Figure 7B) and their mobilities when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis allowed them to be identified in their order of elution as the larger (76,000) fragment (IRS-T-I) and the smaller (41,000) fragment (IRS-T-II). The radioactively labeled *N*-ethylmaleimide and, hence, the reactive cysteinyl residue were found associated with the larger (76,000) fragment.

Discussion

Numerous proteins have been found to be susceptible to limited proteolysis which result in varying effects upon their catalytic activities (Mihalyi, 1972), and several of the aminoacyl-tRNA synthetases are among them. Tryptophanyl-tRNA synthetase of bovine pancreas may undergo limited proteolysis during isolation (Gros *et al.*, 1972) with a retention of catalytic activity. Lysyl-tRNA synthetase of *E. coli* may be similarly degraded during isolation (Dimitrijevic, 1972); partial trypsinolysis mimics this degradation and is accompanied by a loss in catalytic activity. Leucyl-tRNA synthetase of *E. coli* may be partially hydrolyzed during isolation or by trypsin (Rouget and Chapeville, 1971); both modes of proteolysis result in a loss in ability to charge tRNA but not to catalyze the ATP–pyrophosphate isotope exchange reaction. Cleavage of methionyl-tRNA synthetase by trypsin resulted in release of enzymatically inactive fragments and modified enzyme of slightly lowered catalytic activity (Cassio and Waller, 1971). We have found that native isoleucyl-tRNA synthetase of *E. coli* when incubated with a limited concentration of trypsin undergoes a rapid and complete loss of both its ability to catalyze the ATP–[^{32}P]pyrophosphate exchange reaction and its ability to esterify tRNA with isoleucine.

The substrates of isoleucyl-tRNA synthetase, isoleucine, ATP, and tRNA, all have negative charges at neutrality. It is likely that their binding to the enzyme is through the positively charged side chain functional groups of lysyl and/or arginyl residues. Since these residues are also the sites of cleavage of trypsin, it was considered possible that tryptic cleavage occurred adjacent to a residue essential for binding a substrate; thus, cleavage could result in a localized conformational change, and a consequent loss of ability to bind substrate or catalyze reactions. To test this possibility, the effects of substrates on the rate of inactivation of the synthetase by trypsin were studied. If a substrate did bind near the site of tryptic cleavage, its presence would be expected to prevent access to such a bond by trypsin. We found that tRNA had no detectable effect on the rate of inactivation; therefore, the site of tRNA binding is distinct from and does not interact with the site of trypsin cleavage. Isoleucine, ATP, and isoleucyl-AMP formed *in situ* did decrease the rate of inactivation; however, inactivation still occurred at a significant rate. The fragments formed under these conditions when examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were indistinguishable from those formed in the absence of these substrates. Therefore, binding of any of these latter substances to the synthetase makes the bond or bonds to be hydrolyzed less accessible to trypsin possibly through a minor conformational change. It is unlikely that this bond or bonds is at the binding or catalytic sites since the presence of substrates would be expected to fully protect catalytic activity.

Examination of the products of trypsin treatment of isoleucyl-tRNA synthetase by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 3) showed the formation of two stable polypeptide fragments. The formation of these pieces occurred within the same period of time required for loss of catalytic activity. The two fragments were found to have molecular weights of 76,000 and 41,000. The sum of the molecular weights of these two pieces is 117,000, approximately the same as the molecular weight of 114,000 ± 2000 reported for the uncleaved synthetase (Arndt and Berg, 1970), and only slightly greater than the molecular weight of 112,000 derived from the sedimentation equilibrium experiment of this study. Since the method has an experimental uncertainty of ±10% (Weber and Osborn, 1969), these two fragments could easily account for the entire uncleaved protein and be the only polypeptides derived from it. However, these results do not eliminate the possibility that a relatively small peptide or peptides which account for at most a few per cent of the total mass of the protein have also been formed.

The behavior of the trypsin cleaved synthetase on polyacrylamide gel electrophoresis (Figure 4), column chromatography on Sephadex G-150 (Figure 5), and in a sedimentation equilibrium study (Figure 6) suggested that an equilibrium existed between the polypeptide fragments and their associated form and that this equilibrium must lie in the direction of the associated form. The fact that a dissociation can take place implies that the polypeptide fragments of isoleucyl-tRNA synthetase are not linked by covalent disulfide bridges. Furthermore, the fragments must associate only by the mediation of noncovalent, ionic, hydrophobic, and hydrogen bonds.

These results lead to an intriguing speculation on the tertiary structure of the isoleucyl-tRNA synthetase molecule. The molecule may be conceived of as two sections, approximately globular in shape, associating at an interface by noncovalent bonds, and connected by one relatively short segment of polypeptide. Trypsin cleaves a peptide bond within this segment, and this cleavage results in a conformational change. This conformational change would have to be significant enough to disrupt the active site and cause a loss in catalytic ability. Furthermore, cleavage of the one covalent link between the two polypeptide segments would allow dissociation of these pieces. The degree of this dissociation would be dependent upon the strength of the interactions at the interface.

This model for the structure of isoleucyl-tRNA synthetase is not without precedent. A model with similar features has been proposed for leucyl-tRNA synthetase of *E. coli* by Rouget and Chapeville (1971). However, the model for the leucyl-tRNA synthetase differs in that the two major polypeptide fragments are of approximately equal size (mol wt 55,000), and a polypeptide bridge with a molecular weight 3000 is completely excised by trypsin. Furthermore, these aminoacyl-tRNA synthetases are not alone in having distinct structural regions; similar globular intrachain regions have been recognized in the three-dimensional structures of numerous proteins with a wide variety of functions (Wetlaufer, 1973).

Lastly, this study demonstrates the feasibility of using limited proteolytic cleavage as a first step in the determination of the amino acid sequence of isoleucyl-tRNA synthetase. The fragments produced by tryptic cleavage have been sep-

arated in milligram quantities, and the reactive cysteinyl residue has been located in the larger (76,000) fragment. Amino acid sequence analysis of the two separated fragments is now in progress.

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