High-Molecular-Weight Forms of Aminoacyl-tRNA Synthetases and tRNA Modification Enzymes in Escherichia coli

CHARLES L. HARRIS
Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia 26506

Received 28 June 1989/Accepted 19 December 1989

The presence of high-molecular-weight complexes of aminoacyl-tRNA synthetases in Escherichia coli has been reported (C. L. Harris, J. Bacteriol. 169:2718-2723, 1987). In the current study, Bio-Gel A-5M gel chromatography of 105,000 x g supernatant preparations from E. coli Q13 indicated high molecular weights for both tRNA methylase (300,000) and tRNA sulfurtransferase (450,000). These tRNA modification enzymes did not appear to exist in the same multienzymic complex. On the other hand, 4-thiouridine sulfurtransferase eluted with aminoacyl-tRNA synthetase activity on Bio-Gel A-5M, and both of these activities were cosedimented after further centrifugation of cell supernatants at 160,000 x g for 18 h. Despite this evidence for association of the sulfurtransferase with the synthetase complex, isoleucyl-tRNA synthetase and tRNA sulfurtransferase were totally resolved from each other by DEAE-Sephadex chromatography. Subsequent gel chromatography showed little change in their elution positions on agarose. Hence, either nonspecific aggregation occurred here, or the modification enzymes studied are not members of the aminoacyl-tRNA synthetase complex in E. coli. These findings do suggest that some bacterial tRNA modification enzymes are present in multiprotein complexes of high molecular weight.

MATERIALS AND METHODS

Bacteria and cell growth. E. coli Q13 (RNase I') was grown in the M9 medium of Anderson (3) supplemented with methionine and tyrosine at 0.13 and 0.11 mM, respectively. E. coli C6 (a relA Met' Cys' strain) was grown in the same medium but supplemented with methionine (0.13 mM) and cysteine (0.16 mM). This strain was used to prepare methyl-deficient or sulfur-deficient tRNAs for use as substrates for tRNA modification enzymes (14, 16). Growth was limited by using 1/10 of the above-described concentration of methionine and continuing incubation for 6 h after methionine was depleted. Identical conditions were used with cysteine-starved cells, except that cysteine was present at 0.016 mM.

Enzyme preparation. Cell extracts of E. coli Q13 were prepared by sonication as follows. Cell pellets were suspended in 5 volumes (wt/vol) of 0.05 M Tris hydrochloride (pH 7.4) containing 10 mM MgCl2, 1 mM dithiothreitol, and 10% (vol/vol) glycerol (buffer A). The suspension was sonicated at 0 to 4°C with a microtip at a setting of 4 on a Heat-Systems Ultrasonics model 375-W sonicator for 2 min. Extracts were clarified by centrifugation at 12,000 x g for 30 min; the resulting supernatant solutions were further centrifuged at 105,000 x g for 90 min. The final supernatant solutions were carefully removed from the ribosomal pellets and used immediately. Total synthetase activity and gel elution position were found to be unchanged after several months at -20°C with glycerol added at 50% (vol/vol).

Protein was measured by the Bio-Rad method (Bio-Rad Laboratories, Richmond, Calif.) using bovine immunoglobulin G as the standard.

Chromatography. The 105,000 x g solutions were chromatographed on Bio-Gel A-5M columns (Bio-Rad) with buffer A as the elution buffer (see legends to figures for exact conditions). The columns were calibrated by using dextran blue (2,000,000 daltons), beta-galactosidase (510,000 daltons), pyruvate kinase (237,000 daltons), hemoglobin (64,000 daltons), and lactalbumin (35,000 daltons). Beta-galactosidase was obtained by growing a culture of E. coli in M9 medium plus lactose. After sonication and clarification as
Gel described above, the extract was chromatographed on Bio-Gel A-5M, and fractions were assayed for beta-galactosidase as described by Miller (18). Purified pyruvate kinase from yeast was a gift of James Blair, and hemoglobin was obtained from rat erythrocytes by hypotonic lysis.

Thionucleoside analysis. [35S]tRNA was isolated from E. coli C6 grown in M9 medium (13) supplemented with 0.13 M glucose (37°C). Then 200 ml of E. coli alkaline phosphatase (Worthington Diagnostics, Freehold, N.J.) was added. The mixture was incubated for 16 h at 37°C, clarified by centrifugation, and applied to a 2.5- by 50-cm column of Bio-Gel P2. The column was equilibrated and eluted with 0 mol sodium citrate buffer (pH 10.6) by the method of Rao and Cherayil (113, 000 daltons), tRNA was isolated by the method of von Ehrenstein (26). Samples of appropriate fractions are added to scintillation fluid (ACS, Amersham), and radioactivity was determined.

Enzymatic assays. Aminoacyl-tRNA synthetase activity was assayed essentially as described previously (13, 14). Each assay contained the following components in a final volume of 0.5 ml: 25 μmol of Tris hydrochloride (pH 7.3), 5 μmol of magnesium acetate, 2 μmol of ATP (pH 7), 0.1 to 1.0 μCi of a (14C)- or 3H-labeled amino acid, 0.4 mg of E. coli B tRNA, and 0.2 ml of the fraction. After a 30-min incubation at 37°C, 0.1-ml samples removed from the reaction mixture, added to 2.4-cm-diameter Whatman 3MM disks, and plunged in cold 5% trichloroacetic acid. The disks were washed in trichloroacetic acid, ethanol-ether, and ether and then dried and counted as previously described (13).

tRNA modification assays were carried out as previously described (15). Methylation assays contained the following components (in 0.2 ml): Tris hydrochloride (pH 8.0), MgCl2, 0.5 μmol; 2-mercaptoethanol, 0.5 μmol; methyl-deficient tRNA, 0.5 mg; [14CH3]S-adenosyl-L-methionine, 0.1 μCi; and 0.1 ml of each enzyme fraction. Reactions were incubated at 37°C for 30 min, and 0.1-ml samples of the reaction mixture were applied to a 3 MM paper disk and added to 5% trichloroacetic acid. The disks were washed and counted as described above.

Sulfurtransferase assays contained the following components in a final volume of 0.5 ml: Tris hydrochloride (pH 8.5), 25 μmol; MgCl2, 2.5 μmol; ATP, 2.0 μmol; diithiothreitol, 0.5 μmol; pyridoxal phosphate, 0.02 μmol; sulfur-deficient tRNA, 0.4 μmol; [14S]cysteine, 2 μCi; [35S]cysteine, 5 mmol; and 0.3 ml of enzyme fraction. The incubations were as described above; the reaction was terminated by adding 1.5 ml of 20 mM cysteine, 0.5 mg of E. coli B tRNA as the carrier, and 0.05 ml of a 5% cetyltrimethylammonium bromide solution. After 1 h on ice, the tRNA was collected by centrifugation, dissolved, and precipitated with 2 volumes of ethanol. The tRNA was dissolved in 0.5 M Tris hydrochloride-0.005 M NaCl (pH 10) and incubated for 90 min at 37°C to deacylate cysteinyl-tRNA. An equal volume of redistilled phenol was then added to the deacylation mixture, and the emulsion was shaken for 10 min. After centrifugation to separate the phases, 0.1 ml of the aqueous phase (0.25-ml total volume) was applied to paper disks, and the disks were washed and counted as described above. Complete details of this assay procedure have been published (15).

Chemicals and isotope. Bio-Gel A5M, Bio-Gel P2, and Affigel 601 were obtained from Bio-Rad Laboratories. DEAE-Sepharose was a product of Pharmacia Fine Chemicals, Piscataway, N.J. [35S]cysteine (600 Ci/mmol) and [14CH3]S-adenosyl-L-methionine (40 μCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. [3H]isoleucine (113 Ci/mmol) was purchased from Amersham. E. coli B tRNA was purchased from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were of the highest quality commercially available.

RESULTS

We reported the first evidence that aminoacyl-tRNA synthetase activity was associated with a high-molecular-weight complex in E. coli (13). This conclusion was based on data from agarose gel filtration and ultracentrifugation experiments. To see whether tRNA modification enzymes were also members of this complex, a 105,000 X g supernatant (S105) preparation from E. coli Q13 was chromatographed on Bio-Gel A-5M. Isoleucyl-tRNA synthetase (114,000 daltons), tRNA sulfurtransferase, and tRNA methylase (38,000 to 80,000 daltons) activities chromatographed in the same region, between 300,000 and 500,000 daltons (Fig. 1A). Aminoacylation assays were also carried out with a mixture of 15 [14C]-labeled amino acids as substrates, with profiles essentially identical to those observed with isoleucyl-tRNA synthetase (data not shown). Hence, both total and individual synthetase activities eluted similarly on Bio-Gel A-5M, at a position consistent with the existence of a multienzyme complex (13).

We also subjected the S105 preparation to further centrifugation at 160,000 X g for 18 h. Under these conditions, 90% of the synthetase and sulfurtransferase activities were sedimented along with 55% of the methylase activity (Fig. 1B). The isoleucyl-tRNA synthetase eluted in a slightly broader peak after pelleting and resuspension, but in essentially the same position as in the S105 (Fig. 1A). Total synthetase activity (15 labeled amino acids) had the same elution profile before and after pelleting, as did sulfurtransferase and methylase activities.

We investigated whether the presence of glycerol in the buffers could cause aggregation, leading to higher apparent molecular weights for these enzymes. Cell extracts were prepared in buffer A without glycerol and chromatographed on Bio-Gel A-5M in the same glycerol-free buffer. Less protein aggregation occurred under these conditions, as the A280 profile indicates (compare Fig. 1 and 2). However, the elution positions of tRNA sulfurtransferase, isoleucyl-tRNA synthetase, and tRNA methylase were essentially the same as with glycerol. It should be noted that sulfurtransferase eluted somewhat earlier than tRNA methylase (Fig. 1).

The effect of various methods of cell disruption and extraction conditions on the size of these complexes has been investigated. It was previously shown that aminoacyl-tRNA synthetases existed as high-molecular-weight com-
suggest that the high-molecular-weight forms of these enzymes shown in Fig. 1 are not the result of aggregation in the low-salt buffer used to extract the cells.

To further investigate the relationship between the synthetase and sulfurtransferase activities, we chromatographed an S105 preparation on DEAE-Sepharcl (Fig. 3). Isoleucyl-tRNA synthetase activity eluted at an NaCl concentration of 0.15 M, whereas the sulfurtransferase was clearly separated from the synthetase at 0.22 M NaCl. It is possible that exposure to a high salt concentration or contact with the chromatographic matrix resulted in dissociation of the complex. To investigate this, the fractions containing synthetase and sulfurtransferase activities were separately pooled, concentrated by Amicon ultrafiltration, and separately chromatographed on Bio-Gel A-5M, as before. Both the synthetase and sulfurtransferase chromatographed at

FIG. 1. Bio-Gel A-5M chromatography of 105,000 × g supernatant (S105) and 160,000 × g pellet (P160) preparations. (A) A 20-ml sample of an S105 preparation was applied to a preequilibrated 1.5-
by 80-cm column, and the proteins were eluted with buffer A. Fractions of 3.0 ml were collected at a flow rate of 6.5 ml/h. Appropriate fractions were assayed for isoleucyl-tRNA synthetase, tRNA methylase, or tRNA sulfurtransferase as described in Materials and Methods. The data are expressed as counts per minute per 0.1 ml of reaction volume in each case, with the latter two activities plotted using the inset scale. (B) An 8-ml sample of the S105 preparation was centrifuged at 160,000 × g for 18 h. The pellet was rinsed and suspended in 2 ml of buffer A and chromatographed on Bio-Gel A-5M as described above. The column was standardized by using the following proteins (described in Materials and Methods): db, dextran blue; β-gal, beta-galactosidase; pk, pyruvate kinase; Hb, hemoglobin. Symbols: ——, A; ○, isoleucyl-tRNA synthetase activity; ■, tRNA sulfurtransferase activity; □, tRNA methylase activity.

FIG. 2. Bio-Gel A-5M chromatography of an S105 preparation from E. coli Q13 without glycerol in buffers during preparation or chromatography. The conditions were as described in the legend to Fig. 1. In this experiment, the data for isoleucyl-tRNA synthetase assays have been divided by 10. Symbols: ——, A; ○, isoleucyl-tRNA synthetase activity; ■, tRNA sulfurtransferase activity; □, tRNA methylase activity.

FIG. 3. DEAE-Sepharcl chromatography of an S105 preparation from E. coli Q13. A 202-ml sample of protein was applied in 28 ml of buffer A to a 2.5- by 17-cm column equilibrated with the same buffer. After a wash with 145 ml of buffer A, a 400-ml linear gradient from buffer A to 0.3 M potassium phosphate (pH 7.5)–1 mM dithiothreitol–10% glycerol was used to elute proteins. Fractions of 6 ml were collected and assayed for synthetase and sulfurtransferase activities as described in Materials and Methods. In this case synthetase activity is expressed as counts per minute per 0.1 ml of reaction mixture, and sulfurtransferase activity is expressed as counts per minute per total reaction mixture. NaCl concentrations were determined by conductivity measurements. Symbols: ——, A; ○, isoleucyl-tRNA synthetase; ■, tRNA sulfurtransferase activity.
FIG. 4. Bio-Gel A-5M chromatography of pooled fractions of isoleucyl-tRNA synthetase and tRNA sulfurtransferase from DEAE-Sephacel. The fractions containing isoleucyl-tRNA synthetase activity (fractions 74 through 96 in Fig. 3) and sulfurtransferase activity (fractions 91 through 106) were separately pooled, concentrated by Amicon UM10 ultrafiltration, and chromatographed separately on Bio-Gel A-5M as described in the legend to Fig. 1. In each case, 4 ml of concentrated material was chromatographed, amounting to 7 and 3.4 mg of synthetase and sulfurtransferase protein, respectively. Symbols: ○, isoleucyl-tRNA synthetase; ●, tRNA sulfurtransferase.

approximately the same high-molecular-weight region as they had before ion-exchange chromatography (Fig. 4). The isoleucyl-tRNA synthetase assays demonstrated multiple forms of this enzyme. The activity in the void volume was probably due to aggregation, whereas the activity at lower molecular weights may have been due to dissociation of the synthetase from the complex. We observed similar behavior when the synthetase complex was chromatographed in the presence of 0.4 M NaCl (13). Under these conditions, 30% of the total synthetase activity was dissociated, whereas 60% of isoleucyl-tRNA synthetase eluted at the position expected for the monomer. Sulfurtransferase activity eluted in a symmetrical peak but four fractions later than observed for the S105 preparation (compare Fig. 4 with Fig. 1). This suggests a reduction in molecular weight for the sulfurtransferase as a consequence of the above-described treatments. We also observed an 80% loss of activity after the DEAE-Sephacel step, suggesting that dissociation or removal of a stabilizing factor had occurred.

The above data show that one sulfurtransferase peak was observed on both gel and ion-exchange matrices. However, four thionucleosides are known to exist in E. coli tRNA (7), each presumably synthesized by a separate sulfurtransferase (5). To determine which enzymes we observed here, [35S]tRNA was isolated from reaction mixtures by using the S105 preparation as the source of sulfurtransferase. The labeled tRNA was hydrolyzed to the nucleoside level and analyzed on Bio-Gel P2 by the method of Rao and Cheryayl (21). The thionucleoside profiles of [35S]tRNA digests labeled in vivo or in vitro are given in Fig. 5. All four thionucleosides were seen with in vivo [35S]tRNA digests (Fig. 5A): s4U, mmms2U, s2C, and ms2s6A. Peak 1 represented non-nucleoside sulfur (did not bind to Affi-Gel 601, a boronate gel) and could be cysteine (which elutes at 78 ml) or a derivative of this amino acid. Radioactivity was seen at this position for tRNA labeled in vitro also, despite phenol extraction and several ethanol precipitations. With in vitro-labeled [35S]tRNA digests the only detected thionucleoside was s4U. Measurements of the entire 3-ml fraction in regions of the chromatogram where the other thionucleosides were expected to elute gave only background radioactivity. A small amount of sulfurtransferase activity pelleted with the ribosomes but was removed by suspension and recentrifugation of the ribosomal pellet. Analysis of tRNA digests labeled with the ribosomal sulfurtransferase also showed that s4U was the only labeled thionucleoside (data not shown). Hence, we concluded that this enzyme was merely occluded in the ribosomal pellet. We have not been able to locate the other sulfurtransferase activities with sonicated extracts, because the crude extract also had only s4U sulfurtransferase activity.

FIG. 5. Bio-Gel P2 chromatography of nucleoside digests of in vivo- and in vitro-labeled [35S]tRNA. Digests were prepared and chromatographed on Bio-Gel P2 as described in Materials and Methods. The identity of in vivo-labeled thionucleosides is based on previous reports (21) or on known standards (cysteine and s4U). All [35S]peaks except peak 1 bound to Affi-Gel 601 and were judged to represent nucleoside sulfur. Recoveries were 75% with the following amounts applied to Bio-Gel P2: in vivo, 10 x cpn; in vitro, 269,900 cpn.

DISCUSSION

The gel filtration behavior of aminoacyl-tRNA synthetases and tRNA modification enzymes in E. coli was investigated. Despite past evidence to the contrary (19), it was found that aminoacyl-tRNA synthetases were organized in high-molecular-weight complexes in these cells (13). In the current study, it was shown that isoleucyl-tRNA synthetase, with a reported molecular weight of 114,000 (23), elutes from Bio-Gel A-5M columns at a volume expected for a protein with a molecular weight of 400,000. tRNA modification enzymes also appear to be associated in higher-molecular-weight complexes, whose compositions are yet undefined.

It is possible that aggregation is responsible for the occurrence of high-molecular-weight forms of these enzymes. I had previously reported that the aminoacyl-tRNA synthetases were present as apparent complexes when prepared with or without 10% glycerol in buffers, after DNase treat-
ment, or in the presence of 0.15 M NaCl (13). In addition, there was little change in the elution position of tRNA sulfurtransferase under these same conditions. As mentioned above, treatment with 0.4 M NaCl did result in partial dissociation of the synthetase complex and a small drop in the molecular weight of the sulfurtransferase. Hence, electrostatic forces may be involved in formation of these complexes. On the other hand, the complexes do not appear to be formed as a result of aggregation due to the use of low salt concentrations in extraction buffers.

We found that tRNA methyltransferases, a group of enzymes whose reported molecular weights range from 38,000 to 80,000 (5, 12), eluted at a molecular weight of 300,000 on Bio-Gel A-5M. Indeed, tRNA methylase activity was not observed at the position expected for the monomeric enzymes (Fig. 1 and 2). Methylase activity did not elute coincident with the bulk of isoleucyl-tRNA synthetase and sulfurtransferase activities. However, since some synthetase activity was seen in fractions containing methylase activity, such an association cannot be completely ruled out. The finding that tRNA methylase activity elutes as a higher-molecular-weight entity than purified enzymes is in agreement with the work of Hagervall et al., who observed a high-molecular-weight form of mmn<sup>3</sup>U methylase (12). They showed that the high-molecular-weight form of the enzyme was partially converted to the 80,000-molecular-weight active monomer during rechromatography on Ultragel AcA34, suggesting that the high-molecular-weight form of that enzyme is labile.

Both ultracentrifugation and agarose gel chromatography experiments support the conclusion that isoleucyl-tRNA synthetase and sulfurtransferase are associated with one another. However, these enzymes are completely resolved during gradient elution from DEAE-Sepharacel. After ion-exchange chromatography, isoleucyl-tRNA synthetase eluted at its original elution position on Bio-Gel A-5M. tRNA sulfurtransferase now eluted at 300,000 daltons, a drop in size of 100,000 daltons, accompanied by a dramatic loss of activity. In other experiments, a similar decrease in size and activity after precipitation with ammonium sulfate was observed (Harris, unpublished observations). One interpretation of these results is that isoleucyl-tRNA synthetase and tRNA sulfurtransferase are present in complexes that are separate but of similar sizes. Alternatively, ion-exchange chromatography could have disrupted these two enzymes from a complex, followed by subsequent formation of nonspecific aggregates similar in size to the original complex.

The above-described results also suggest that the sulfurtransferase is stabilized in crude extracts, possibly by association with other factors. The stabilizing factor(s) is removed by ion-exchange chromatography or ammonium sulfate treatment, suggesting that electrostatic forces are important in maintaining the active form of this enzyme. It is also possible that the holoenzyme is composed of several active subunits of active but separately unstable monomers. Similar observations were made by Abrell et al. (1), who found that the majority of the s<sup>4</sup>U sulfurtransferase activity was too unstable to further purify after ammonium sulfate fractionation. I previously reported that the size of the synthetase complex was increased when cells were disrupted by glass-bead homogenization or freeze fracture (13). In contrast, tRNA sulfurtransferase was not altered in size in these preparations (Harris, unpublished results). I also characterized the [<sup>35</sup>S]tRNA reaction product to see which sulfurtransferase(s) was detected. Thionucleoside analysis showed only one major product, 4-thiouridine. Hence, I can only conclude that the s<sup>4</sup>U sulfurtransferase exists in a high-molecular-weight form in E. coli.

These findings suggest that aminoacyl-tRNA synthetases and tRNA modification enzymes do not simply exist as monomeric enzymes in the bacterial cytoplasm. The synthetases appear to be associated with each other in complexes of moderate size, perhaps composed of five or six members. In freeze-fractured E. coli extracts, complexes as large as 1 megadalton were observed (13), suggesting that even larger multienzyme complexes may exist. The physiological significance of the association of aminoacyl-tRNA synthetases in high-molecular-weight complexes is not known at present. It is possible that the activities of several synthetases could be regulated together through a physical association. In eukaryotes, entry of extracellular amino acids may occur directly to groups of synthetases associated with the cell membrane (10). Extracellular amino acids are shunted directly to aminoacyl-tRNA, rather than mixing with intracellular substrates. The eucaryotic synthetase complex may be associated with membranes in the endoplasmic reticulum, suggesting a role in directing newly formed aminoacyl-tRNA to regions of the cell that are actively making proteins. In this regard, previous reports of tRNA modification enzymes with these complexes (2, 15, 23) leads to speculation that final steps in tRNA modification might occur just before aminoacylation. Although speculative at present, the bacterial aminoacyl-tRNA synthetases may be organized in a larger protein synthesis apparatus inside the cell. This putative apparatus is disrupted when the cell is broken, dissociated into a group of smaller complexes. In this regard, I show here that two classes of tRNA modification enzymes may also be associated in high-molecular-weight complexes in the bacterial cell, although not with each other. It is not known whether the members of these complexes are other tRNA modification enzymes, or proteins with different functions. These complexes should be purified and further characterized to learn which other cellular components exist in these complexes and to determine the physiological role for this association.

ACKNOWLEDGMENTS

I thank James Blair, Kent Vrana, and George Wirtz for their suggestions and Carol Molisee for typing the manuscript. This work was supported by Public Health Service grant GM-32807 from the National Institute of General Medical Sciences.

LITERATURE CITED


