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Tyrosyl Transfer Ribonucleic Acid Synthetase from *Bacillus stearothermophilus*. Preparation and Properties of the Crystallizable Enzyme†

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ABSTRACT: A procedure was developed for the purification of 100–200 mg of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. Although thermostable, the enzyme is very sensitive to proteolysis and conditions were developed to prevent fragmentation during purification and storage. The adoption of these precautions was important for the crystallization of the enzyme (B. R. Reid, G. L. E. Koch, Y. Boulanger, B. S. Hartley, and D. M. Blow (1973), *J. Mol. Biol. 80*, 199). The enzyme is a dimer and is composed of two identical protomers with molecular weights of about 45,000 each. The enzyme contains four cysteine residues which could be useful in the preparation of heavy atom derivatives for the crystallography. Only one of these residues appears to react with 5,5′-dithiobis(2-nitrobenzoic acid) at room temperature and even this is very slow.

Aminoacyl-tRNA synthetases catalyze the esterification of amino acids to tRNA in a highly specific manner and ensure the correct translation of the genetic code. The mechanism by which these enzymes effect their bispecificity has therefore attracted a substantial amount of attention. A further source of interest in these enzymes emanates from the fact that each enzyme indulges in a very specific interaction with a well-defined polynucleotide and the system therefore lends itself readily to studies on the forces involved when proteins combine with specific nucleic acids. Since a proper understanding of either of the above processes will ultimately require knowledge of the three-dimensional structures of the enzymes, a high priority has been placed on the preparation of crystals of the enzymes suitable for study by X-ray diffraction. Before the present study three enzymes, lysyl-tRNA synthetase (Rymo *et al.*, 1970), leucyl-tRNA synthetase from yeast (Chirikjian *et al.*, 1972), and methionyl-tRNA synthetase from *Escherichia coli* (Waller *et al.*, 1971), have been crystallized in either the native or modified form.

In an attempt to overcome some of the problems experienced with enzymes from mesophiles I carried out an investigation on the aminoacyl-tRNA synthetases from the thermophile *Bacillus stearothermophilus* with the intention of preparing suitable crystalline preparations of one or more of these enzymes. This organism was chosen because it produces proteins of considerable thermal stability which can be a major asset in crystallization. Since it was not possible to decide *a priori* which protein was likely to yield suitable crystals several of the enzymes were isolated and their amenability to crystallization was tested. One of the enzymes so isolated, tyrosyl-tRNA synthetase, has yielded crystals of exceptional quality (Reid *et al.*, 1973) and we have commenced studies on the primary and tertiary structure of the protein. In this report the isolation of the crystallizable enzyme and some of its characteristics are described.

Experimental Section

Materials. Cells of *B. stearothermophilus* NCA 1503 were grown at the Microbiological Research Establishment, Porton, Wilts., U. K. tRNA was prepared by phenol extraction of cells of *B. stearothermophilus* NCA 1503 according to the procedure of Stanley and Bock (1965). Radioactive amino acids and [^{32}P]PP_i were obtained from the Radiochemical Centre, Amersham, Bucks., U. K. Activated charcoal (Noritol) was washed with distilled water and stored as a 2% solution in water. ATP, $PhCH_2SO_2F$, and Nbs_2[1] were from the

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[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Sigma Chemical Co., Ltd. and sodium dodecyl sulfate (special grade) from British Drug Houses. Hydroxylapatite was a kind gift from Dr. A. Atkinson of the M.R.E., Porton.

Methods. [^{32}P]PP$_i$–ATP Exchange Assay. The method of Calender and Berg (1966) was used to measure amino acid activation. The standard mixture contained 100 μmol of Tris-HCl (pH 8.0), 5 μmol of $MgCl_2$, 10 μmol of KF, 10 μmol of 2-mercaptoethanol, 2 μmol of ATP, 2 μmol of $Na_4[^{32}P]P_2O_7$ (*ca.* 250,000 cpm), and 2 μmol of amino acid in 1 ml. Aliquots of 200 μl of this mixture were incubated with limiting amounts of enzyme at 37° for 10 min. The reaction was stopped by the addition of 100 μl of 7% (v/v) perchloric acid followed by 200 μl of an aqueous suspension of 2% (w/v) charcoal. The suspension was thoroughly mixed and filtered onto a 2-cm Whatman GF/C disc and washed with deionized water. The filter was dried in an oven at 100° for 5 min and counted in a thin window gas flow counter (Nuclear Chicago). The measurement of initial velocities was ensured by limiting the pyrophosphate incorporation to <10% of the total. One unit of enzyme activity is that amount which incorporates 1 μmol of [^{32}P]PP$_i$ into ATP in 10 min at 37°.

Aminoacylation of tRNA. The standard reaction mixture contained 5 μM Na_2ATP, 10 μM [^{14}C]amino acid, 100 μg of unfractionated tRNA, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM KOAc, and 100 mM Tris-HCl (pH 7.4) in a total volume of 200 μl. The reaction was started by the addition of enzyme and the mixture was incubated at 37° for 5 min. The reaction was stopped by the addition of 200 μl of cold 10% trichloroacetic acid; the precipitate was collected on Whatman GF/C glass filters and subjected to scintillation counting.

Estimation of Protein. The amount of protein in samples obtained during the purification was calculated from the absorbance at 280 nm assuming that an absorbance of 1 corresponded to a concentration of 1 mg/ml. The absorbance coefficient for the pure enzyme was determined using amino acid analysis to estimate the weight of protein. Protein concentrations were then determined from absorbance measurements at 280 nm using the absorbance coefficient $A_{1\%}^{1\,cm} = 13$.

Amino Acid Analyses. Samples were hydrolyzed in 6 N HCl for 22 hr at 110° *in vacuo* and analyses were carried out in duplicate. Cysteic acid was estimated from hydrolysates of the oxidized protein (Hirs, 1956) and tryptophan was measured spectrophotometrically according to the method of Edelhoch (1967).

Polyacrylamide Gel Electrophoresis. Electrophoresis of the native protein was carried out in 0.4 × 10 cm glass tubes with 7.5% acrylamide gels using the system described by Davis (1964). Samples were applied in 50% glycerol, electrophoresed at 2 mA/tube, and stained with freshly prepared 0.2% Coomassie Blue in methanol–acetic acid–water (50:7:43 v/v) for 16 hr and destained electrophoretically in 7% acetic acid. Gels containing sodium dodecyl sulfate were prepared and used according to the method described by Laemmli (1970); 10% gels were poured in 0.6 × 11 cm glass tubes and polymerized with amonium persulfate. About 10 μg of protein was used in each sample which was immersed in boiling water for 5 min before use to ensure complete dissociation of the protein. Staining and destaining were as described above.

Gel Filtration. A column of Sephadex G-150 (3 × 140 cm) was equilibrated with 100 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, and 2.5 × 10^{-4} M $PhCH_2SO_2F$, and eluted with the same buffer. Samples were prepared in the column buffer with 50% glycerol and pumped upwards through the gel at a rate of 17 ml/hr. The column was operated at 4° and 11-ml fractions were collected and assayed.

Mapping of Tryptic Peptides. Samples of the enzyme were oxidized with performic acid according to the method of Hirs (1956). The oxidation mixture was diluted with four volumes of water and dialyzed against a large excess of 0.5% NH_4HCO_3. This procedure was used because it yielded a clear solution of the oxidized protein in the buffer used for trypsin digestion and improved the digestion significantly. Tryspin was added (1:100, w/w); the mixture was incubated at 37° for 4 hr, lyophilized, and resuspended in 0.2 M pyridine–acetate (pH 2.9). The slight precipitate which formed was removed by centrifugation and the clear supernatant adsorbed onto a column of SP-Sephadex C-25 equilibrated with 0.2 M pyridine acetate (pH 2.9). The column was eluted with a gradient of pyridine acetate using the conditions described by Schroeder (1972). The effluent was collected in fractions which were dried down with a stream of air and subjected to paper electrophoresis at pH 6.5. The peptides were detected by spraying the sheets with 0.01% fluorescamine (Udenfriend *et al.*, 1972) in acetone followed by 0.5% pyridine in acetone. After storage at room temperature overnight the fluorescence from the peptides had disappeared completely and the sheets were sprayed with *o*-phenanthroline for arginine peptides and diazotized sulfanilic acid for histidine peptides.

Titration of Sulfhydryl Groups (Ellman, 1959). A sample of protein was dialyzed extensively against completely degassed 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA, and maintained under vacuum during the dialysis. The solution was then diluted to a concentration of approximately 2 mg/ml and placed in a cuvet to which was added 20 μl/ml of a 2.5 mM solution of Nbs_2 in acetone. Control reactions were carried out with a solution containing 20 nmol of dithiothreitol. Titrations in the presence of sodium dodecyl sulfate were carried out by adding sodium dodecyl sulfate to the cuvet, to a final concentration of 1%. No color was obtained with sodium dodecyl sulfate alone. The reactions were all carried out at room temperature and the amount of protein used in each sample was checked by amino acid analysis.

Carboxymethylation (Crestfield et al., 1963). The lyophilized protein was dissolved in 98% formic acid and lyophilized. It was resuspended in 100 mM Tris-HCl (pH 8.3) containing 10 mM EDTA and 6 M guanidine hydrochloride. Oxygen-free nitrogen was bubbled through the solution for about 0.5 hr to ensure complete removal of oxygen and dithiothreitol added to a concentration of 1 mM. Reduction was carried out overnight at room temperature after which radioactive iodoacetate (5000 cpm/nmol) was added to a concentration of 10 mM iodoacetate and the reaction allowed to proceed for 4 hr at 37°. The reaction was stopped by the addition of 1% 2-mercaptoethanol; the mixture was dialyzed extensively against 0.5% NH_4HCO_3 and lyophilized.

Purification of the Enzyme. All operations were carried out at about 4°, and all buffers were made up to 0.1 mM $PhCH_2SO_2F$ before use. Unless otherwise stated 10 mM 2-mercaptoethanol was added to all buffers just before use. Protein solutions were concentrated by ultrafiltration in Amicon cells and stored at −20° in 50% glycerol. Even impure preparations of the enzyme could be stored in this manner for periods of several months without seriously affecting activity. Enzyme activity was only monitored by the pyrophosphate exchange assay during the purification. The procedure used is summarized in Table I. Steps 1, 2, and 3 were carried out at the Microbiological Research Establishment with Dr. A. Atkinson.

TABLE I: Purification of Tyrosyl-tRNA Synthetase from *B. stearothermophilus*.

Step	Fraction	Total Units	Total Protein (g)	Specific Activity	Yield (%)
1	Crude extract	80,000	600	0.13	100
2	DEAE-cellulose (batch)	80,000	300	0.26	100
3	Hydroxylapatite I	60,000	50	1.2	75
4	DEAE-Sephadex I	50,000	25	2	60
5	Ammonium sulfate fractionation	50,000	20	2.5	60
6	Hydroxylapatite II	35,000	3	12	45
7	DEAE-Sephadex II	28,000	0.5	56	35
8	Sephadex G-150	20,000	0.2	100	25

Step 1. Preparation of Cell Extracts. Frozen cells (10 kg wet weight) were thawed, suspended in 10 l. of 0.1 M imidazole-HCl (pH 8.0) with 2 mM EDTA and 8 μg/ml of DNase, and disrupted by passage through a Manton-Gaulin homogenizer. Cell debris was removed by centrifugation at 13,700*g* for 80 min.

Step 2. Batch Elution from DEAE-Cellulose. The supernatant from step 1 was mixed with DEAE-cellulose (2 kg of dry powder, preequilibrated with 20 mM imidazole-HCl (pH 7.0) with 90 mM NaCl and 2 mM EDTA). The slurry was stirred for 30 min and the resin spun down. It was then washed with 0.4 M Cl^- (2 × 12 l.) and all the enzyme activity was eluted from the resin.

Step 3. Hydroxylapatite Chromatography I. The product from step 2 was stirred with 5 l. of hydroxylapatite equilibrated with 10 mM potassium phosphate (pH 6.8) and poured onto a column (diameter 37 cm, height 8 cm). The column was washed with the following range of potassium phosphate (pH 6.8) buffers: 70 mM (12 l.), 200 mM (12 l.), 300 mM (12 l.), and 500 mM (12 l.). The bulk of the enzyme activity was eluted in the 300 mM wash. The active fractions were pooled, concentrated, and diluted to a phosphate concentration below 50 mM.

Step 4. DEAE-Sephadex I (pH 8.0). The enzyme fraction was adjusted to pH 8.0 with 10 N KOH and adsorbed onto a column of DEAE-Sephadex A50 (14 × 40 cm) equilibrated with 50 mM potassium phosphate (pH 8.0). After washing with 4 l. of the same buffer the column was eluted with a linear gradient from 50 to 400 mM phosphate. The active fractions were pooled and concentrated down to about 1 l.

Step 5. Ammonium Sulfate Precipitation. Solid ammonium sulfate was added slowly up to 70% saturation; the mixture was allowed to stand for 30 min and then centrifuged for 30 min at 10,000 rpm. The precipitate was dissolved in 250 ml of 20 mM potassium phosphate (pH 6.8) and dialyzed overnight against the same buffer. A slight precipitate which appeared during dialysis was discarded after centrifugation.

Step 6. Hydroxylapatite Chromatography II. The ammonium sulfate precipitate was subjected to chromatography on hydroxylapatite (9.5 × 15 cm) equilibrated with 20 mM potassium phosphate (pH 6.8). The column was washed with 1 l. of the same buffer and eluted with a linear gradient of phosphate (pH 6.8) from 20 to 200 mM in a total volume of 8 l. The enzyme activity was eluted between 110 and 140 mM phosphate and was pooled, concentrated, and dialyzed against 0.1 M Tris-HCl (pH 7.5).

Step 7. DEAE-Sephadex II (pH 7.5). A column of DEAE-Sephadex A-50 (5 × 25 cm) was equilibrated with 100 mM Tris-HCl (pH 7.5) and the product from step 5 was adsorbed directly onto it. After washing with 0.5 l. of the same buffer the column was eluted with a linear gradient of chloride from 100 to 300 mM in a total volume of 5 l. The enzyme activity which eluted at about 190 mM chloride was pooled and concentrated.

Step 8. Gel Filtration. The concentrated sample was subjected to gel filtration on Sephadex G-150 as described above. The active fractions were pooled, concentrated, and dialyzed against 100 mM Tris-HCl (pH 7.5) with 50% glycerol.

Results and Discussion

Purification. The procedure used for the purification of the enzyme was developed for the isolation of several proteins including four other aminoacyl-tRNA synthetases. Therefore some of the steps have not been optimized for the tyrosyl-tRNA synthetase and could bear significant improvement. However, even in its present form the procedure is adequate for most purposes and yields over 100 mg of enzyme which is pure on the basis of its homogeneity on polyacrylamide gels in the presence or absence of sodium dodecyl sulfate (Figure 1). The overall purification is about 600-fold in protein and the final yield about 25%. The pure enzyme can be stored for several months at −20° in 50% glycerol without loss of activity. The major disadvantage of this scheme is that it takes several weeks to complete, but this can be reduced considerably if the only objective is the isolation of this enzyme.

Molecular Weight and Subunit Structure. The molecular weight of the native enzyme was determined from studies on the behavior during gel filtration on Sephadex G-150. A single peak of protein which was symmetrical and completely coincident with the enzyme activity was obtained. There was no evidence for dissociation or aggregation of the enzyme at the temperature used (*ca.* 4°). The average of the elution volume from four runs was used to estimate the molecular weight of the enzyme from the standard curve prepared with several well-characterized proteins (Figure 2) and yielded an estimate of 95,000 ± 5000 for the molecular weight of the native enzyme. The same value was obtained at a variety of enzyme concentrations (1–30 mg/ml) so there does not appear to be any concentration dependence in the molecular weight.

The molecular weight of the subunits of the enzyme was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Only a single band of protein was obtained after electrophoresis in either 7.5 or 10% gels and no alteration in the mobility of the band could be detected after reduction and carboxymethylation or performic acid oxidation of the protein (Figure 1). Comparisons of the mobility of the protein with those of several proteins of known size yielded an estimate of 40,000–45,000 for the molecular weight of the subunits of the enzyme (Figure 3). It was

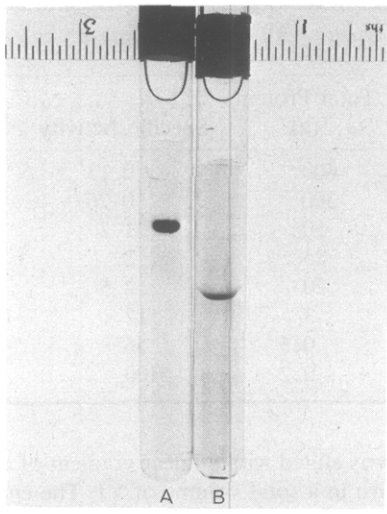

FIGURE 1: Polyacrylamide gel electrophoresis of purified tyrosyl-tRNA synthetase: (A) electrophoresis of 70 μg of enzyme in a 7.5% gel by the method of Davis (1964); (B) electrophoresis of 20 μg of enzyme in a 10% gel contaning sodium dodecyl sulfate by the method of Laemmli (1970).

concluded that the native enzyme contains two subunits of almost identical size.

In order to determine whether or not the subunits were also similar in amino acid sequence an investigation was carried out on the tryptic peptides of the protein. The oxidized protein was employed in these studies because the tryptic digestion of the protein in this form was very efficient and essentially all of the products were soluble and therefore amenable to separation by conventional techniques. The peptides were first separated by ion-exchange chromatography and then by paper electrophoresis at pH 6.5 (Figure 4). This combination gave a good spread of the peptides and it was possible to obtain estimates of the total number of peptides yielded by the protein. The estimates ranged from 40 to 50 tryptic peptides of which about half contained arginine and about 10 contained histidne. Since all these numbers are close to half those predicted by the amino acid analysis (Table II) of the intact dimer it appears that the two protomers have very similar amino acid sequences.

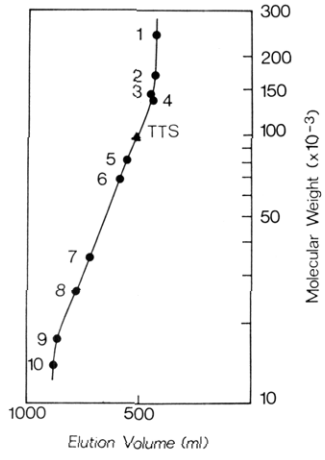

FIGURE 2: Molecular weight of native tyrosyl-tRNA synthetase by gel filtration. The conditions used are described in the Experimental Section and the proteins used to construct the standard curve were: (1) catalase, (2) *E. coli* methionyl-tRNA synthetase, (3) glyceraldehyde-3-phosphate dehydrogenase, (4) bovine serum albumin dimer, (5) creatine kinase, (6) bovine serum albumin, (7) myoglobin dimer, (8) chymotrypsinogen, (9) myoglobin, (10) lysozyme.

TABLE II: Amino Acid Composition of Tyrosyl-tRNA Synthetase from *B. stearothermophilus*.

Amino Acid	Residues/Mole of Enzyme	Amino Acid	Residues/Mole of Enzyme
Cya	4	Met	22
Asp	58	Ile	52
Thr	46	Leu	82
Ser	36	Tyr	34
Glu	88	Phe	36
Pro	12	Trp[a]	22
Gly	66	His	18
Ala	66	Lys	54
Val	46	Arg	56

[a] Estimated spectrophotometrically (Edelhoch, 1967).

Thus all the available evidence indicates that tryosyl-tRNA synthetase is a dimer made up of two protomers of identical amino acid sequence and resembles the tyrosyl-tRNA synthetase of *E. coli* (Calender and Berg, 1966) in being an enzyme of the α_2 type. However, it differs from the tyrosyl-tRNA synthetase from yeast (Beicherich *et al.*, 1972) which is reported to be a monomer with a molecular weight of about 45,000 and is one of the smallest known aminoacyl-tRNA synthetases.

Sulfhydryl Groups. The free cysteine residues in a protein are potentially the most promising sites for the attachment of heavy atoms in the preparation of isomorphous replacements for X-ray crystallographic studies. Consequently a detailed investigation of these residues is of considerable value in the preparation of heavy-atom derivatives. The amino acid analysis of tyrosyl-tRNA synthetase which had been oxidized with performic acid gave 4 mol of cysteic acid per mol of enzyme (Table II). Since this is a relatively small amount of cysteic acid special care was taken to ensure the accuracy of the analyses. Hydrolysates of oxidized chymotrypsinogen were used to check the color constant for cysteic acid and hydrolysates of oxidized *B. amyloliquifaciens* ribonuclease (Hartley and Barber, 1972) were used to ensure that no other hydrolysis product emerged at the same position as cysteic acid. Analyses of the ribonuclease (which contains no half-cysteine) gave a completely flat base line for cysteic acid showing that the analyses for cysteic acid are not likely to be overestimates.

Titrations of the free sulfhydryl groups in the native enzyme were carried out at room temperature with Nbs_2 at pH 7.5. Under these conditions 20 nmol of dithiothreitol was completely reacted in 5 min. In contrast a solution containing the equivalent of 80 nmol of protein half-cysteine reacted very slowly and after 6 hr the equivalent of 1 mol of –SH per mole of enzyme had been reacted (Figure 5). However, the addition of 1% sodium dodecyl sulfate to the reaction mixture resulted in a rapid exposure of more –SH groups and the final titration corresponded to four –SH groups per mole of enzyme. This is in good agreement with the number of cysteic acid residues obtained by amino acid analysis. It is also in agreement with the detection of only two radioactive peptides in tryptic digests of the reduced and ^{14}C-carboxymethylated enzyme. One of the radioactive peptides was obtained in high yield in the soluble fraction from the tryptic digest while the second was isolated from the insoluble tryptic peptides after digestion with thermolysin. No other

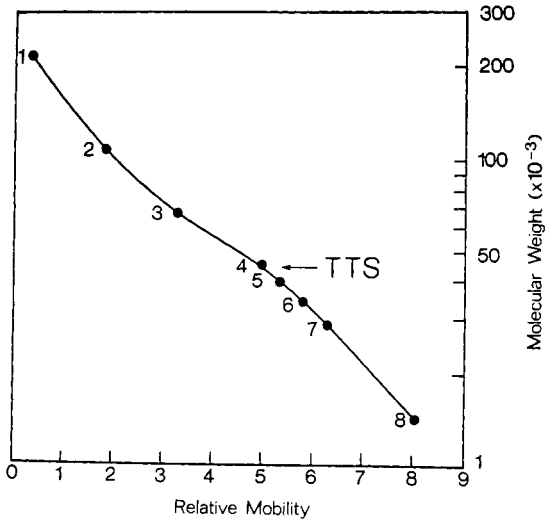

FIGURE 3: Molecular weight of the protomers of tyrosyl-tRNA synthetase by sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970). The proteins used to construct the standard curve were: (1) myosin, (2) oyster paramyosin, (3) bovine serum albumin, (4) muscle actin, (5) muscle aldolase, (6) glyceraldehyde-3-phosphate dehydrogenase, (7) carbonic anhydrase, (8) lysozyme.

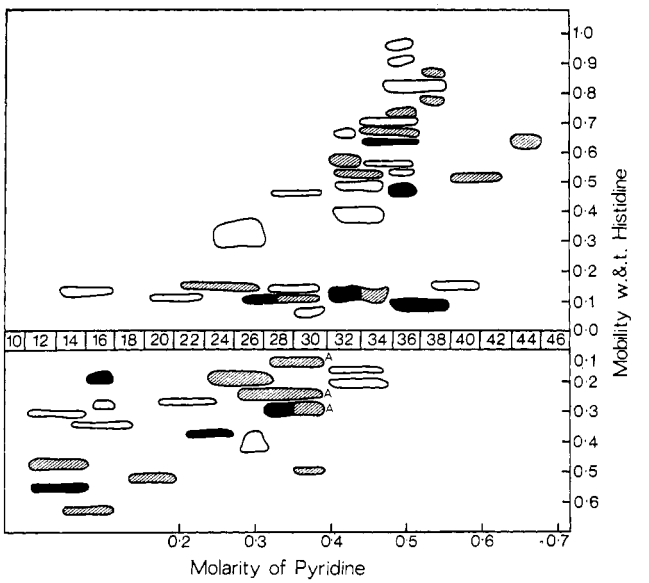

FIGURE 4: Separation of the tryptic peptides from oxidized tyrosyl-tRNA synthetase. The procedures used are described in the Experimental Section. Peptides which contained arginine are shaded lightly while those containing histidine are shaded heavily.

peptides contained a significant amount of radioactivity and it was concluded that there are only two unique cysteine sequences in the enzyme which is further evidence for the identity of the protomers. Although the rate of reaction of the native enzyme with Nbs_2 is relatively slow, the fact that at least 1 mol of -SH can be reacted per mole of enzyme suggests that mercurial reagents which are likely to be more reactive than Nbs_2 could provide suitable heavy-atom derivatives for the determination of the tertiary structure of the enzyme by X-ray crystallography.

Stability and Crystallization. The main objective in isolating the tyrosyl-tRNA synthetase from a thermophilic bacterium was to exploit its extra stability to obtain suitable crystals of the enzyme. Tests on the thermal stability of the pure enzyme confirmed the expected stability since it was found that the enzyme could be heated at 65° for 1 hr without any decrease in its activity in either the pyrophosphate exchange assay or in tRNA charging. Polyacrylamide gel electrophoresis or gel filtration of the heated enzyme showed no evidence for dissociation or aggregation. Thus there is little doubt that the enzyme possesses the desired stability. However, in spite of this it was found in early experiments on the crystallization of the enzyme that the small crystals which were formed with relative ease did not grow beyond about 20 μ. During these studies it was observed that in contrast to the thermal stability, stored samples of the enzyme, including those which had been used for crystallization, steadily lost activity and even showed signs of precipitation. Electrophoresis in sodium dodecyl sulfate gels showed that preparations which had been completely homogeneous just after purification had acquired several new polypeptide chains upon storage at 0° in solution. The pattern of disproportionation suggested that the heterogeneity had been generated by proteolysis during storage and crystallization. Consequently such samples were discarded and a combination of procedures adopted to prevent the proteolysis during preparation and storage. The cells used in the purification were harvested at mid-log phase to avoid the large amount of proteinases released at later stages of the growth cycle. The proteinase inhibitor $PhCH_2SO_2F$ was added to all buffers while solutions which had to be stored were made 50% in glycerol and kept at −20°. The adoption of these measures prevented the disproportionation and their value is evident from the facility with which the enzyme was then crystallized in a form which is very suitable for X-ray crystallography (Reid *et al.*, 1973).

Specificity of the Enzyme. A detailed study of the kinetic properties of the enzyme has not yet been completed but preliminary investigations clearly show that the enzyme catalyzes the esterification of tyrosine alone to tRNA. Under the conditions described in the Experimental Section (which are by no means optimal) about 2 μmol of tyrosine were esterified per min per mg of protein and it was not possible to detect the esterification of any other amino acid to unfractionated tRNA. It was interesting to note that the enzyme also charges tyrosine to unfractionated *E. coli* tRNA at a rate comparable to that obtaned with the homologous tRNA.

Concluding Remarks

The results of this study show that tyrosyl-tRNA synthetase from *B. stearothermophilus* can be isolated in quantities suf-

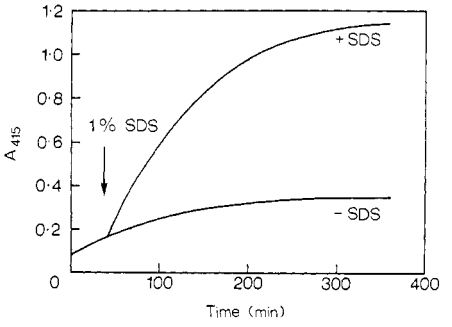

FIGURE 5: Titration of the sulfhydryl groups of tyrosyl-tRNA synthetase with Nbs_2; 20 μmol of protein were used in each experiment and under the conditions used a change in absorbance at 415 nm of 0.28 corresponds to the reaction of 20 μmol of -SH groups. In the experiments shown the change in absorbance with the undenatured enzyme was 0.27 while that in the presence of sodium dodecyl sulfate was 1.06. These correspond to the titration of one and four -SH groups per mole of protein, respectively.

ficient for structural analyses. The procedure described in this paper has recently been scaled up further and yields almost 1 g of protein. Although thermostable, the enzyme must be protected against proteolysis, otherwise crystallization is difficult to achieve. The native enzyme is composed of two very similar protomers with molecular weights of 45,000 each. This is consistent with the unit cell symmetry in the enzyme crystals which indicates a moiety of 45,000 daltons as the asymmetric unit (Reid *et al.*, 1973). One equivalent of cysteine can be reacted with Nbs_2 albeit quite slowly at room temperature although the full complement of half-cystine in the protein can be titrated upon denaturation with sodium dodecyl sulfate, showing that the enzyme does not contain disulfide bonds.

The convenient size of this enzyme, its substantial thermal stability, and the high quality of the crystals it yields render it an excellent candidate for the elucidation of the complete three-dimensional structure of an aminoacyl-tRNA synthetase and such studies are now in progress. Since a major source of interest in these enzymes is the structural basis of their bispecificity it is conceivable that this system will prove useful for the preparation and investigation of the complex formed between the enzyme and its cognate tRNA.

Acknowledgments

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Purification of Rat Urinary Kallikreins and Their Specific Antibody†

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ABSTRACT: Four biologically active and immunologically identical rat urinary kallikreins (B_1–B_4) have been obtained in highly purified form by pressure dialysis, chromatography on DEAE-Sephadex A-50 and hydroxylapatite columns, and electrofocusing. Molecular weights of 35,300, 33,600, 33,100, and 32,300 were estimated for kallikreins B_1, B_2, B_3, and B_4, respectively, by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis, and of 38,500 for B_3 by Bio-Gel P-200 gel filtration. A sheep was immunized against B_3, and an immune precipitate was prepared from antiserum and a crude urine concentrate. Antigen and antibody were dissociated in 8 M urea and separated on a column of Sephadex G-100. Both antigen and antibody were recovered in highly purified form by this simple procedure.

Mammalian glandular kallikreins are peptidylhydrolases (EC 3.4.4.21) of unique specificity. They are unusual in being able to break two dissimilar peptide bonds, Met–Lys and Arg–Ser, in plasma kininogens to liberate the extremely biologically active decapeptide, kallidin (lysylbradykinin: Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (see Pierce, 1968, 1970). The urinary kallikreins are of further interest because of their possible involvement in the regulation of local blood flow (Hilton, 1970), blood pressure and sodium balance (Croxatto and San Martín, 1970; Adetuyibi and Mills, 1972; Margolius *et al.*, 1972; Geller *et al.*, 1972).

To elucidate the origin and physiological function(s) of urinary kallikrein, it was deemed necessary to isolate the pure enzyme and its specific antibody. The present study describes the purification of rat urinary kallikreins, the preparation of specific antiserum, and the simple purification of rat urinary kallikreins and anti-kallikrein from an immune precipitate. Rat urine was found to contain four kallikreins distinguishable by both electrofocusing and polyacrylamide gel electrophoresis.

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