Evidence That the Folate-Requiring Enzymes of de Novo Purine Biosynthesis Are Encoded by Individual mRNAs†

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ABSTRACT: Isolation of the mRNAs encoding for the three folate-requiring enzymes involved in de novo purine biosynthesis followed by their in vitro translation resulted in three separate proteins electrophoretically identical with those previously isolated. The three enzymes are glycinamide ribonucleotide transformylase, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, and 5,10-methenyl-, 5,10-methylene-, and 10-formyltetrahydrofolate synthetase. Thus these enzymes do not appear to be derived from large multifunctional proteins that are then subject to proteolysis in vivo or during in vitro purification. The levels of these enzymatic activities were increased by approximately 2-fold after raising the concentration of protein in the chicken's diet. The observed response is similar to that noted for glutamine phosphoribosylpyrophosphate amidotransferase, the presumed rate-limiting enzymatic activity for this pathway. For 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and the trifunctional synthetase but not glycinamide ribonucleotide transformylase the increase in enzymatic activity correlates with higher mRNA levels.

Glycinamide ribonucleotide transformylase (GAR TFase, EC 2.1.2.2)[1] and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR TFase, EC 2.1.2.3) are the two transformylases involved in purine biosynthesis de novo. The reduced folate cofactor required by these enzymes is supplied by 5,10-methenyl-, 5,10-methylene-, and 10-formyltetrahydrofolate synthetase (combined) (trifunctional protein, EC 3.5.4.9, EC 1.5.1.5, and EC 6.3.4.3). These enzymes have been individually purified from chicken liver (Young et al., 1984; Smith et al., 1980; Mueller & Benkovic, 1981) but under certain conditions all will copurify through several steps of the procedure, including affinity chromatography.

Since the transformylases and the trifunctional protein are functionally related, other types of association between them have been investigated. Physical association between the trifunctional protein and proteolyzed GAR TFase has been observed (Smith et al., 1980). That observation, coupled with our recent finding that GAR TFase is rapidly cleaved into two fragments of similar molecular weight that retain the specific activity of the parent enzyme (Young et al., 1984), led us to search for enzymes that might combine transformylase and synthetase activities, using protocols unaffected by proteolysis. In addition, Henikoff et al. (1983) have reported that in *Drosophila* GAR TFase is part of a larger polypeptide domain. In order to avoid posttranslational modifications, we have isolated and partially characterized the messenger RNAs coding for these proteins and translated them in vitro. In the course of these experiments we discovered incidentally that the levels of these enzyme activities could be influenced by dietary manipulations and have made preliminary observations relating mRNA and protein levels for these three enzymes.

Experimental Procedures

Materials

Methylmercury hydroxide (1 M in H_2O) was purchased from Alfa, Danvers, MA. SDS, sucrose (RNase free), and agarose (standard low molecular weight) were obtained from Bio-Rad, Richmond, CA. Human placental RNase inhibitor, *Escherichia coli* MRE-600 ribosomal RNA, λ DNA cleaved by *Hin*dIII, and phage MS-2 RNA are products of Boehringer-Mannheim, Indianapolis, IN. Tobacco mosaic virus RNA and NCS tissue solubilizer were from Amersham, Arlington Heights, IL. IgGsorb is a product of The Enzyme Center, Inc., Boston, MA. Translation cocktail, rabbit reticulocyte lysate, L-[^{35}S]methionine, [^{14}C]phosphorylase B, [^{14}C]globulins, and ^{125}I-labeled protein A (9.2 μCi/μg) were purchased from New England Nuclear, Boston, MA. PPO, POPOP, and TCA were from Fisher Scientific Co., Fairlawn, NJ. Acridine orange and 40% glyoxal are products of Aldrich Chemical Co., Milwaukee, WI. Kodak XAR-5 film was obtained from Picker International, Highland Heights, OH. Aminothiophenol paper was prepared by the method of Seed (1982). Bovine liver tRNA, ribonuclease A, phenylmethanesulfonyl fluoride, Nonidet P-40, sodium deoxycholate, 2-mercaptoethanol, amino acids, grade V chicken egg albumin type II-O ovomucoid, α-1-antitrypsin, aprotinin, and pepstatin were purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Protein Purification and Antiserum Production. AICAR TFase was purified by the procedure of Mueller & Benkovic (1981). Trifunctional protein was purified by the procedure of Smith et al. (1980). GAR TFase was purified by the procedure of Young et al. (1984).

For trifunctional protein and GAR TFase additional purification was achieved by preparative SDS gel electrophoresis of 1 mg/mL samples on a 12 × 13 × 0.3 cm slab gel following the procedure of Laemmli (1970). After the protein bands were visualized by 5–10 min of staining (Weber et al., 1972), the appropriate sections of the gel were removed and the

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[1] Abbreviations: SDS, sodium dodecyl sulfate; GAR TFase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosylglycinamide transformylase; AICAR TFase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosyl-5-aminoimidazole-4-carboxamide transformylase; trifunctional protein, 5,10-methenyltetrahydrofolate, 5,10-methylenetetrahydrofolate, and 10-formyltetrahydrofolate synthetase (combined); H_4folate, tetrahydrofolate; TCA, trichloroacetic acid; S, Svedberg unit; PPO, 2,5-diphenyloxazole; Me_2POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; EDTA, ethylenediaminetetraacetic acid; CH_3HgOH, methylmercury hydroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; poly(A), polyadenylate; poly(U), polyuridylate.

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proteins were eluted from the polyacrylamide by agitation in 0.2% SDS overnight. The SDS/protein solution was concentrated by lyophilization and used without further treatment for preparation of antisera.

Antisera were prepared in rabbits from SDS-treated trifunctional protein and GAR TFase and also from purified AICAR TFase with complete Freund's adjuvant by the method of Crowle (1973).

^{14}C Labeling of Proteins. AICAR TFase, trifunctional protein, GAR TFase, and bovine serum albumin were labeled with [^{14}C]formaldehyde according to the procedure of Dottavio-Martin & Ravel (1978). The dialysis step was replaced by the centrifuge desalting technique of White (1979).

Tissue Preparation. One-day-old chicks were placed on either a high-protein or low-protein diet. The high-protein diet was the same as the carbohydrate-free diet of Evans & Scholz (1971) containing 850 g of isolated soybean protein/kg of feed, whereas the low-protein diet was a modification of the control diet of Evans & Scholz (1971) in which isolated soybean protein was reduced to 200 g/kg, DL-methionine was 4.0 g/kg, and glycine was 638 g/kg. After 15 days on their respective diets, the chickens were sacrificed and their livers were quickly frozen in liquid nitrogen.

Measurement of Enzyme Levels. Samples of the frozen chicken livers were homogenized in 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 0.25 M sucrose containing 250 mg of type II-O ovomucoid, 25 mg of α-1-antitrypsin, 2.5 mL of aprotinin, and 15 mg of pepstatin per liter in a glass Teflon homogenizer according to the procedure of Mueller & Benkovic (1981). The homogenization was done by employing 10 mL of buffer/g of tissue. The 100000*g* supernatants were used without further purification for the enzyme assays and gel electrophoresis. AICAR TFase was assayed by the procedure of Mueller & Benkovic (1981). GAR TFase was assayed by the procedure of Smith et al. (1981) with 75 μM 10-formyl-5,8-dideazafolate as the cofactor. 10-Formyl-H_4folate synthetase was assayed by the procedure of Caperelli et al. (1980). Protein was measured by using the Bio-Rad protein assay kit, with ovalbumin as a standard.

The quantity of the enzymes (in milligrams) in the supernatants was estimated from comparison of the densitometer scans of SDS gel electrophoresed (Laemmli, 1970) samples after staining with Coomassie blue (Weber et al., 1972). Purified protein controls were used to calibrate the densitometer scans. Integration of the densitometer scans was accomplished by weighing the excised peaks.

Measurement of Enzyme Levels by Western Transfer. The 100000*g* supernatants from livers obtained from chickens fed either high- or low-protein diets were electrophoresed in an 8.75% acrylamide gel according to the SDS procedure of Neville (1971). Purified proteins were run in adjacent lanes to serve as controls. When the bromphenol blue tracking dye approached the bottom of the gel, the gel was removed from the apparatus and washed 3 times with 15 mM sodium phosphate, pH 6.5, for 5 min each. The proteins in the gel were then transferred to diazotized aminothiophenol paper for 1 h at 2 A, 20 °C, in 15 mM sodium phosphate (Reiser & Stark, 1983). The transfer of proteins less than 150000 daltons was judged to be quantitative by Coomassie blue staining (Weber et al., 1972).

The rest of the procedure was the same as described by Young et al. (1984). After transfer, the diazotized paper was deactivated by treatment with a solution of 0.1 M Tris·HCl, pH 9.0, containing 0.25% gelatin overnight. The paper was treated with 80 μL of each antiserum (against GAR TFase, trifunctional protein, or AICAR TFase) in 5 mL of antibody buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris·HCl, pH 7.4, 0.25% gelatin, 0.05% NP-40) for 1 h and then washed with several changes of antibody buffer for 4 h at room temperature.

The paper was then treated with 2×10^6 cpm of ^{125}I-labeled protein A in 5 mL of antibody buffer for 1 h at room temperature and then washed for 1 h with several changes of 0.65 M NaCl, 5 mM EDTA, 50 mM Tris·HCl, pH 7.4, and 0.4% *N*-lauroylsarcosine at room temperature. Autoradiography was done with a maximum exposure time of 10 h at −70 °C with Du Pont Cronex Lighting-Plus intensifier screens.

Immunotitration of Trifunctional Protein. Preimmune serum and antiserum to trifunctional protein were partially purified by 45% ammonium sulfate precipitation followed by three additional precipitations at 40% saturation. The final precipitate was dissolved in one-third the original serum volume in 10 mM sodium phosphate and 150 mM NaCl, pH 7.2. Lipoproteins were removed by extensive dialysis of the solution at 4 °C against four alternating changes of H_2O and 50 mM sodium acetate, pH 5, followed by centrifugation at 10000 rpm and 4 °C for 10 min. The final supernatant was dialyzed against 0.9% NaCl and 0.02% sodium azide at 4 °C and stored at −80 °C. The concentrations of protein in the final solutions were quantitated by the Bio-Rad protein assay.

Immunotitrations were carried out on liver homogenates isolated from chickens fed a high- or low-protein diet and on purified trifunctional protein (Smith et al., 1980). Protein concentrations were determined by either the Bio-Rad protein assay or a UV–biuret assay (Zamenhof, 1957). Titrations were performed by incubating a constant amount of trifunctional protein activity units with increasing amounts of preimmune serum or antiserum in a total volume of 100 μL of 100 mM sodium phosphate, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 150 mM 2-mercaptoethanol, pH 7.2. Solutions were maintained at 4 °C for 72–96 h and then centrifuged for 30 min at 150000 rpm and 4 °C.

Supernatants were assayed for the 10-formyl-H_4folate synthetase activity of the remaining trifunctional protein by using the spectrophotometric assay procedure of Smith et al. (1980). Appropriate controls were carried out to ensure that none of the components of the immunotitration mixture contributed to the absorbance in the enzyme activity assay. Data were plotted as units of activity remaining in the supernatant against milligrams of antiserum added.

Preparation of Total RNA and Poly(A)-Containing RNA. Total RNA was extracted by the method of Chirgwin et al. (1979) as modified by Ricca et al. (1981) from the frozen livers obtained from chickens fed on either the high- or low-protein diet. Poly(A)-containing RNA was extracted from total RNA by two passages through a poly(U)–Sepharose column (Ricca et al., 1981). Poly(A)-containing RNA was heated at 65 °C for 10 min, quickly cooled to 25 °C, and sedimented through a 5–29.9% isokinetic sucrose gradient (McCarty et al., 1974) containing 1% SDS, 25 mM HEPES (pH 7.4), and 5 mM EDTA in a Beckman SW41 rotor at 27000 rpm for 10 h at 20 °C. The gradients were collected in 0.45-mL fractions, and the RNA was precipitated twice with ethanol. The RNA samples were redissolved in 5 mM HEPES (pH 7.4) containing 0.1 mM EDTA and were used for subsequent translation analysis.

Cell-Free Translation. Poly(A)-containing RNA was pretreated with 10 mM methylmercury hydroxide according to the procedure of Payvar & Schimke (1979). The methylmercury hydroxide concentration in the translation mixture

Table I: Enzyme Activity and Concentration in Chicken Liver Supernatants

	(a) ratio of enzymatic activity[a]			(b) ratio of enzyme concentration[b]		
	10-formyl-H_4-folate synthetase	AICAR TFase	GAR TFase	trifunctional protein	AICAR TFase	GAR TFase
high- vs. low-protein diet	2.6 ± 0.7	2.4 ± 0.6	2.7 ± 0.7	2.9 ± 0.6	2.2 ± 0.5	5.3 ± 1.3

[a] Average values from two independent experiments from 10 liver samples per diet per experiment. [b] Values normalized per milligram of total protein in the sample.

was diluted to 0.25 mM to prevent inhibition of the translation reaction. Pretreated RNA was translated according to the procedure of Pelham & Jackson (1976). Total poly(A)-containing RNA was used at a concentration of 10 μg/mL in the assay while gradient fractionated poly(A)-containing RNA was at 2 μg/mL. These concentrations proved to be optimal for translation of GAR TFase, trifunctional protein, and AICAR TFase. Translation assays (37.5 μL) contained 80 mM potassium acetate, 1 mM magnesium acetate, 0.03 mM amino acid except methionine, 50 μg/mL bovine liver tRNA, 1 mCi/mL L-[^{35}S]methionine (1175 Ci/mmol), 3 μL of translation cocktail, and 15 μL of rabbit reticulocyte lysate. After incubation for 60 min at 37 °C, assays were quenched with 11 μL of a mixture containing 84 μg/mL ribonuclease A, 44 mM methionine, 4.4 mM phenylmethanesulfonyl fluoride, and 0.11 M EDTA. Quenched solutions were incubated at 37 °C for 15 min and then centrifuged at 15 000 rpm for 10 min in an Eppendorf Model 5414 centrifuge.

Duplicate samples (1 μL) were removed to assay total protein synthesis by measuring the radioactivity incorporated into the TCA precipitate. In this assay samples were spotted on 1-cm^2 Whatman 3MM paper, boiled 10 min in 10% trichloroacetic acid, and washed with water, ethanol, and acetone. After the paper was dried, squares were placed in 20-mL scintillation vials, incubated 30 min at 55 °C in 0.5 mL of NCS tissue solubilizer, and then counted for radioactivity in 10 mL of a toluene-based scintillation cocktail (4 g of PPO + 0.1 g of POPOP per 1000 mL of toluene).

The ability of the translation assay mixture to synthesize high molecular weight proteins was demonstrated with 0.12 μg/mL tobacco mosaic virus RNA. Human placental ribonuclease inhibitor (10 μg/mL) had no effect on the translation of chicken liver poly(A)-containing RNA (Scheele & Blackburn, 1979).

Identification of Translation Products. Translation products from the above procedure were immunoprecipitated with 2 μL of antiserum, followed by treatment with 100 μL of 10% (w/v) IgGsorb as described by Kessler (1976) with some modifications. Initial incubations of antigen and antiserum were performed at 37 °C for 30 min followed by 4 °C overnight in the immunoprecipitation buffer containing 1% NP-40, 1 mM phenylmethanesulfonyl fluoride, 5 mM leucine, 100 mM NaCl, 50 mM LiCl, 0.1% SDS, and 1% sodium deoxycholate. The protein A bound complex was washed 4 times with 300 μL of immunoprecipitation buffer, and the antigen was solubilized by resuspension of the pellet in 50 μL of 0.125 M Tris·HCl, pH 6.8, 20% glycerol (v/v), 6% SDS (w/v), and 10% 2-mercaptoethanol (v/v), followed by heating at 95 °C for 5 min. All centrifugations were for 5 min at 15 000 rpm.

SDS gel electrophoresis of [^{35}S]methionine-labeled translation products and their immunoprecipitates was performed according to Laemmli (1970) on 8.75% polyacrylamide gels. Radioactive bands were detected by fluorography (Ricca et al., 1981) using Kodak XAR-5 film. In some cases the translation mixture was pretreated with IgGsorb (Su et al., 1981), followed by precipitation with specific immune serum in order to lower extraneous background.

Analysis of Poly(A)-Containing RNA on Agarose Gels. Nucleic acid samples were concentrated to dryness in 1.5-mL polypropylene micro test tubes by using a Savant vacuum concentrator. RNA samples were denatured with glyoxal followed by electrophoresis at 100 V for 4 h in a 1% agarose gel using a horizontal slab gel apparatus (McMaster & Carmichael, 1977). After the gel was stained with acridine orange, the gel was destained for 24 h and then photographed under UV light with Polaroid type 667 film; a red filter was used. A standard curve was established by use of the following RNA and DNA molecular weight markers: phage MS-2 RNA, *E. coli* MRE-600 ribosomal RNA, and *Hin*dIII-treated λ DNA. Prior to electrophoresis, DNA samples were denatured with Me_2SO and glyoxal as described by Liao et al. (1981).

Results

Production of Antiserum. With trifunctional protein and GAR TFase, the SDS-treated proteins were sufficiently immunogenic for the production of antisera in rabbits. The same procedure was tried twice with AICAR TFase with no success. Other modifications of the protein such as cross-linking with glyoxal (Reichlin, 1980) or performic acid oxidation (Van Eldik & Watterson, 1981) also led to species that were apparently not immunogenic in rabbits. It was only when native AICAR TFase was used that satisfactory antiserum was produced.

Diet Selection. In order to simplify isolation and characterization of the mRNAs coding for the two transformylases and the trifunctional protein, an investigation was conducted to determine conditions that might result in increased concentrations of their respective mRNAs. The assay procedure was initially to determine the activity of a given enzyme in a crude cytosol fraction of the liver, on the basis of the assumption that higher levels of the enzyme would be associated with an increased concentration of mRNA. Chickens use purine biosynthesis and purine degradation to excrete ammonia formed by catabolism of amino acids (Henderson, 1982). Two enzymes of purine biosynthesis, glutamine synthetase and glutamine phosphoribosyl pyrophosphate amidotransferase, are reported to be elevated in response to a high-protein diet (Katunuma et al., 1970) as is the last enzyme catalyzing purine degradation to uric acid, xanthine dehydrogenase (Evans & Scholz, 1973). Consequently, dietary protein levels were manipulated in order to alter the enzymatic levels of GAR TFase, trifunctional protein, and AICAR TFase.

The measurements of relative enzyme activities in the supernatants are summarized in Table Ia. The same supernatants were used for SDS gel electrophoresis, with the results of a typical gel shown in Figure 1. Densitometer scans of the gels were used to compare the concentrations of protein present as AICAR TFase, GAR TFase, and trifunctional protein in the livers of chickens raised on both types of diets. The results are expressed as a ratio of the two concentrations (Table Ib). This method of analysis presumes that (i) Coomassie blue staining of a given protein is linear with concentration (an accepted practice) and (ii) each band represents only a single

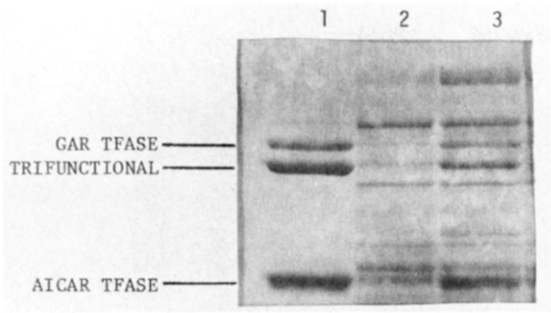

FIGURE 1: SDS–polyacrylamide gel electrophoresis of liver supernatants from chickens fed low- and high-protein diets after staining with Coomassie blue. Lane 1 is purified protein controls as indicated. The following amounts were loaded on the gel: GAR TFase, 1.5 μg; trifunctional protein, 3.0 μg; AICAR TFase, 5.0 μg. Lane 2 is the supernatant from livers of chickens fed the low-protein diet. Total protein loaded on the gel was 100 μg. Lane 3 is the supernatant from the livers of chickens fed the high-protein diet. Total protein loaded on the gel was 162 μg.

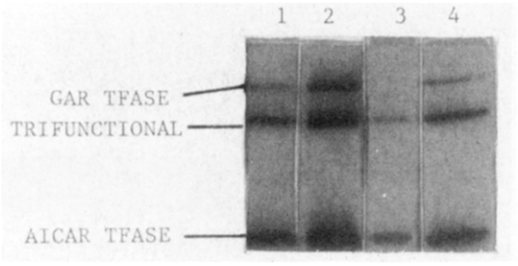

FIGURE 2: Western transfer analysis of 100000*g* liver supernatants from chickens fed with low- or high-protein diets. Equal amounts of liver supernatants (39 μg of protein or 12 μg of protein) from chickens fed with low- (lane 1 or 3) or high- (lane 2 or 4) protein diets were analyzed by SDS–polyacrylamide gel electrophoresis followed by electrophoretic transfer and analysis as described under Methods.

protein. Western transfer analysis of the same gels (Figure 2) revealed protein levels that qualitatively paralleled those from the densitometer scans. Further justification for assumption ii is obtained by calculating the specific activities for all three enzymes (the synthetase assay was used to measure the trifunctional protein level), which are within 20% of the specific activities of the purified proteins.

Immunotitration of Trifunctional Protein. In order to confirm independently that the measured increase in activity units of the trifunctional protein (Table I) was due to an elevated protein level instead of a change in the specific activity, both types of liver homogenates were titrated with trifunctional protein antiserum. As illustrated in Figure 3, the synthetase activity in both supernatants titrated with the same amount of antiserum and was equivalent to 5.89×10^{-2} unit of activity/mg of antiserum (1 unit = 1 μmol/min). In a separate experiment, when purified trifunctional protein was titrated, 6.09×10^{-2} unit was inactivated per milligram of antiserum. Since the same amount of antibody titrated identical units of enzyme activity from both types of liver homogenates, the increase in activity units in the high-protein liver results from an increased protein level and not a change in the specific activity of the trifunctional protein.

Translation of Total Chick Liver Poly(A)-Containing RNA. Despite our expectations of having measurable quantities of mRNA isolated from the livers of chickens fed a high-protein diet all initial attempts to detect in vitro translation products with AICAR TFase antisera failed. Although addition of chicken liver poly(A)-containing RNA stimulated in vitro protein synthesis, when the products were analyzed on SDS–

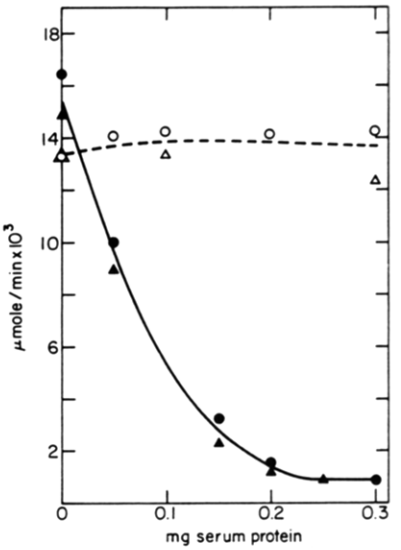

FIGURE 3: Immunotitration of 10-formyl-H_4folate synthetase activity in chicken liver homogenates. A fixed amount of synthetase activity was titrated with 0–0.3 mg of partially purified immune serum as described under Experimental Procedures. The solid line represents the activity remaining in the titration supernatant of liver homogenates from chickens fed high- (▲) or low- (●) protein diets after treatment with trifunctional protein antibody. The dotted line shows the results of sample treatment with preimmune serum (high-protein diet, △; low-protein diet, ○).

polyacrylamide gels the translation mixtures contained numerous labeled products having molecular weights up to about 67 000 and proportionally very few products larger than this. No proteins comigrating with [^{14}C]GAR TFase, ^{14}C-labeled trifunctional protein, or [^{14}C]AICAR TFase were present in the immunoprecipitates of the translation reaction. Translations performed without added poly(A)-containing RNA produced a single protein of approximately 48 000 daltons that was not immunoreactive with any of the antisera.

The translation of mRNAs for GAR TFase, trifunctional protein, and AICAR TFase in the total poly(A)-containing RNA fraction was realized when the RNA was pretreated with CH_3HgOH prior to translation and tRNA plus amino acids were added to the translation mixture. Equal amounts of poly(A)-containing RNA from the livers of chickens fed either a low- or a high-protein diet were translated and immunoprecipitated as described above. The fluorograph of the immunoprecipitates after SDS gel electrophoresis is shown in Figure 4.

The abundant product at 67 000 daltons is serum albumin whose mRNA constitutes a major portion of the total poly(A)-containing RNA fraction. The products that precipitated with GAR TFase, trifunctional protein, and AICAR TFase antisera in each case contained a protein that comigrated with the purified ^{14}C-labeled protein. When they were compared to the preimmune serum, none of the immune sera reacted with any unique products having a molecular weight greater than the purified standards. In addition, the preimmune serum did not react with any proteins comigrating with the ^{14}C-labeled standards except in the case of AICAR TFase. However, since a constant background was noted in all lanes, it was subtracted in the calculation of band intensities. Densitometer scans of the fluorographs provided ratios of the band intensities for GAR TFase, AICAR TFase, and the trifunctional enzyme for the two diet types (Table II) that represent the ratio of the specified mRNA for the two conditions.

Determination of Molecular Weight of GAR TFase, Trifunctional Protein, and AICAR TFase mRNAs. The fractions

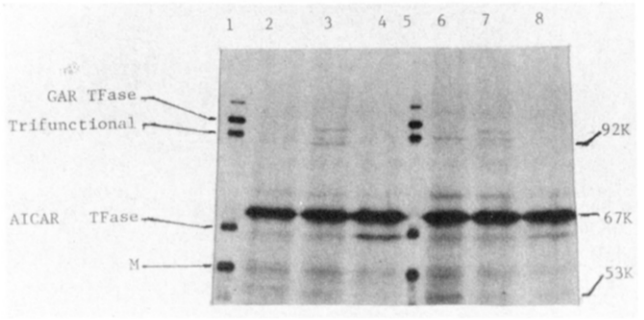

FIGURE 4: Fluorograph of [^{35}S]methionine-labeled in vitro translation products. Total poly(A)-containing RNA from livers obtained from chickens fed a high- or low-protein diet was purified, pretreated with CH_3HgOH, and translated as described under Methods. Total TCA-precipitable material (5×10^5) was reacted with immune serum and the immunoprecipitation analyzed by SDS–polyacrylamide gel electrophoresis. The gel was exposed for 3 days. Lanes 1 and 5 show the migration of ^{14}C-labeled GAR TFase, trifunctional protein, AICAR TFase, and a marker protein M. Lanes 2–4 show the results of immunoprecipitation of the proteins translated by poly(A)-containing RNA derived from livers of chickens fed the high-protein diet. Lanes 6–8 show the results of immunoprecipitation of the proteins translated by poly(A)-containing RNA derived from livers of chickens fed the low-protein diet. Lanes 2 and 6 are immunoprecipitations with preimmune serum. Lanes 3 and 7 are immunoprecipitations with a mixture of GAR TFase and trifunctional protein antisera. Lanes 4 and 8 are immunoprecipitations with AICAR TFase antiserum. Molecular weight markers, as indicated, are phosphorylase B (M_r 92000), bovine serum albumin (M_r 67000), and globulins (M_r 53000).

Table II: Ratio of Proteins Produced by Translation of High- and Low-Protein Diet Messenger RNAs[a]

protein	ratio of densitometer peak areas[b]
GAR TFase	1.1
trifunctional protein	3.8
AICAR TFase	2.4

[a] Fluorograph used is shown in Figure 4. [b] High protein/low protein. Integration of densitometer peak area was done by weighing the peaks.

of the sucrose density gradient containing mRNAs for GAR TFase, trifunctional protein, and AICAR TFase were determined by translation of a small portion of each fraction. Analysis of the immunoprecipitates by gel electrophoresis (Figure 5) revealed that fraction 14 was enhanced in the mRNA coding for GAR TFase and the trifunctional protein, whereas fraction 16 contained most of the AICAR TFase mRNA. The molecular weight range of the RNA contained in these fractions was determined by their mobilities on 1% agarose gels vs. known molecular weight DNA and RNA standards. The agarose gel in Figure 6 illustrates the decrease in molecular weight of RNA between fraction 14 and fraction 16 of the sucrose density gradient. According to the molecular weight values given on the side of Figure 6, the maximum size of mRNA in fractions 16 and 14 is 1.07×10^6 and 1.43×10^6 daltons, respectively. The average size is 7×10^5 and 1×10^6 daltons, respectively. As calculated in Table III, the maximum size protein that could be encoded by mRNAs of these sizes is at most 1.5 times the size of the proteins found in the respective fractions.

Discussion

There are several recent examples where folate-requiring enzymes have been isolated that combine several activities on a single protein including the avian trifunctional enzyme of this study, the porcine formiminoglutamate transferase:formiminotetrahydrofolate cyclodeaminase (Drury et al., 1975), and the dihydrofolate reductase:thymidylate synthetase (Ferone & Roland, 1980), to cite a partial listing. Moreover,

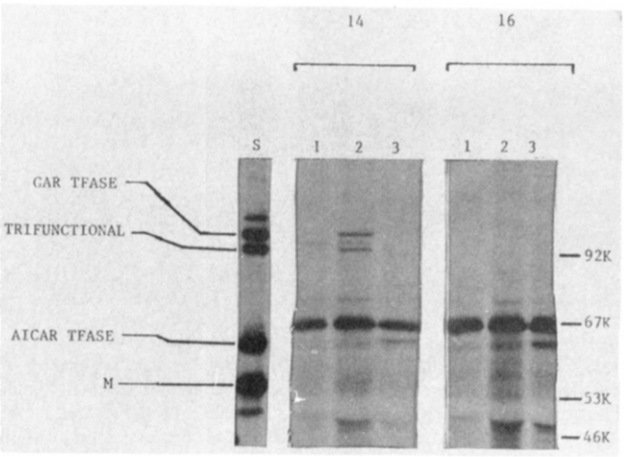

FIGURE 5: Fluorograph showing in vitro translation products from sucrose density fractions of poly(A)-containing RNA. Equivalent amounts of RNA from each fraction were translated as described under Methods. A total of 2×10^5 cpm of TCA-precipitable material was reacted with immune serum, and the immunoprecipitates were analyzed by SDS gel electrophoresis. The gel was exposed for 96 h. Molecular weight markers, as indicated, are phosphorylase B (M_r 92000), bovine serum albumin (M_r 67000), globulins (M_r 53000), and ovalbumin (M_r 46000). Lane S shows the migration of [^{14}C]GAR TFase, trifunctional protein, AICAR TFase, and protein M standards. The uppermost numbers are the fraction numbers of the sucrose density gradient. For both fractions 14 and 16, lanes 1–3 represent the translation products after immunoprecipitation with the following antisera: (1) preimmune serum, (2) a mixture of GAR TFase and trifunctional protein antisera, and (3) AICAR TFase antiserum.

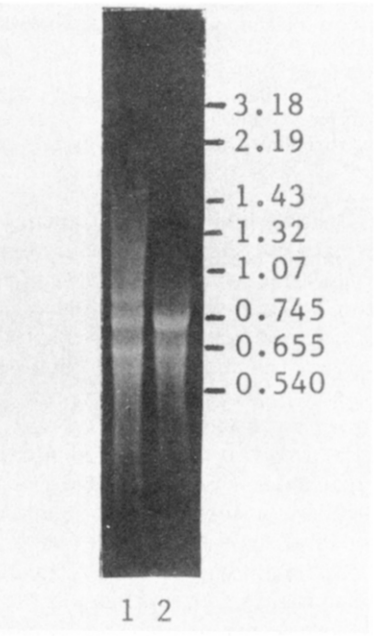

FIGURE 6: Electrophoresis of glyoxal-denatured poly(A)-containing RNA sucrose density gradient fractions in a 1% agarose slab according to a procedure from McMaster & Carmichael (1977) with some modifications as described in the text. Lane 1 is 5 μg of RNA from gradient fraction 14. Lane 2 is 5 μg of RNA from gradient fraction 16. The numbers at the side indicate the size of molecular weight markers in megadaltons. The molecular weight markers are *E. coli* MRE-600 ribosomal RNA, phage MS-2 RNA, and λ DNA that had been cut with *Hin*dIII restriction enzyme.

in eukaryotes a single mRNA that codes for several enzyme activities is usually associated with a multifunctional protein, such as the one catalyzing UMP biosynthesis (Padgett et al., 1979).

The results presented here show directly that AICAR TFase is coded by a mRNA that is independent of trifunctional protein or GAR TFase since its mRNA is separable from the

Table III: Comparison of Protein Subunit Molecular Weights to Coding Capacity of mRNA Fractions

	subunit M_r ($\times 10^{-3}$)[a]	peak fraction for mRNA of protein[b]	max M_r of RNA in fraction ($\times 10^{-6}$)[c]	max coding capacity of fraction M_r protein ($\times 10^{-3}$)[d]
GAR TFase	106	14	1.43	140
trifunctional protein	97	14	1.43	140
AICAR TFase	64	16	1.07	105

[a] Determined by comparison with molecular weight standards in 8.75% SDS–polyacrylamide gels (Laemmli, 1970). [b] Determined by immunoprecipitation of in vitro translation of sucrose density gradient RNA fractions as described under Methods. [c] Determined by comparison with molecular weight standards in a 1% agarose gel. [d] Calculated by assuming 3.4×10^5 daltons/1000 nucleotides and a minimum of 3000 nucleotides/100 000-dalton protein (Padgett et al., 1979).

mRNAs of the other two proteins by sucrose density gradient centrifugation. Furthermore, the mRNAs for the trifunctional protein and GAR TFase are likewise separate species since the largest mRNA contained within their gradient fraction is insufficient to code for both proteins in the form of a fused precursor protein. The absence of high levels of proteolytic activity in the translation system makes further protein processing unlikely (Pelham & Jackson, 1976). Significantly, the proteins translated from the rabbit reticulocyte lysate system migrate identically with the purified protein controls in an SDS gel, indicating that no major posttranslational modifications occur after in vitro synthesis, in further support of our argument for separate mRNAs encoding the trifunctional and GAR TFase enzymes.

When the chicken's diet is changed from one low to one high in protein, there is a 2–3-fold elevation in the enzymatic activities of GAR TFase, trifunctional protein, and AICAR TFase as the result of elevation in protein levels. Evidence for this increase in protein concentration is derived from immunological titration of the trifunctional enzyme activity and more qualitatively from densitometer scans of gel slabs as well as the comparison of Western blots for the given proteins. These results are very similar to those reported for three other chicken liver enzymes. The elevation of AICAR TFase and trifunctional protein activity appears to be due to changes in the protein's concentration brought about by elevation of their respective mRNAs. Interpretation of the data for GAR TFase is more complex. While the enzymatic activity of GAR TFase does increase about 2–3-fold, the concentration of the enzyme appears to increase roughly 5-fold, but the increase is not accompanied by any change in its mRNA level.

It is highly probable that the lack of correlation between enzyme activity and concentration as measured by SDS gel electrophoresis for GAR TFase is caused by proteolytic degradation during homogenization of the liver (Young et al., 1984). If the rate of proteolysis is independent of GAR TFase concentration, then a greater percentage of GAR TFase would be lost from livers of chickens fed the low rather than the high-protein diet. Since the proteolyzed GAR TFase is still active but with a molecular weight of 55 000, the change in enzyme concentrations would appear to be much larger than the change in enzyme activity. Measurement of mRNA levels by in vitro translation is subject to a number of experimental variables that affect its precision. Nevertheless, comparison of mRNA levels within each diet type should be less subject to error and suggests that the change in GAR TFase mRNAs in response to an increase in dietary protein is less than for the two other enzymes. Whether the level of GAR TFase is significantly controlled by its proteolytic degradation requires more rigorous analysis of its mRNA concentration under differing dietary conditions.

In conclusion, at least the folate-requiring transformylase and synthetase activities in avian de novo purine biosynthesis are not tightly linked since they reside in separate polypeptide chains and are derived from separate appropriately sized mRNAs. However, their levels seem to be coordinately regulated but apparently through different mechanisms. In contrast, the GAR TFase from *Drosophila* is encoded by 1 kb of a 4.7-kb mRNA so that in this species it is the carboxyl terminal domain of a much larger polypeptide (Henikoff, 1983).

Registry No. GAR TFase, 9032-02-4; AICAR TFase, 9032-03-5; EC 3.5.4.9, 37318-64-2; EC 1.5.1.5, 9029-14-5; EC 6.3.4.3, 9023-66-9; folic acid, 59-30-3.

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tRNA Binding Sites of Ribosomes from *Escherichia coli*†

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ABSTRACT: 70S tight-couple ribosomes from *Escherichia coli* were studied with respect to activity and number of tRNA binding sites. The nitrocellulose filtration and puromycin assays were used both in a direct manner and in the form of a competition binding assay, the latter allowing an unambiguous determination of the fraction of ribosomes being active in tRNA binding. It was found that, in the presence of poly(U), the active ribosomes bound two molecules of *N*-AcPhe-tRNA^{Phe}, one in the P and the other in the A site, at Mg^{2+} concentrations between 6 and 20 mM. A third binding site in addition to P and A sites was observed for deacylated tRNA^{Phe}. At Mg^{2+} concentrations of 10 mM and below, the occupancy of the additional site was very low. Dissociation of tRNA from this site was found to be rather fast, as compared to both P and A sites. These results suggest that the additional site during translocation functions as an exit site, to which deacylated tRNA is transiently bound before leaving the ribosome. Since tRNA binding to this site did not require the presence of poly(U), a function of exit site bound tRNA in the fixation of the mRNA appears unlikely. Both the affinity and stability of binding to the additional site were found lower for the heterologous tRNA^{Phe} from yeast as compared to the homologous one. This difference possibly indicates some specificity of the *E. coli* ribosome for tRNAs from the same organism.

The generally accepted model of the ribosomal elongation cycle is based upon the existence of two tRNA binding sites on the ribosome: one for peptidyl-tRNA (P site) and another for aminoacyl-tRNA (A site). From the P site bound peptidyl-tRNA, the peptide is transferred to the aminoacyl-tRNA to yield peptidyl-tRNA in the A site and deacylated tRNA in the P site. During the subsequent translocation step, the A site bound peptidyl-tRNA is translocated to the P site, and the deacylated tRNA is released.

Additional tRNA binding sites other than the two canonical ones have been discussed repeatedly. For instance, an entry (Hardesty et al., 1969) or recognition (Lake, 1977) site has been proposed, from which the aminoacyl-tRNA reaches the A site only after GTP hydrolysis and codon recognition have taken place. Since at least the anticodon region of the tRNA in both recognition and A sites is bound to the same site on the ribosome, such a recognition site does not constitute an independent, nonoverlapping site. A third site functioning as an exit site, from which deacylated tRNA leaves the ribosome after translocation, has been reported for eucaryotic ribosomes (Wettstein & Noll, 1965).

For *Escherichia coli* ribosomes, the existence of a third, independent site being accessible only for deacylated tRNA has been reported recently by Rheinberger et al. (1981). Furthermore, these authors reported that binding of the peptidyl-tRNA analogue *N*-AcPhe-tRNA^{Phe} to the P site excluded binding of *N*-AcPhe-tRNA^{Phe} to the A site and vice versa ("exclusion principle"). While the existence of the third binding site for deacylated tRNA was confirmed by some authors (Grajevskaja et al., 1982; Kirillov et al., 1983), it was doubted by others, because the additional binding could not be detected in equilibrium centrifugation experiments (Schmitt et al., 1982) and because stoichiometric release of deacylated tRNA was observed within one round of translocation in a column-bound poly(U)-ribosome system (Spirin, 1984). Concerning the binding of peptidyl-tRNA, Kirillov & Semenkov (1982) presented evidence for the simultaneous binding of two molecules of *N*-AcPhe-tRNA^{Phe} to one ribosome.

Since the exact knowledge of the number of tRNA binding sites is fundamental for any study of ribosome function, we reexamined the controversial issue and studied the tRNA binding properties of tight-couple ribosomes from *E. coli* in some detail. For comparisons, some experiments were also performed with ribosomes prepared according to Rheinberger & Nierhaus (1980). Binding experiments were carried out with deacylated tRNA^{Phe} and *N*-AcPhe-tRNA^{Phe} from both *E. coli* and yeast. To escape a possible misinterpretation of saturation titration plateaus, a competition binding assay was developed which allowed an independent determination of the

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