Yeast Phenylalanyl Transfer Ribonucleic Acid Synthetase. Purification, Molecular Weight, and Subunit Structure*

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ABSTRACT: A simple and rapid procedure for the isolation of Phe-tRNA synthetase from commercial baker's yeast is reported. It consists of ammonium sulfate fractionation, gel filtration on Sephadex G-200, chromatography on DEAE-Sephadex, and chromatography on hydroxylapatite. A 450-fold purification of the enzyme is achieved with a recovery of over 30%. The purified enzyme appears homogeneous on gel filtration, sedimentation equilibrium analysis, and gel electrophoresis. Its molecular weight is 236,000, based on ultracentrifugation studies, and 276,000 as deduced from Sephadex G-200 chromatography and subunit gel electrophoresis. The enzyme consists of two types of subunits with molecular weights of 63,000 and 75,000, respectively, as determined by sodium dodecyl sulfate gel electrophoresis.

During the past few years a number of amino acid activating enzymes from various sources have been isolated and characterized (for reviews, see Moldave, 1965, Novelli, 1967, Lengyel and Söll, 1969, and Yarus, 1969). These enzymes and their tRNA substrates are interesting examples for the specific interaction of proteins and nucleic acids. One of the most thoroughly studied tRNA species is $tRNA^{Phe}$ from yeast (RajBhandary and Chang, 1968; Philippsen *et al.*, 1968; Cramer *et al.*, 1969; Beardsley and Cantor, 1970). Unfortunately, relatively little is known about the cognate enzyme Phe-tRNA synthetase.

Partial purification of this enzyme was originally reported by Lagerkvist and Waldenström (1964). As a by-product of their work on Ser-tRNA synthetase, Makman and Cantoni (1965) obtained a preparation of yeast Phe-tRNA synthetase that was 180-fold purified and about 90% homogeneous according to sedimentation equilibrium studies. A 350-fold purification of the enzyme was described by Schlimme *et al.* (1969); however, the specific activity of their purified material was more than an order of magnitude lower than that reported by Makman and Cantoni. Recently, Fasiolo *et al.* (1970), using laboratory-grown yeast, succeeded in purifying the enzyme to apparent homogeneity with recoveries of 8–12%.

In the course of studies on the enzymic recognition of $tRNA^{Phe}$ (Schmidt *et al.*, 1970; Schmidt and Reid, 1971) we attempted to purify Phe-tRNA synthetase following the procedure outlined by Makman and Cantoni. Due to the unexpectedly high lability of the enzyme their method proved rather difficult to reproduce. Based on a systematic study of the stabilization of this synthetase, and on further studies on large-scale preparation of the enzyme, we have devised a simple and rapid procedure for the isolation of Phe-tRNA synthetase in high yield from commercial bakers' yeast. This communication reports on the purification procedure as well as the molecular weight and subunit structure of the enzyme.

Materials and Methods

Fleischmann's pressed bakers' yeast was purchased locally (Standard Brands, Inc.) and stored at −20°. Unfractionated brewers' yeast tRNA was purchased from Boehringer-Mannheim and had a phenylalanine acceptance of 2.5–3.0% under standard assay conditions. Sephadex G-200 (lot no. 1680) and DEAE-Sephadex A-25 (lot no. 5727) were obtained from Pharmacia Fine Chemicals, Inc. Hydroxylapatite was purchased from Bio-Rad Laboratories (Bio-Gel HTP, control no. 006709). L-[^{14}C]Phenylalanine (455 mCi/mmole, >99% radiochemical purity) was a product of Schwarz BioResearch. All other chemicals were reagent grade compounds.

Assay Procedures. In column eluates protein was determined by absorbance at 280 and 260 nm, correcting for nucleic acids according to Warburg and Christian (1941) using the table of Layne (1957). Protein concentrations in the pooled fractions at each stage of purification were determined by the method of Lowry *et al.* (1951). It is important that aliquots of the crude extracts in the early stages of purification be dialyzed before analysis to remove low molecular weight compounds which interfere with the Lowry assay and may give spuriously high apparent protein concentrations.

Enzyme activity was assayed at the pH optimum for aminoacylation in a reaction mixture which contained (in 0.2 ml) 20 μmoles of Tris-Cl (pH 8.0) (titrated at 35°), 10 μmoles of $MgCl_2$, 2 μmoles of ATP, 2 μmoles of reduced glutathione, 0.04 mg of bovine serum albumin, 2 mg of yeast tRNA, 0.016 μmole of L-[^{14}C]phenylalanine (6.17 μCi/μmole; 1 nmole is equivalent to 8530 cpm at the 62.3% counting efficiency employed), and an appropriate aliquot of enzyme containing 1–2 units. The reaction was incubated at 37° and 40-μl aliquots were withdrawn at 1, 4, 7, and 10 min, pipetted onto filter paper disks (Schleicher & Schuell no. 593-A) which were dropped immediately into ice-cold 10% trichloroacetic acid, and washed and counted as previously described (Schmidt and Reid, 1971). One unit is defined as that amount of enzyme which incorporates 1 nmole of phenylalanine into tRNA in 10 min at 37° under these conditions. When the enzyme concentration was too high to give enzyme-limited rate assays, aliquots were diluted with 0.5 M ammonium sulfate to give approximately 50 enzyme units/ml.

Gel Electrophoresis. Enzyme preparations were dialyzed into 5 mM potassium phosphate (pH 7.2), 2.5 mM mercaptoethanol, 0.5 mM EDTA, and 10% glycerol at a protein concentration of 1.3 mg/ml and then dissociated into subunits by incubating in 10 mM sodium phosphate (pH 7.0), 1% sodium dodecyl sulfate, and 1% mercaptoethanol according to Weber

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and Osborn (1969). Electrophoresis was carried out essentially as described in the above reference with the following minor changes. The molarity of the gel buffer was decreased by a factor of 2, gels were polymerized with one-half the normal amount of cross-linker, and gels of 1.4-ml volume were poured in tubes of 5-mm i.d. Separation was performed at 5–6 mA/gel (60–70 V) for 2.5 hr using bovine serum albumin, ovalbumin, pepsin, and trypsin as markers. For subunit molecular weight determinations samples were electrophoresed individually and also coelectrophoresed with the standard markers. When greater separation of the subunits was desired the electrophoresis was carried out for 5 hr under the same conditions (allowing the bromophenol blue to migrate out of the end of the gel) before removing and staining the gels.

Analytical Ultracentrifugation. The purified enzyme was dialyzed for 24 hr against two 1-l. portions of 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 1 mM EDTA, and 200 mM ammonium sulfate at 2° at a protein concentration of 0.3 mg/ml. The solution (0.130 ml) was placed in the sample compartment of a 12-mm double-sector aluminum-filled Epon cell and an equal amount of the final dialysate was placed in the reference compartment. High-speed sedimentation equilibrium analysis was carried out in the An-D rotor of a Model E analytical ultracentrifuge for 36 hr at 10° and 11,272 rpm using Rayleigh interference optics. Conditions for sedimentation equilibrium were calculated according to Teller (1965). Establishment of equilibrium was judged by no further change in the position of the fringes. Fringe displacements were measured at 0.25-mm increments using a Nikon Model 6C microcomparator and results were calculated according to Yphantis (1964) using an APL/360 computer program.

Results

Procedure for the Purification of Phe-tRNA Synthetase. All operations are carried out at 0–4°, unless otherwise noted.

EXTRACTION PROCEDURE. Six 1-lb cakes of frozen bakers' yeast are placed in a bucket containing 1350 ml of 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, and 10 mM mercaptoethanol, and left at 2–4° overnight. The thawed preparation is processed by a single passage through a Gaulin Model 15M laboratory homogenizer (Gaulin Corp., Everett, Mass.) at a pressure of 8000 psi and a rate of approximately 5 min/l. The crude extract (*ca.* 4 l.) is centrifuged at 14,000*g* for 1 hr in the GS-3 rotor of a Sorvall RC2-B refrigerated centrifuge. The postmitochondrial supernatant (1.85 l.) is titrated to pH 8.0 by the addition of concentrated ammonium hydroxide and, after removal of a small aliquot for dialysis and activity assay, immediately subjected to ammonium sulfate fractionation.

AMMONIUM SULFATE FRACTIONATION. The volume of the postmitochondrial supernatant is measured, and solid ammonium sulfate is added (over a period of 15–30 min) to 50% saturation at 0° (*i.e.*, 29.8 g of ammonium sulfate/100 ml of solution). After the addition, stirring is continued for 30 more min before centrifugation (14,000*g* for 1 hr). The supernatant is then adjusted to 70% saturation by the gradual addition of a further 12.5 g of ammonium sulfate/100 ml of the 50% saturated solution. After stirring for 30 more min the suspension is centrifuged at 14,000*g* for an hour. The precipitate is redissolved in a small volume of 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol (v/v). The turbid solution (*ca.* 200 ml) is dialyzed against the same buffer for 2–3 hr to remove excess salt.

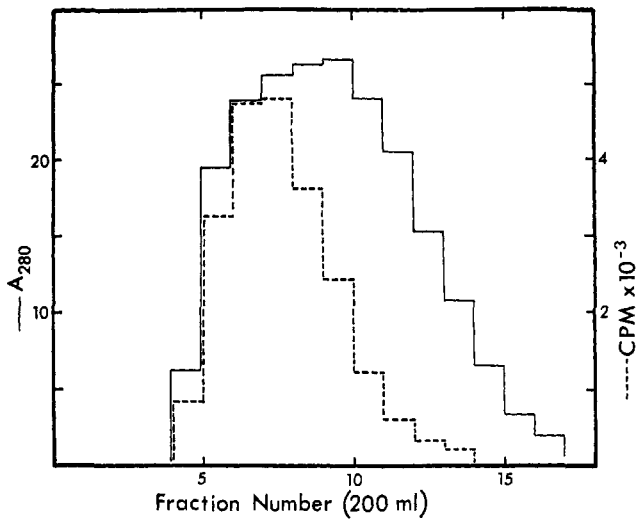

FIGURE 1: Gel filtration of ammonium sulfate fraction on Sephadex G-200. Ammonium sulfate fraction (20,600 mg; 170 EU/mg) in 350 ml of buffer was chromatographed on Sephadex G-200 as outlined in the text. The eluate was assayed for absorbance at 280 nm and for enzyme activity as described in Methods.

SEPHADEX G-200 SIEVING STEP. The dialyzed material is applied to a large Sephadex G-200 column (13.2 × 22 cm) equilibrated with the same glycerol-containing phosphate buffer in which the enzyme was dissolved. Attempts to apply the dissolved ammonium sulfate fraction directly to the G-200 column failed due to the high density of the solution which caused it to sink immediately through the gel bed. The column is eluted with the same buffer at a flow rate of 350 ml/hr, and fractions of 200 ml are collected and assayed for enzyme activity. The enzyme elutes in the turbid first half of the protein peak (see Figure 1).

DEAE-SEPHADEX CHROMATOGRAPHY. The enzyme-containing fractions from the Sephadex G-200 column are immediately applied to a column of DEAE-Sephadex A-25 (6.6 × 45 cm) equilibrated with the buffer used for the gel filtration step (10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 2 mM EDTA, and 10% glycerol). The column is eluted with a linear chloride gradient, with 4 l. of the above buffer in the mixing vessel and 4 l. of the same buffer containing 0.5 M NaCl in the reservoir. Fractions of 20 ml are collected at a flow rate of 1000 ml/hr and assayed for enzyme activity. The enzyme emerges well separated from the early-eluting protein peak (see Figure 2).

HYDROXYLAPATITE CHROMATOGRAPHY. All buffers in this step are adjusted to 20% glycerol. The enzyme-containing fractions of the DEAE-Sephadex eluate (*ca.* 750 ml at a NaCl concentration of about 0.2 M) are pooled and immediately applied to a column of 6.5-cm diameter which is packed to a height of 4.2 cm with a mixture of hydroxylapatite and powdered cellulose (4:1, w/w) and equilibrated with 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, and 1 mM EDTA. By raising the hydrostatic head to 200 cm, sample application can be completed in 2 hr. The column is then eluted with 2000 ml of a linear gradient from 100 to 300 mM potassium phosphate (pH 7.2), both buffers containing 5 mM mercaptoethanol and 1 mM EDTA. Flow rates are maintained at approximately 100 ml/hr using a hydrostatic pressure drop of 80–100 cm. The bulk of the protein elutes early while the

TABLE I: Purification of Phe-tRNA Synthetase.

Fraction	Protein (mg)	Sp Act. (eu/mg)	Total Act. (eu)	Purifcn	Recov (%)
I. Postmitochondrial supernatant[a]	51,850	77	4.00×10^6		100
II. Ammonium sulfate fraction (50–70% saturation)	20,600	180	3.72×10^6	2.33	93
III. G-200 filtrate	8,000	379	3.03×10^6	4.9	76
IV. DEAE-Sephadex fraction	415	3,590	1.49×10^6	45.2	37
V. Hydroxylapatite fraction[b]	36	35,500	1.28×10^6	460	32

[a] The analysis was performed on a dialyzed aliquot. [b] The specific activity of the pooled hydroxylapatite fraction was slightly over 40,000 before the final ammonium sulfate precipitation which caused some activity loss.

enzyme appears later in the gradient as a discrete peak (see Figure 3). The active fractions are mixed with an equal volume of glycerol-free phosphate buffer (10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, and 1 mM EDTA), solid ammonium sulfate is added to 85% saturation (56 g/100 ml), and the suspension is stirred for 4 hr at 0°. The protein precipitate is centrifuged, redissolved in 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, and 1 mM EDTA containing 10% glycerol, and either used for analysis or stored at −20° after mixing with an equal volume of glycerol.

A summary of the purification procedure from 6 lb of yeast is given in Table I.

ASSESSMENT OF PURITY AND DETERMINATION OF MOLECULAR WEIGHT. The purified enzyme obtained after hydroxylapatite chromatography was subjected to analysis by gel filtration, electrophoresis, and equilibrium sedimentation to assess its homogeneity. Figure 4 shows the elution profile obtained after chromatography on a column of Sephadex G-200 using buffers containing 0.5 M ammonium sulfate. The preparation appears to be quite homogeneous as evidenced by a single protein peak of constant specific activity. The small peak observed around fraction 80 is a small molecular weight non-protein contaminant as indicated by its 280:260 ratio of 0.4. Also shown in Figure 4 are the elution positions of known molecular weight standard proteins after chromatography on the same column. Estimation of the molecular weight of Phe-tRNA synthetase from these data according to the method of Andrews (1965) yields a value of 280,000 ± 15,000. The enzyme is evidently larger than rabbit muscle pyruvate kinase of mol wt 230,000–237,000 (the latter enzyme was reported by Warner (1958) to have a molecular weight of 237,000, and by Steinmetz and Deal (1966) to consist of two 115,000 molecular weight protomers).

The purified enzyme was also subjected to sedimentation equilibrium analysis as described in Materials and Methods. The results of this experiment are shown in Figure 5 and the preparation appears to be homogeneous as judged by the linearity of the ln c *vs.* r^2 plot (the single point deviation at low fringe displacement may represent traces of a lower molecular weight component). From the buffer density and an assumed partial specific volume of 0.740, the weight average molecular weight was calculated to be 236,000 from seven-point sets at the bottom of the cell, and 230,300 using the whole cell data.

The preparation was also subjected to analytical polyacrylamide gel electrophoresis at pH 7.0 and showed a single protein component.

SUBUNIT STRUCTURE. Purified yeast Phe-tRNA synthetase was dissociated into subunits in sodium dodecyl sulfate-

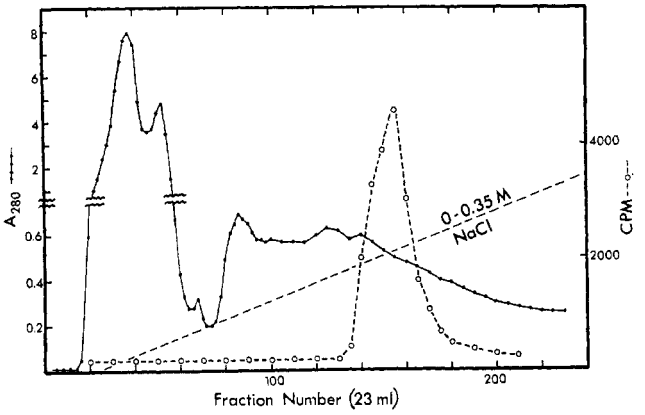

FIGURE 2: Gradient elution of Sephadex G-200 fraction from DEAE-Sephadex. A G-200 eluate (800 ml) containing 6300 mg of protein (364 EU/mg) was chromatographed on DEAE-Sephadex A-25 as described in the text. Collection of 23-ml fractions was started after about half of the sample had entered the resin. The eluate was assayed for absorbance at 280 nm and for enzyme activity as described in Methods.

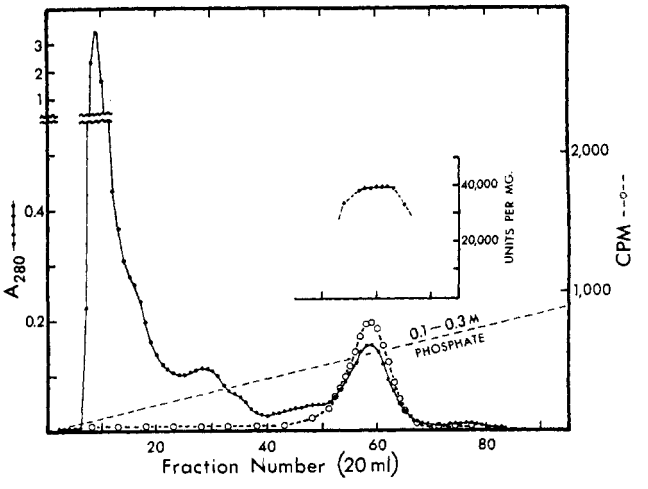

FIGURE 3: Gradient elution of DEAE-Sephadex fraction from hydroxylapatite. DEAE-Sephadex effluent (800 ml) containing 384 mg of protein (3220 EU/mg) was applied to a column of hydroxylapatite and eluted with a linear phosphate gradient as described in the text. Fractions of 18 ml were collected at a flow rate of *ca.* 100 ml/hr and assayed for absorbance at 280 nm and enzyme activity as described in Methods.

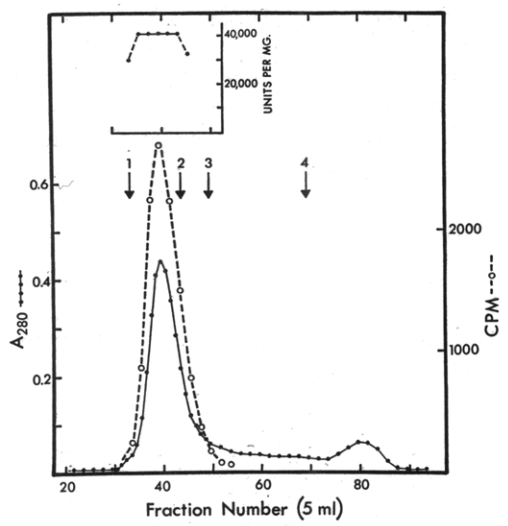

FIGURE 4: Sephadex G-200 chromatography of purified Phe-tRNA synthetase. Purified enzyme (24 mg) in 2.3 ml of 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol was applied to a column of Sephadex G-200 (2.5 × 95 cm) equilibrated in the same buffer containing 0.5 M ammonium sulfate. At a hydrostatic head of 15–20 cm and a flow rate of 15 ml/hr, 4.7-ml fractions were collected, monitored for absorbance at 280 nm, and assayed for Phe-tRNA synthetase activity as described in the text. The arrows refer to the elution positions of: 1, Blue Dextran; 2, rabbit muscle pyruvate kinase (mol wt 230,000–237,000); 3, lactate dehydrogenase (mol wt 144,000); 4, ovalbumin (mol wt 43,000).

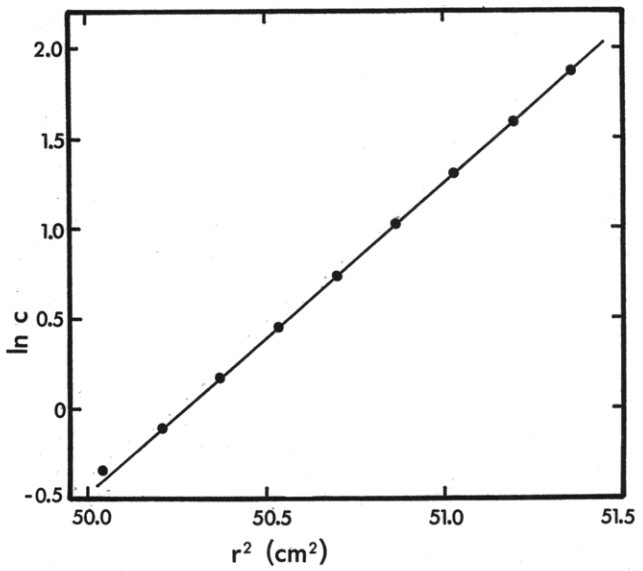

FIGURE 5: High-speed sedimentation equilibrium centrifugatin of yeast Phe-tRNA synthetase. Ln c (as fringe displacement) *vs.* r^2 plot from data obtained at 10° in 0.2 M ammonium sulfate containing buffers (pH 7.2) at an enzyme concentration of 0.3 mg/ml (rotor speed 11,272 rpm).

mercaptoethanol and subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate using the method of Weber and Osborn (1969) with the modifications described in Materials and Methods. As seen in Figure 6, two discrete subunits were readily discernible under standard electrophoretic conditions. Comparison of their electrophoretic mobilities with standard proteins indicated that they were quite similar in size to bovine serum albumin (mol wt 68,000). In order to more precisely determine their mobilities with respect to bovine serum albumin, samples were also co-electrophoresed with bovine serum albumin and ovalbumin standards for twice the normal period. As can be seen from the gels on the right, bovine serum albumin is intermediate in size between the larger and smaller subunits of yeast Phe-tRNA synthetase. Calculation of the molecular weights of the two subunits by comparison to the relative mobilities of several standard proteins according to Weber and Osborn (1969) yields values of 75,000 for the larger subunit and 63,000 for the smaller subunit. The native enzyme thus appears to be a tetramer of the $\alpha_2\beta_2$ type and the apparent molecular weight calculated from the subunit molecular weights is 276,000.

Discussion

The purification procedure described in this communication offers several advantages over methods worked out in other laboratories.

It makes use of commercially available yeast, thus saving the time consumed growing and harvesting cultures in the laboratory. Successful utilization of commercial yeast for the partial purification of Phe-tRNA synthetase has so far only been reported by Makman and Cantoni (1965); Schlimme *et al.* (1969) achieved unsatisfactory results. Substituting the Gaulin homogenizer for the MSK apparatus (as used by Fasiolo *et al.*, 1970) greatly reduces the time required for the cell disruption step and allows one to work at a scale that is limited by centrifuge capacities only.

Removal of ribosomes by high-speed centrifugation is a standard step in many synthetase purification procedures; however it frequently results in considerable activity losses. We have found that it can be omitted without detrimental effect since the bulk of the ribosomes are removed during ammonium sulfate fractionation and the remainder do not seem to interfere with either gel filtration or DEAE-Sephadex chromatography.

The gel filtration step was originally introduced into the purification scheme for the removal of ammonium sulfate in preparation for the first ion-exchange step; Sephadex G-200 rather than G-25 was used in order to effect a crude protein

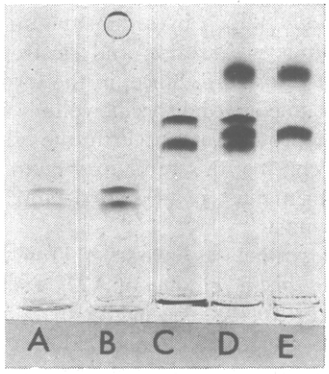

FIGURE 6: Electrophoresis of purified Phe-tRNA synthetase in polyacrylamide gels containing sodium dodecyl sulfate. The enzyme was dissociated into subunits and subjected to electrophoresis under the conditions described in Methods. Gels A and B were electrophoresed for 2.5 hr while C, D, and E were electrophoresed for 5 hr. (A) 5–10 μg of enzyme, (B) 15–20 μg of enzyme, (C) 20 μg of enzyme, (D) 20 μg of enzyme with 10 μg of bovine serum albumin plus 20 μg of ovalbumin, and (E) 10 μg of bovine serum albumin plus 20 μg of ovalbumin. Migration was from the bottom (cathode) to the top (anode).

fractionation. Although the purification achieved is only twofold, subsequent chromatography on DEAE-Sephadex appears to be greatly improved by this procedure; if dialyzed ammonium sulfate fraction is used directly, the enzyme frequently fails to adsorb to the resin.

An important aspect of the present procedure is the fact that no special concentration step is required until the enzyme is purified. Furthermore, since there is no need for any extended dialysis the whole enzyme purification can be completed in 2 days; this in turn contributes toward satisfactory enzyme recoveries. Yields of purified Phe-tRNA synthetase (6 mg/lb of yeast) compare favorably with those obtained utilizing log-phase cultures (Fasiolo *et al.*, 1970).

The purified enzyme appears to be homogeneous by three independent criteria; however, it differs in several respects from the Phe-tRNA synthetase described by Fasiolo *et al.* (1970). The specific activity reported by this group corresponds to 0.91 μmole of phenylalanine incorporation into tRNA per min at 37° per mg of protein (this value rises to 1.40 μmoles after prolonged storage at −20°) whereas our data indicate a value of 4.0 μmoles of phenylalanine incorporated per min per mg for the pure enzyme. Although the possibility remains that the enzyme exhibits strain-specific variation in turnover number, the discrepancy can perhaps more likely be explained by the different Mg^{2+}:ATP ratio and the suboptimal pH used in their aminoacylation rate assays. Based on our data the turnover number (molecular activity) of the pure enzyme at 37°, pH 8, is 1100 min^{-1}.

These authors also report a molecular weight for the purified enzyme of 167,000–234,000 as determined by sucrose gradient centrifugation whereas our data indicate a molecular weight of 280,000 from gel filtration chromatography and 276,000 based on sodium dodecyl sulfate gel electrophoresis of the subunits. On the other hand, sedimentation equilibrium studies performed in our laboratory yielded a molecular weight of 236,000 which is in close agreement with their preliminary value of 237,000, obtained by this method (Fasiolo *et al.*, 1970). Although the enzyme is obviously a tetramer containing two types of subunits, the reported molecular weights of 56,000 and 63,000 differ from our values of 63,000 and 75,000. It is somewhat unlikely but nevertheless possible that these values again reflect strain differences between *Saccharomyces cerevisiae* C_{836} and the commercial bakers' yeast used in our work. The discrepancy between our molecular weight values obtained by sedimentation equilibrium on the one hand and gel filtration and electrophoresis on the other is difficult to explain. Since, in the former method, we have used an assumed partial specific volume and since partial hydration of the protein may occur in the salt solutions used to stabilize the enzyme, this value may be in error; the solution of this problem must await more detailed experiments on the purified subunits.

The aa-tRNA synthetases have several functional properties in common, *e.g.*, specific binding of ATP, a single amino acid, and the tRNA (or tRNAs) specific for that amino acid; however, despite these common functional features, a uniform pattern in the structure of these enzymes is difficult to discern. These enzymes may consist of single polypeptide chains (Arndt and Berg, 1970; Hayashi *et al.*, 1970), or identical subunits (Bruton and Hartley, 1970; Katze and Konigsberg, 1970; Lee and Muench, 1969; Kosakowski and Böck, 1970), or nonidentical subunits of the same size (Preddie, 1969), or nonidentical subunits of different sizes (Ostrem and Berg, 1970). Yeast Phe-tRNA synthetase is a member of this latter group. This presents an interesting aspect of the evolution of aa-tRNA synthetases since *Escherichia coli* Phe-tRNA synthetase, which is also a tetramer, consists of identical subunits (Kosakowski and Böck, 1970).

Acknowledgments

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