Purification and Structural Characterization of Rat Liver Threonyl Transfer Ribonucleic Acid Synthetase†

John David Dignam,‡ David G. Rhodes, and Murray P. Deutscher*

ABSTRACT: Threonyl-tRNA synthetase was purified ~500-fold from a high-speed supernatant fraction of rat liver. The purified enzyme was estimated to be >95% pure from acrylamide gel electrophoresis under denaturing and nondenaturing conditions. Based on a native molecular weight from sedimentation equilibrium of 154 000 and a subunit molecular weight of 85 000 obtained by sodium dodecyl sulfate gel electrophoresis, the protein appears to be an α_2 dimer. The α_2 structure was also supported by cross-linking studies of the native enzyme. The purified protein has an $s_{20,w}$ of 7.2 and an isoelectric point of 6.4. Amino acid analyses revealed no unusual features, but attempts at automated sequence analyses suggested that both amino termini are blocked. Preliminary carbohydrate analyses suggested that the enzyme is a glycoprotein. Antibodies were raised against the purified protein which could inactivate and precipitate threonyl-tRNA synthetase.

The aminoacyl-tRNA synthetases catalyze the first step in protein biosynthesis and play an important role in the specificity of this process. Extensive study of these enzymes from prokaryotes and lower eukaryotes has been carried out and has revealed a surprising diversity of structure among this group of proteins [see Schimmel & Söll (1979) for references]. In contrast, relatively little is known about the structure of individual aminoacyl-tRNA synthetases from higher organisms (Schimmel & Söll, 1979). Generally, synthetase activities from mammalian cells are found in high molecular weight complexes (Bandyopadhyay & Deutscher, 1971; Vennegoor & Bloemendal, 1972; Hampel & Enger, 1973; Som & Hardesty, 1975; Denny, 1977; Dang & Yang, 1979), but the structural basis for this aggregation is unknown. As a prelude to understanding the complexity of the high molecular weight forms of the aminoacyl-tRNA synthetases, examination of the structure of the individual components appears warranted.

Accordingly, we have begun to study the properties of individual, highly purified mammalian aminoacyl-tRNA synthetases. In this paper we describe the purification and structural characterization of threonyl-tRNA synthetase from rat liver and the preparation of antibodies directed against this purified protein. Our results indicate that threonyl-tRNA synthetase is an α_2 dimer with a molecular weight of ~170 000. Studies such as these are a necessary first step for a more complete understanding of the supramolecular organization of aminoacyl-tRNA synthetases in higher organisms.

Experimental Procedures

Materials. Alumina Cγ gel, calcium phosphate gel, agarose, ATP,[1] horse apoferritin, bovine liver catalase, *Escherichia coli* β-galactosidase, rabbit muscle phosphorylase *b*, dithiothreitol, PMSF,[1] amino acids, and sodium dodecyl sulfate were obtained from Sigma Chemical Co. *E. coli* alkaline phosphatase was from Worthington Biochemical Corp. Dimethylsuberimidate and 3,3′-dithiobis(propionimidate) were purchased from Pierce Chemical Co. Ammonium sulfate and guanidine hydrochloride were from Schwarz/Mann. Sephacryl S-300 was from Pharmacia and ampholines for isoelectric focusing were from LKB. DEAE-cellulose (DE-52) was obtained from Reeve-Angel. Ovalbumin and bovine serum albumin were obtained from Calbiochem and *E. coli* RNA polymerase was obtained from Boehringer Mannheim. Spectrin was a gift from Dr. M. Sheetz. Rabbit liver tRNA was prepared as described previously (Deutscher, 1972). Radioactive amino acids were obtained from New England Nuclear. Freund's complete adjuvant was obtained from Difco.

Animals. Long–Evans strain rats (125–150 g each) were obtained from Blue Spruce Farms and New Zealand White rabbits (7 lb each) were from Jenks.

Enzyme Assays. Aminoacyl-tRNA synthetases, including threonyl-tRNA synthetase, were assayed as described previously (Bandyopadhyay & Deutscher, 1971). One unit is that amount of activity that will incorporate 1 nmol of amino acid into acid-insoluble material in 1 min.

Analytical Methods. Protein concentration was determined spectrophotometrically according to Warburg & Christian (1941). Tryptophan and tyrosine were determined by the method of Edelhoch (1967) and $E_{280}^{1\%}$ was determined as described by Scopes (1974). Protein for amino acid analysis was dialyzed extensively against 0.1 M NH_4HCO_3 (pH 9.0), lyophilized, and acid-hydrolyzed for either 24 or 72 h, and 50 μg was analyzed on a Beckman 121 automatic amino acid analyzer. Cysteine and methionine were determined as cysteic acid and methionine sulfoxide after performic acid oxidation followed by acid hydrolysis of 150 μg of protein for 24 h.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing. The Tris–Gly $NaDodSO_4$ system of Laemmli (1970) was used for assessment of purity of the protein samples and in cross-linking experiments. The phosphate–$NaDodSO_4$ buffer system of Weber & Osborn (1969) was used to determine subunit molecular weight. Electrophoresis under

† From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032 (J.D.D. and M.P.D.), and the Department of Biophysics and Biochemistry, University of Connecticut, Storrs, Connecticut 06268 (D.G.R.). *Received March 7, 1980.* Supported by Grant NP-165 from the American Cancer Society, Grant GM 16317 from the National Institutes of Health, and Grant PCM 76-21847 from the National Science Foundation (D.G.R.). J.D.D. was a Postdoctoral Fellow (GM 06981) of the U.S. Public Health Service.

‡ Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, MO 63110.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate.

0006-2960/80/0419-4978$01.00/0

nondenaturing conditions was performed according to Davis (1964). Gels were stained overnight with 0.1% Coomassie brilliant blue R-250 in methanol–acetic acid–water (45:10:45) and destained in the same solvent (2 h for slab gels and overnight for the tube gels). Isoelectric focusing was performed as described by Godson (1970).

Preparation of Antibodies. Rabbits were inoculated subdermally at eight spots on the flanks with purified threonyl-tRNA synthetase (1 mg of protein per rabbit in 50 mM $NaPO_4$, pH 7.4, and 0.1% $NaDodSO_4$ combined by sonication with an equal volume of Freund's complete adjuvant). Rabbits were bled through the main ear artery prior to inoculation to obtain preimmune serum. Four weeks later an additional 0.5 mg of protein was given through the lateral ear vein. The rabbits were bled on the seventh and tenth days following the boost. The sera were titred and the most active samples were combined. The IgG fractions from immune and preimmune sera were purified by ammonium sulfate fractionation and DEAE-cellulose chromatography.

Ouchterlonyl Immunodiffusion. Immunodiffusion was carried out on microscope slides coated with 1 mm of 1% agarose containing 50 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA. The slides were incubated at 4 °C for 3 days in a humid atmosphere until precipitin lines became clearly visible. The slides were washed by gentle swirling in 50 mM potassium phosphate (pH 7.4) and 0.3 M sodium chloride for 3 days (the buffer was changed twice daily) to remove unprecipitated serum and antigen. The slides were stained for 2 h in 5% acetic acid containing 0.1% naphthol blue black and washed with 5% acetic acid to remove unbound stain.

Determination of f, f_0, M, and D. Values of f, f_0, and D were calculated from the following relationships.

$$f = 6\pi\eta R_s$$

where η = 0.01 poise and R_s (Stokes radius) = 57 Å (determined by gel filtration).

$$f_0 = 6\pi\eta\left(\frac{3M_r\bar{\nu}}{4\pi N}\right)^{1/3}$$

where $N = 6.02 \times 10^{23}$ mol^{-1}, $\bar{\nu} = 0.73$ cm^3/g (calculated from the amino acid composition), and M_r = 154 000 (determined by sedimentation equilibrium).

$$D = kT/f$$

where $k = 1.38 \times 10^{-16}$ erg/deg and T = 293.20 K.

$$M_r = \frac{RTs}{D(1 - \bar{\nu}\rho)}$$

where $R = 8.3144 \times 10^7$ erg/deg, $\rho = 1.0$ g/cm^3, and $s_{20,w}$ = 7.2 S (determined by ultracentrifugation).

The value of f was also calculated from the measurements of M_r and s according to the equation

$$f = \frac{M_r(1 - \bar{\nu}\rho)}{Ns}$$

Sedimentation Equilibrium and Sedimentation Velocity Experiments. Sedimentation equilibrium experiments were performed in a J rotor in a Beckman Model E ultracentrifuge at 4 °C by using pulsed laser interferometry (Paul & Yphantis, 1972; Yphantis, 1979) according to Yphantis (1964), employing a short column and four-channel cell (Yphantis, 1960) run at 14 000 rpm. Sedimentation velocity experiments were performed in an An-D rotor at 40 000 rpm and 4 °C by employing interference optics. Samples of the purified enzyme (~1 mg/mL) were extensively dialyzed against 0.1 M Tris-HCl (pH 7.5), 1% sucrose, 0.1 mM DTT, and 0.1 mM EDTA prior to ultracentrifugation.

Results and Discussion

Purification of Threonyl-tRNA Synthetase. Preparation of Homogenate. Fifty rats (125–150 g each) were decapitated and their livers removed and placed in cold homogenization buffer (0.1 M Tris–acetate, pH 7.4, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF,[2] and 10% glycerol). All subsequent procedures were carried out at 0–4 °C. The livers (~300 g) were washed with buffer to remove blood and homogenized in 2.5 volumes of buffer with 10 strokes of a loose-fitting Dounce homogenizer and 3 strokes of a tight-fitting one. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 11 500 rpm in a GSA rotor (Sorvall) for 30 min. The supernatant fluid from the low-speed centrifugation was then centrifuged at 38 000 rpm in a Ti 50.2 rotor (Beckman) for 90 min.

Alumina Cγ Adsorption. Alumina Cγ gel was added to the high-speed supernatant fraction (0.4 mg of gel/mg of protein) and stirred for 20 min. The gel was collected by centrifugation and washed 4 times with 400-mL portions of 0.08 M sodium phosphate, pH 7.4, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 10% glycerol. Threonyl-tRNA synthetase activity was eluted with three 130-mL portions of the same solution containing 0.2 M sodium phosphate and 10% ammonium sulfate (adjusted to pH 7.4). The combined eluates (390 mL) were dialyzed against 6 L of the same solution containing 0.05 M sodium phosphate (pH 7.4); the buffer was changed twice and dialyzed 12 h each time.

Calcium Phosphate Gel Adsorption. Calcium phosphate gel (5 mg/mg of protein) was added to the dialyzed material and stirred for 30 min. The gel was collected by centrifugation and washed with four 250-mL portions of the same solution as the dialysis buffer containing 0.13 M sodium phosphate (pH 7.4). Threonyl-tRNA synthetase was eluted with two 150-mL portions of buffer containing 0.1 M sodium phosphate (pH 7.4) and 10% ammonium sulfate (the other components being the same). The combined eluates were dialyzed against 6 L of the same solution containing 0.015 M sodium phosphate (pH 7.7) and 20% glycerol for 12 h; the buffer was changed and dialysis was continued for 12 more h. At this stage of purification the preparation contained aminoacyl-tRNA synthetase activity for glutamic acid, arginine, lysine, methionine, aspartic acid, isoleucine, leucine, proline, serine, and valine, as well as threonine. The preparation at this stage could be stored at −80 °C for at least 1 year with retention of 60–80% activity for most of the synthetases; threonyl-tRNA synthetase retained ~70% of its activity.

DEAE-cellulose Chromatography. The dialyzed calcium phosphate gel fraction was applied to a column of DEAE-cellulose (2.0 × 30 cm) equilibrated with the dialysis buffer. The column was run at a flow rate of 120 mL/h. After addition of the sample, the column was washed with the same buffer until the A_{280} was down to base line. Threonyl-tRNA synthetase was eluted with a linear gradient (8 column volumes containing the same solution with 0.015–0.10 M sodium phosphate). Threonyl-tRNA synthetase was eluted early in the gradient ahead of the other synthetase activities. Fractions containing >20 units/mL were combined (185 mL) and concentrated to a small volume (5.3 mL) by using an Amicon ultrafiltration cell with a PM 30 membrane.

[2] PMSF was added to buffers immediately before use.

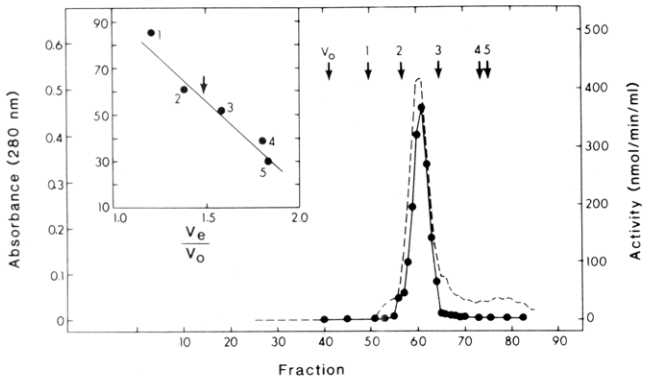


FIGURE 1: Sephacryl S-300 chromatography of threonyl-tRNA synthetase. The concentrated DEAE-cellulose fraction was run as described in the text and assayed for threonyl-tRNA synthetase activity (●) and A_{280} (---). The elution positions of the following standard proteins are noted by the arrows: (1) thyroglobulin (85 Å); (2) apoferritin (61 Å); (3) catalase (52 Å); (4) alkaline phosphatase (39 Å); (5) ovalbumin (30 Å). The Stokes radii of these proteins are given in parentheses and are presented in the inset to the figure from which the R_s of threonyl-tRNA synthetase was determined (indicated by arrow). Stokes radii for thyroglobulin and apoferritin were calculated from data given in Edelhoch (1960) and Rothen (1944), respectively. Stokes radii for the other proteins were taken from Tanford et al. (1974).

Table I: Purification of Threonyl-tRNA Synthetase

step	total act. (units)	total protein (mg)	sp act. (units/mg)	recovery (%)
high-speed supernatant	24465	18080	1.35	100
alumina Cγ gel	17331	1580	11.0	71
calcium phosphate gel	11050	436	25.3	45
DEAE-cellulose	6603	18.5	357	27
Sephacryl S-300	6248	9.0	~700	26

Sephacryl S-300 Chromatography. The concentrated enzyme preparation was applied to a column of Sephacryl S-300 (2.5 × 94 cm) equilibrated with 50 mM sodium phosphate (pH 7.4), 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 10% glycerol. The column was run at a flow rate of 19 mL/h. Threonyl-tRNA synthetase activity was eluted as a single peak coincident with the peak of protein (Figure 1). Active fractions (tubes 56–64) were frozen in a dry ice–ethanol bath and stored at −80 °C. The purified enzyme was stable for at least 3 months under these conditions and could be frozen and thawed many times with little loss in activity.

The purification procedure described here results in an ~500-fold enrichment of threonyl-tRNA synthetase from the high-speed supernatant fraction with an overall recovery of ~25% (Table I). In contrast to the results of Allende et al. (1966), we did not observe a second form of the enzyme at any stage of purification. Although the explanation for this difference is not completely understood, it may be attributable to our use of the protease inhibitor PMSF throughout the purification.

The specific activity of the purified rat liver threonyl-tRNA synthetase under standard assay conditions (700 units/mg) is about sixfold higher than that reported for a previous preparation of this enzyme (Allende et al., 1966). By use of more optimal conditions of assay (5 mM ATP, 10 mM $MgCl_2$, 1.3 mM threonine, and 4 mg/mL tRNA), the specific activity of the purified enzyme can be increased about threefold which corresponds to a turnover number of ~280/min based on a molecular weight for the enzyme of 170000 (see below). This value for the turnover number also compares favorably with the value of 104/min recently reported for the threonyl-tRNA synthetase from yeast (Yamada, 1978).

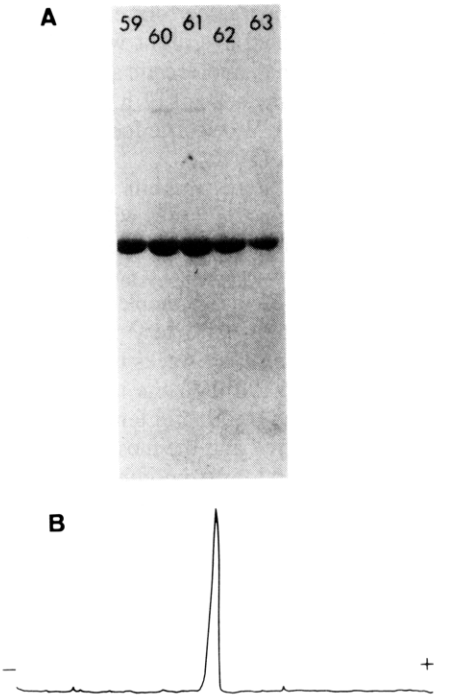


FIGURE 2: $NaDodSO_4$–acrylamide gel electrophoresis of fractions from Sephacryl S-300. (A) Aliquots (5 μL) of peak fractions 59–63 were run on an 8% acrylamide gel according to Laemmli (1970) for 4 h at 30 mA. (B) A densitometer tracing of the lane with fraction 61 from a stained gel.

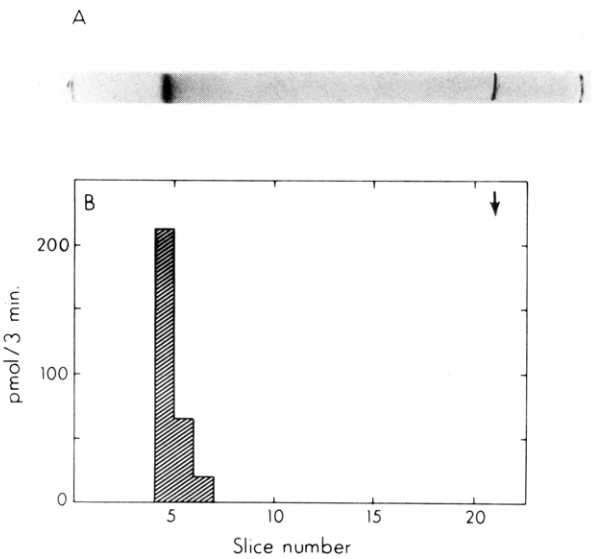


FIGURE 3: Acrylamide gel electrophoresis of threonyl-tRNA synthetase under nondenaturing conditions. Purified enzyme (8 μg) was applied to each of two 5% acrylamide gels and run according to Davis (1964) at 4 °C with 20% glycerol in the resolving gel. When the tracking dye had migrated 8.3 cm, one gel was stained with Coomassie brilliant blue (A) and the other was cut into 4-mm slices (B). Each slice was crushed into 100 μL of 0.3 M potassium phosphate, pH 6.8, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol. After 12 h of standing at 4 °C, 2 μL of each fraction was assayed for threonyl-tRNA synthetase under standard conditions. The arrow indicates the dye front.

Purity of Threonyl-tRNA Synthetase. The coincident elution of protein and threonyl-tRNA synthetase activity on Sephacryl S-300 (Figure 1) suggested that the enzyme is quite pure. This was confirmed by $NaDodSO_4$–acrylamide gel electrophoresis of several fractions from the peak of activity on Sephacryl S-300 (Figure 2A). Based on a densitometer

tracing of a stained gel (Figure 2B), the major species in fraction 61 represents at least 96% of the stained material. Further analysis of an extremely overloaded gel (50 μg of protein) revealed the presence of several distinct minor bands (data not shown) which together account for <5% of the protein on the gel. Gel electrophoresis of the purified enzyme under nondenaturing conditions in 7 (not shown) or 5% acrylamide gels (Figure 3A) again showed only one major species stained with Coomassie brilliant blue. Slicing and assay of fractions from a duplicate gel revealed that threonyl-tRNA synthetase activity comigrates with the major protein species (Figure 3B). These results, as well as the sedimentation equilibrium data described below, indicate that the threonyl-tRNA synthetase has been purified to near homogeneity.

Assay of the purified enzyme for other aminoacyl-tRNA synthetases demonstrated that is is free (<0.3% relative to threonine) of activity for alanine, arginine, asparagine, aspartic acid, glycine, glutamic acid, isoleucine, lysine, methionine, phenylalanine, serine, tyrosine, and valine. However, significant levels of activity for proline (4.6%) and leucine (1.7%) could be detected. These latter activities probably correspond to the minor bands seen after electrophoresis of the purified enzyme since many of the aminoacyl-tRNA synthetases tend to copurify in the early steps of the purification procedure and may be incompletely resolved in the final two steps.

Molecular Weight and Subunit Structure. The subunit molecular weight of threonyl-tRNA synthetase in the phosphate buffer–$NaDodSO_4$ gel system of Weber & Osborn (1969) was 85 000 with ovalbumin (45 000), catalase (58 000), bovine serum albumin (68 000), and phosphorylase *b* (94 000) as standards. A slightly lower value of 80 000 was obtained by using the Tris–Gly system of Laemmli (1970). No evidence for more than one type of subunit was obtained in either gel system. Differences in apparent molecular weight in the two gel systems have been reported previously (Swaney et al., 1974; Camacho et al., 1975; Lowe et al., 1979). Since the binding of $NaDodSO_4$ to proteins has been better characterized at pH 7.0 (Reynolds & Tanford, 1970) and the $NaDodSO_4$–phosphate system appears to be less prone to anomalous mobilities, we have used the subunit molecular weight of 85 000 for various calculations in this paper. This value is very close to that (82 000) reported by Yamada (1978) for the enzyme from yeast.

Estimation of the molecular weight of the native enzyme by gel filtration and sucrose gradient centrifugation using several globular molecular weight markers gave discrepant values. Thus, chromatography on Sephacryl S-300 gave an apparent molecular weight close to 300 000 (Figure 1), whereas sucrose gradient centrifugation suggested a molecular weight of ~120 000 (data not shown). The Stokes radius of threonyl-tRNA synthetase determined from the gel filtration experiment (Figure 1, inset) was 57 Å, and the sedimentation coefficient from the centrifugation experiment varied from 6.9 to 7.6 (relative to alcohol dehydrogenase and catalase standards) in several experiments.

The purified enzyme was analyzed by sedimentation equilibrium in order to resolve the discrepancy between these molecular weight determinations. A typical experiment is shown in Figure 4 and reveals a small amount of a low molecular weight contaminant at the top of the cell. The molecular weight calculated from the linear portion of the ln fringe displacement vs. r^2 graph, and representing most of the material in the cell, was 154 000. This result is consistent with threonyl-tRNA synthetase existing as a dimer of subunits of ~85 000 molecular weight. The sedimentation coefficient

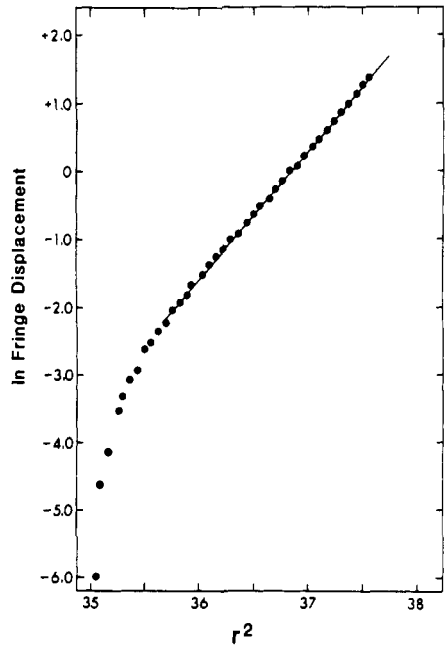


FIGURE 4: Sedimentation equilibrium centrifugation of threonyl-tRNA synthetase. Centrifugation was carried out as described under Experimental Procedures. The molecular weight was calculated from the linear portion of the ln fringe displacement vs. r^2 curve between 35.7 and 37.5 r^2.

Table II: Summary of Physical Properties of Threonyl-tRNA Synthetase

property	methods	value
partial sp vol ($\bar{\nu}$)	amino acid composition	0.73 cm^3/g
Stokes radius (R_s)	gel filtration	57 Å
diffusion coeff ($D_{20,w}$)	calcd from R_s	3.77×10^{-7} cm^2/s
sedimentation coeff ($s_{20,w}$)	sedimentation velocity	7.2 S
subunit M_r	$NaDodSO_4$ gel electrophoresis	85 000
native M_r	sedimentation equilibrium	154 000
native M_r	calcd from $s_{20,w}$ and $D_{20,w}$	174 000
native M_r	$NaDodSO_4$ gel electrophoresis of cross-linked protein	~140 000–200 000
f/f_0	calcd from R_s	1.6
f/f_0	calcd from $s_{20,w}$ and M_r	1.4

($s_{20,w}$) determined in the analytical ultracentrifuge was 7.2, in close agreement with the results from the sucrose gradient experiment. The molecular weight calculated from this sedimentation coefficient and the diffusion coefficient (obtained from the Stokes radius) was 174 000. These data are summarized in Table II. The results from the sedimentation equilibrium, sedimentation velocity, gel filtration, and gel electrophoresis experiments strongly suggest that threonyl-tRNA synthetase is an α_2 dimer, most likely with an elongated shape (f/f_0 = 1.6 calculated from the Stokes radius and 1.4 calculated from the sedimentation coefficient and molecular weight). This latter result may explain the discrepancy between the molecular weights determined by gel filtration and sucrose gradient centrifugation.

The dimeric nature of threonyl-tRNA synthetase is further supported by cross-linking experiments employing bifunctional imidates (Davies & Stark, 1970). Exposure of threonyl-tRNA synthetase to dimethylsuberimidate, followed by $NaDodSO_4$ gel electrophoresis, as shown in Figure 5, revealed a broad band which migrated between the β and β′ subunits of RNA polymerase (145 000 and 150 000) and the smaller subunit of spectrin (220 000), consistent with a dimer. A similar result

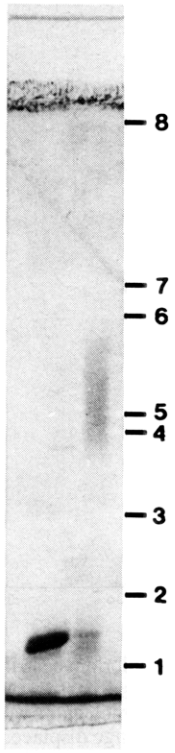


FIGURE 5: $NaDodSO_4$–acrylamide gel electrophoresis of cross-linked threonyl-tRNA synthetase. Purified enzyme (10 μg) was incubated in the presence (right lane) or absence (left lane) of 2.5 mg/mL dimethylsuberimidate in 0.25 M triethanolamine hydrochloride, pH 8.4, 0.05 mM DTT, 0.05 mM EDTA, 0.05 mM PMSF, and 5% glycerol in a total volume of 40 μL. After incubation at 24 °C for 3.5 h, samples were treated with 2% $NaDodSO_4$–10% β-mercaptoethanol for 30 s in a boiling water bath and dialyzed for 1 h against 0.125 M Tris-HCl, pH 6.5, 1% $NaDodSO_4$, and 1% β-mercaptoethanol. Samples were applied to a 5% acrylamide gel run according to Laemmli (1970). The numbers at the right indicate the position of standard proteins run on the same gel: (1) bovine serum albumin (68 000); (2) phosphorylase *b* (94 000); (3) β-galactosidase (130 000); (4) and (5) β and β' subunits of RNA polymerase (145 000 and 150 000); (6) and (7) subunits of spectrin (220 000 and 250 000); (8) spectrin dimer prepared by cross-linking with dimethylsuberimidate.

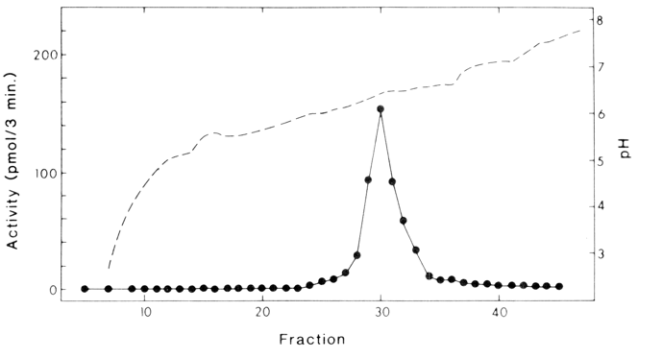


FIGURE 6: Isoelectric focusing of threonyl-tRNA synthetase. A sample of purified enzyme (70 μg) was subjected to isoelectric focusing in the pH 5–8 range at 300 V for 18 h at 4 °C as described by Godson (1970). Fractions of 0.17 mL were collected, and 2-μL aliquots were assayed for activity (●). Recovery of activity was 114%. The pH of each fraction was measured at 0 °C (---).

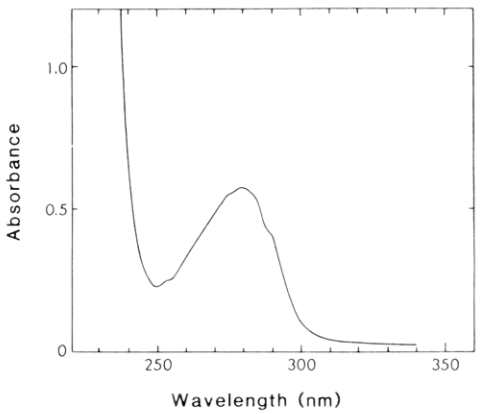


FIGURE 7: Ultraviolet absorption spectrum of threonyl-tRNA synthetase. Purified enzyme (0.6 mL) was dialyzed against 1 L of 50 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol for 24 h at 4 °C. The absorbance of the protein solution was measured from 240 to 340 nm by reading against the dialysis buffer.

was observed with the cleavable reagent 3,3′-dithiobis(propionimidate) (results not shown); in this case, treating the cross-linked protein with β-mercaptoethanol regenerated the monomer. The rather broad band obtained when the protein was treated with these reagents is probably due to the formation of intramolecular as well as intermolecular cross-links, as has been observed with erythrocyte catalase (Hajdu et al., 1976), human liver aldehyde reductase (Wermuth et al., 1979), and glycogen phosphorylase *b* (Hajdu et al., 1979). The absence of cross-linked species that would correspond to a trimer or a tetramer of 85 000 is in accord with our conclusion that threonyl-tRNA synthetase is a dimer.

These results indicate that the mammalian threonyl-tRNA synthetase is very similar in molecular weight and subunit structure to the prokaryotic (Hennecke et al., 1977) and lower eukaryotic enzymes (Yamada, 1978). The functional significance of this retention of structure remains to be determined.

Isoelectric Point. Isoelectric focusing of threonyl-tRNA synthetase according to Godson (1970) in the pH range of 5–8 gave a single active species with a pI of 6.4 (Figure 6). Likewise, only one peak of activity was obtained in the 3–10 pH range. These isoelectric focusing experiments emphasize that the purification procedure described here leads to a single active form of threonyl-tRNA synthetase. The isoelectric point of the rat liver enzyme is similar to that for the yeast enzyme (6.2–6.3), although in the latter case two forms could be separated (Yamada, 1978).

Spectral Properties. The ultraviolet absorbance spectrum of threonyl-tRNA synthetase is presented in Figure 7. The protein has an A_{280}/A_{260} ratio of 1.72, indicating the absence of significant contamination by nucleic acids or nucleotides. The extinction coefficient ($E_{280}^{1\%}$) was determined as described by Scopes (1974) and found to be 14.0. Appropriate dilutions were used to determine the absorbance at 205 nm. The pronounced shoulder in the absorption spectrum at 290 nm indicated the presence in the protein of appreciable amounts of tryptophan, and this was confirmed by analysis of tryptophan and tyrosine in 6 M guanidine hydrochloride as described by Edelhoch (1967). The tryptophan content was calculated to be 27 residues/170 000 molecular weight, and the tyrosine content was 63 residues. The latter value is close to that determined from amino acid analysis (see below and Table III).

Amino Acid Composition and N-Terminal Analysis. The amino acid composition of threonyl-tRNA synthetase is given in Table III. No unusual features are evident except, perhaps, for the relatively high content of half-cystine residues. An attempt was made to determine the amino-terminal sequence of the enzyme by automated sequence analysis of 1.6 mg of purified enzyme. However, no amino acids could be detected through five cycles of analysis. A myoglobin standard analyzed

Table III: Amino Acid Composition of Threonyl-tRNA Synthetase

amino acid	residues/ 170000	amino acid	residues/ 170000
lysine	121	alanine	82
histidine	47	half-cystine	45[b]
arginine	55	valine	115
aspartic acid	127	methionine	45[c]
threonine	67[a]	isoleucine	68
serine	88[a]	leucine	117
glutamic acid	184	tyrosine	53, 63[d]
proline	68	phenylalanine	72
glycine	109	tryptophan	27[d]

[a] Calculated from extrapolation to zero time of hydrolysis. [b] Determined as cysteic acid after performic acid oxidation and hydrolysis in 6 N HCl. [c] Determined as methionine sulfoxide after performic acid oxidation and hydrolysis in 6 N HCl. [d] Determined spectrophotometrically according to Edelhoch (1967).

immediately after gave the expected *N*-terminal sequence. Although not conclusive, this result suggests that the amino termini of both subunits are blocked.

Carbohydrate Analysis. Preliminary analysis of purified threonyl-tRNA synthetase for carbohydrate by the anthrone reagent indicated ~1% hexose by using glucose as a standard. Furthermore, staining of $NaDodSO_4$–acrylamide gels with periodic acid–Schiff reagent as described by Fairbanks et al. (1971) revealed a band that was coincident with the material staining with Coomassie brilliant blue and which was dependent on periodate oxidation of the protein. Further studies of the carbohydrate content of the enzyme are under way. With the assumption that these preliminary data can be confirmed by other types of carbohydrate analysis, these results, together with the recent report of Glinski et al. (1979) that partially purified liver arginyl- and lysyl-tRNA synthetases contain carbohydrate, raise the interesting possibility that mammalian aminoacyl-tRNA synthetases are glycoproteins. The importance of these findings for studies of the structure of high molecular weight aminoacyl-tRNA synthetase complexes are apparent.

Catalytic Properties. Apparent K_m values for threonine, ATP, and liver tRNA determined from double-reciprocal plots of saturation experiments were 0.5 mM, 0.4 mM, and 12 μM, respectively. The K_m values for threonine and ATP are higher than those previously reported for the liver threonyl-tRNA synthetase (Allende et al., 1966). At present, we have no explanation for these differences.

Preparation and Properties of Antibodies Directed against Threonyl-tRNA Synthetase. Injection of purified rat liver threonyl-tRNA synthetase into rabbits led to the production of antibodies directed against this enzyme. As shown in Figure 8, serum from injected animals could inactivate the enzyme to ~80%, whereas preimmune serum was inactive. Furthermore, double immunodiffusion studies indicated that the antibody was capable of forming precipitin bands with the enzyme (Figure 9). Also, $NaDodSO_4$–acrylamide gel electrophoresis of immunoprecipitates indicated that antibody prepared from immune serum precipitated threonyl-tRNA synthetase from a high-speed supernatant fraction of liver, whereas antibody prepared from preimmune serum did not show such activity (data not shown). The antibody directed against threonyl-tRNA synthetase did not cross-react with a mixture of other aminoacyl-tRNA synthetases (Figure 9). The antibody preparation should prove extremely useful for further structural studies of threonyl-tRNA synthetase and for examination of its interaction with other aminoacyl-tRNA synthetases.

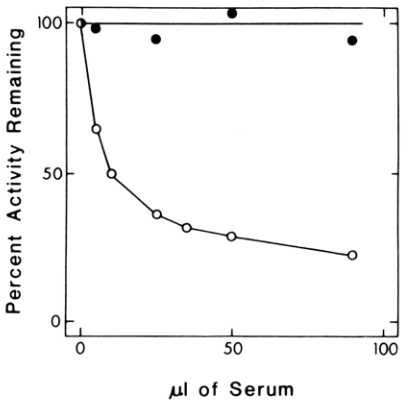


FIGURE 8: Inactivation of threonyl-tRNA synthetase by immune serum. Threonyl-tRNA synthetase (2 μg) in 50 mM potassium phosphate, pH 7.4, was incubated with increasing amounts of immune (○) or preimmune (●) serum in a final volume of 100 μL. Samples were incubated for 90 min on ice, and 1 μL was withdrawn for assay. Data are expressed as percent activity remaining compared to a sample to which no serum was added.

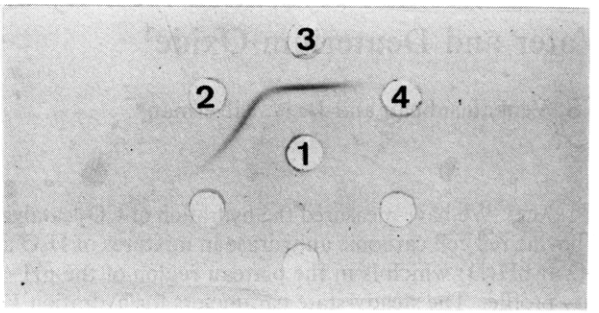


FIGURE 9: Ouchterlony immunodiffusion of threonyl-tRNA synthetase. Immunodiffusion experiments were carried out as described under Experimental Procedures. (Well 1) 5 μL of immune serum; (wells 2 and 3) 5 μL of purified threonyl-tRNA synthetase; (well 4) 5 μL of DEAE-cellulose fraction containing activity for other aminoacyl-tRNA synthetases.

Acknowledgments

The excellent technical assistance of Rong Chang Ni is greatly appreciated.

References

Allende, C. C., Allende, J. E., Gatica, M., Celis, J., Mora, G., & Matamala, M. (1966) *J. Biol. Chem. 241*, 2245.
Bandyopadhyay, A. K., & Deutscher, M. P. (1971) *J. Mol. Biol. 60*, 113.
Camacho, A., Carrascosa, J. L., Vinuela, E., & Salas, M. (1975) *Anal. Biochem. 69*, 395.
Dang, C. V., & Yang, D. C. H. (1979) *J. Biol. Chem. 254*, 5350.
Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.
Denny, R. M. (1977) *Arch. Biochem. Biophys. 183*, 156.
Deutscher, M. P. (1972) *J. Biol. Chem. 247*, 459.
Edelhoch, H. (1960) *J. Biol. Chem. 235*, 1226.
Edelhoch, H. (1967) *Biochemistry 6*, 1948.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.
Glinsky, R. L., Gainey, P. C., Mawhinney, T. P., & Hilderman, R. H. (1979) *Biochem. Biophys. Res. Commun. 88*, 1052.
Godson, G. N. (1970) *Anal. Biochem. 35*, 66.
Hajdu, J., Bartha, F., & Friedrich, P. (1976) *Eur. J. Biochem. 68*, 373.

Hajdu, J., Dombrâdi, V., Bot, G., & Friedrich, P. (1979) *Biochemistry 18*, 4037.
Hampel, A., & Enger, M. D. (1973) *J. Mol. Biol. 79*, 285.
Hennecke, H., Böck, A., Thomale, J., & Nass, G. (1977) *J. Bacteriol. 131*, 943.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344.
Paul, C. H., & Yphantis, D. A. (1972) *Anal. Biochem. 48*, 588.
Reynolds, J. A., & Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002.
Rothen, A. (1944) *J. Biol. Chem. 152*, 679.
Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601.
Scopes, R. K. (1974) *Anal. Biochem. 59*, 277.
Som, K., & Hardesty, B. (1975) *Arch. Biochem. Biophys. 166*, 507.
Swaney, J. B., Vande Woude, G. F., & Bachrach, H. L. (1974) *Anal. Biochem. 58*, 337.
Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2369.
Vennegoor, C., & Bloemendal, H. (1972) *Eur. J. Biochem. 26*, 462.
Warburg, O., & Christian W. (1941) *Biochem. Z. 310*, 384.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Wermuth, B., Münch, J. D. B., Hajdu, J., & Wartburg, J. P. (1979) *Biochim. Biophys. Acta 566*, 237.
Yamada, H. (1978) *J. Biochem. (Tokyo) 83*, 1583.
Yphantis, D. A. (1960) *Ann. N.Y. Acad. Sci. 88*, 586.
Yphantis, D. A. (1964) *Biochemistry 3*, 297.
Yphantis, D. A. (1979) *Methods Enzymol. 61*, 3.

Carbon Dioxide Hydration Activity of Carbonic Anhydrase in Mixtures of Water and Deuterium Oxide†

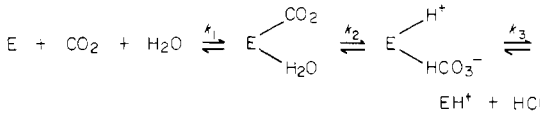
K. S. Venkatasubban and D. N. Silverman*

ABSTRACT: We have measured the hydration of CO_2 catalyzed by bovine red cell carbonic anhydrase in mixtures of H_2O and D_2O at pH(D) which is in the plateau region of the pH–activity profile. The steady-state parameters for hydration V_{max} and K_m both show an exponential dependence on the atom fraction of deuterium in solvent water. These observed solvent isotope effects cannot be fit to a model involving one hydrogen but must arise from the contributions of two or more hydrogens which change their fractionation factor in the transition state of the rate-limiting step of the catalysis. On the basis of previous evidence, this step is taken to be an intramolecular proton transfer which our results suggest involves water bridges. The dependence of the rate constant for the uncatalyzed reaction between CO_2 and water has a nonlinear dependence on the atom fraction of deuterium in the solvent. We conclude that the observed isotope effect must arise from more than one hydrogen in the transition state.

The zinc-containing metalloenzyme carbonic anhydrase catalyzes the reaction of eq 1. Current understanding of the

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

catalytic mechanism of the human carbonic anhydrase II (the high-activity isozyme) and the kinetically similar bovine carbonic anhydrase from red cells is based in part on steady-state kinetic studies carried out in H_2O and D_2O. Steiner et al. (1975) and Pocker & Bjorkquist (1977a) observed a kinetic solvent isotope effect of magnitude between 3 and 4 for both k_{cat} and K_m for hydration, whereas the isotope effect on k_{cat}/K_m was very close to unity. The corresponding values for dehydration were rather similar. Steiner et al. (1975) used these results to suggest a catalytic mechanism in which the hydration of CO_2 and a rate-limiting, intramolecular proton transfer occur in distinct stages. Elements of the proposed mechanism are given in eq 2–4. Equation 2 represents the catalytic interconversion of CO_2 and HCO_3^- in which EH^+ designates the form of enzyme with a protonated, activity-controlling group of pK_a near 7. Equation 3 describes the transfer of a proton between the activity-controlling group and a proton acceptor in the enzyme, designated by HE^+, which acts as a proton shuttle group. Histidine-64 has been suggested as a possible proton shuttle group (Steiner et al., 1975); with a pK near 7 and ~6 Å from the zinc, it is suited to act as either a donor or acceptor of protons from the activity-controlling group. The transfer of a proton between the enzyme and buffer in solution (eq 4) has been found to limit the rate of catalysis of the hydration of CO_2 only at buffer concentrations less than ~10 mM (Silverman & Tu, 1975; Jonsson et al., 1976).



(2)

$$EH^+ \overset{k_4}{\rightleftharpoons} HE^+ \quad (3)$$

$$HE^+ + B \rightleftharpoons E + BH^+ \quad (4)$$

We report here an extension of the work of Steiner et al. (1975) and Pocker & Bjorkquist (1977a,b) by measuring the kinetic solvent isotope effect as a function of the atom fraction of deuterium in solvent water for the catalyzed and uncatalyzed hydration of CO_2. These results provide information, through the Gross–Butler relationship, concerning the number and fractionation factor of hydrogenic sites which undergo bonding changes in the transition state of the rate-limiting event in catalysis (Schowen, 1978). Since proton transfer is believed

† From the Department of Pharmacology, University of Florida, College of Medicine, Gainesville, Florida 32610. *Received March 25, 1980.* This work was supported by Research Grant GM 25154 from the National Institutes of Health.

0006-2960/80/0419-4984$01.00/0