Biochimica et Biophysica Acta, 613 (1980) 514—525
© Elsevier/North-Holland Biomedical Press

BB 68969

PURIFICATION AND CHARACTERIZATION OF HISTIDYL-TRANSFER RNA SYNTHETASE FROM *NEUROSPORA CRASSA*

CHANG CHIU CHEN * and ETHEL W. SOMBERG **

Biochemistry Laboratory, Department of Zoology and Physiology, Rutgers, The State University, Newark, NJ 07102 (U.S.A.)

(Received July 11th, 1979)

Key words: Histidyl-tRNA synthetase; $tRNA^{His}$; (N. crassa)

Summary

Histidyl-tRNA synthetase (L-histidine:$tRNA^{His}$ ligase (AMP-forming), EC 6.1.1.21) has been purified 921-fold from crude extracts of lyophilized mycelia of *Neurospora crassa*. Sodium dodecyl sulfate gel electrophoresis at pH 8.9 of the purified enzyme yields one band with an apparent M_r of 62 500. The estimated M_r by Sephadex gel filtration is 125 000. Thus the native histidyl-tRNA synthetase of *N. crassa* is a dimer, composed of two identical subunits.

The K_m values determined in the enzyme-catalyzed esterification of [^{14}C]-histidine to $tRNA^{His}$ are: for histidine, $5.8 \cdot 10^{-6}$ M, for ATP, $5.9 \cdot 10^{-4}$ M, and for $tRNA^{His}$, $1.2 \cdot 10^{-7}$ M.

Effects of various intermediates of the histidine, tryptophan and arginine biosynthetic pathways on histidyl-tRNA synthetase activity were studied. The K_i values for imidazoleglycerol phosphate and histidinol (histidine intermediates and competitive inhibitors of the enzyme) are $1.1 \cdot 10^{-2}$ M, $1.3 \cdot 10^{-6}$ M, respectively. The K_i for indoleglycerol phosphate (a tryptophan intermediate and non-competitive inhibitor) is $1.2 \cdot 10^{-3}$ M.

Introduction

The aminoacyl-tRNA synthetases are complex enzymes [1—3]. They play a central role in protein synthesis, and they, or their products (aminoacyl-

* Present address: Department of Biochemical Nutrition, Hoffmann-La Roche, Nutley, NJ 07110, U.S.A.

** To whom correspondence should be addressed.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

tRNAs) are also involved in many other biosynthetic and regulatory processes [4—8].

Histidyl-tRNA synthetase (L-histidine:tRNA^{His} ligase (AMP-forming), EC 6.1.1.21) is of particular interest. Its role in the regulation of the histidine operon in *Salmonella typhimurium* is well known [9,10]. In many eucaryotic cells, a phenomenon called 'cross-pathway regulation' has been reported [11—15], in which starvation for any one of the three amino acids (tryptophan, histidine, and arginine) derepresses all three sets of the amino acid biosynthetic enzymes, and the products of histidyl-, tryptophanyl- and arginyl-tRNA synthetase: histidyl-tRNA, tryptophanyl-tRNA and arginyl-tRNA are required for repression [16].

Spurgeon and Matchett [17] has demonstrated in in vivo experiments that accumulation of indoleglycerol phosphate (a tryptophan intermediate) or imidazoleglycerol phosphate (a histidine intermediate) causes an increase in the synthesis of certain biosynthetic enzymes of the tryptophan, histidine and arginine pathways. These accumulated intermediates inhibited the activity of crude preparations of histidyl- and tryptophanyl-tRNA synthetases. The inhibitions noted were consistent with the decreased level of charged tRNA^{Trp} and tRNA^{His} in vivo.

We have purified histidyl-tRNA synthetase from *Neurospora crassa* and studied its structural and kinetics properties. The effects of various intermediates of the histidine, tryptophan and arginine pathways were also determined. We believe this is the first histidyl-tRNA synthetase to be isolated in pure form from an eucaryotic cell. A partially purified enzyme has been reported from yeast [18].

Materials and Methods

Growth of cultures

Stock cultures of *N. crassa* (wild type, Fungal Genetics Stock Center, No. 352-25a, Humboldt State College, Arcata, CA) were maintained on Vogel's medium N [19]. Mycelia for extraction of histidyl-tRNA synthetase were grown in shake cultures in 2-l Erlenmeyer flasks containing 1 l of Vogel's medium N supplemented with 2% sucrose at 30°C. Mycelia were harvested after 16 h of growth, washed several times with glass-distilled water, shredded into small pieces and lyophilized. The freeze-dried material was ground to a fine powder at room temperature with a mortar and pestle and stored at −20°C until further use.

Materials

$(NH_4)_2SO_4$ (enzyme grade) and L-[U-^{14}C]histidine in 50% C_2H_5OH (282 Ci/mol) were purchased from New England Nuclear. Protamine sulfate, unfractionated tRNA from Baker's yeast, L-histidinol and argininosuccinic acid from Sigma. Hydroxyapatite (Biogel HTP), reagents for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Affi-gel blue were from Bio-Rad Laboratories. Sephadex G-100 superfine and G-150 were from Pharmacia Fine Chemicals. L-Ornithine and D-*erythro*-imidazoleglycerol phosphate were obtained from Calbiochem-Boehring Company. DNA-agarose,

prepared according to the method of Schaller et al. [20], was a gift from Dr. John Germershausen. Indoleglycerol phosphate was a gift from Dr. W. Matchett. All other chemicals used were of reagent grade and were obtained from either Sigma or Merck and Company.

Buffers

Buffer A contained 10 mM Hepes (pH 7.4), 1 mM magnesium acetate, 1 mM KCl, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol. Buffer B contained 0.15 M Tris-HCl (pH 8.4), 1 mM sodium EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, 0.05 M NaCl and 10% glycerol. Phosphate buffers were designated as buffer 20, containing 20 mM potassium phosphate (pH 7.4); buffer 50, containing 500 mM potassium phosphate (pH 7.4). Other buffers used are specified in the text.

Preparation of tRNA from N. crassa

Total RNA was prepared from lyophilized mycelial powder by suspending 2 g in 28 ml 0.01 M Tris-HCl buffer (pH 7.2)/10 mM $MgCl_2$ at 4°C (all solutions were sterile). To this, 3 ml 5% SDS and 1.5 ml 1 M NaCl were added. The suspension was stirred at room temperature for 2 min, and an equal volume of redistilled phenol/$CHCl_3$/isoamyl alcohol (12 : 12 : 1, v/v) was added. Total RNA was extracted using the method of Perry et al. [21]. 83 A_{260} units of total RNA were applied to a 50 ml 5—20% sucrose gradient in 0.01 M Tris-HCl buffer (pH 7.9)/10 mM $MgCl_2$ and centrifuged for 19.5 h at 25 000 rev./min in a Beckman SW 25.2 rotor at 4°C. Fractions of 1.7 ml were collected from the bottom of the gradient and A_{260} determined. Three peaks corresponding to 4, 18, and 28 S were observed. The 4 S fraction was pooled and 2 vols. of cold 95% ethanol added. The precipitated tRNA was kept at −20°C overnight, collected by centrifugation and suspended in 0.5 M Tris-HCl buffer (pH 8.9) for 45 min at 37°C to remove endogenous amino acids. This was followed by dialysis for 12 h against glass-distilled water [22]. The solution containing the tRNA was frozen in small batches and stored at −20°C. For every 83 A_{260} units of total RNA, 9.5 A_{260} units of tRNA were obtained. In order to estimate the amount of $tRNA^{His}$ in the sample, we assumed 1 mg tRNA/ml equals 22 A_{260} units. The relative concentration of specific $tRNA^{His}$ was estimated after acylating total tRNA with L-[^{14}C]histidine in the presence of excess purified histidyl-tRNA synthetase. From these results it was calculated that 1 A_{260} unit of total tRNA contained 44.4 pmol $tRNA^{His}$.

Protein assay

Protein was determined according to the method of Shaffner and Weissman [23].

Histidyl-tRNA synthetase assay

Assay a. During the purification process, activity of the crude enzyme was routinely determined by measuring the esterification of [^{14}C]histidine to unfractionated yeast tRNA at 34°C. The reaction mixture contained in a final volume of 0.1 ml: 100 mM Hepes (pH 7.4), 10 mM magnesium acetate, 10 mM KCl, 4 mM disodium ATP (neutralized to pH 7.4), 39 μM [^{14}C]histidine, 0.4

mg yeast tRNA, 7 mM 2-mercaptoethanol and enzyme at various concentrations. After incubation at 34°C for 10 min the reaction was stopped by addition of 3 ml cold 10% trichloroacetic acid and the resulting suspension was chilled on ice. The precipitate was collected on a Metrical GN 6 membrane filter (pore size 0.45 μm) and washed several times with 10% trichloroacetic acid, dried and counted.

Assay b. Purified enzyme was used for kinetic studies, and standard assay conditions were selected so as to ensure optimal and linear reaction rats. The enzyme concentration was 1.4 ng in a 0.1 ml reaction volume. Incubations were at 34°C for 3 min. The reaction mixtures contained 100 mM Hepes (pH 6.5), 20 mM magnesium acetate, 10 mM KCl and 2 μg bovine serum albumin. Fixed (saturating) substrate concentrations were: ATP, 2.0 mM, [^{14}C]histidine, 24.5 μM, *Neurospora* tRNA, 53 μg. K_m and K_i values were calculated from Lineweaver-Burk plots [24].

Determination of the apparent M_r by gel filtration

A column of Sephadex G-150 (2.3 × 79 cm) was equilibrated and eluted with buffer 50 (pH 6.5). Calibration of the column was accomplished using catalase (M_r 245 000), aldolase (M_r 161 000), serum albumin (M_r 68 000), ovalbumin (M_r 45 000), myoglobin (M_r 18 800) and blue dextran 2000 (M_r 2 000 000).

Amino acid composition analysis

A sample containing 2 μg of purified histidyl-tRNA synthetase was hydrolyzed with 5.7 N HCl containing 0.05% (v/v) each of 2-mercaptoethanol and phenol at 110°C for 24 h and the sample analyzed in a Beckman 120 C Amino Acid Analyzer equipped with an automated fluorescamine detection system, according to the method of Stein et al. [25]. 200 pmol norleucine was used as an internal standard.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate

Enzyme purity was analyzed by SDS slab gel electrophoresis in 10% acrylamide. The gel buffer contained 0.35 M Tris-HCl (pH 8.9) and 0.1% SDS. A 13.5 × 10.5 × 0.2 cm gel was formed. Protein samples (5—20 μg) in 25 μl were adjusted to a final concentration of 0.025 M Tris-HCl (pH 6.8), 1% SDS, 10% glycerol and 1% 2-mercaptoethanol, and heated in boiling water for 2 min or longer. After electrophoresis, protein was visualized by staining with 0.3 g Coomassie blue in 500 ml of 50% CH_3OH and 7% acetic acid for 16 h at room temperature, and destained with 7.5% acetic acid and 5% CH_3OH. The mobility was calculated as

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

and plotted against the molecular weights on a semilogarithmic scale. Protein standards, serum albumin, catalase, γ-globulin, ovalbumin, carbonic anhydrase were electrophoresed at the same time.

Purification of the enzyme

All steps of the purification procedure were carried out at 4°C.

Crude extract. 60 g lyophilized mycelial powder was suspended in 1.5 l buffer A and homogenized for twice 1 min at 0°C in a Kontes glass homogenizer. The extract was centrifuged for 30 min at 30 000 × *g*. The supernatant (fraction I) containing enzyme activity was collected for further purification.

Protamine sulfate step. 17 mg/ml protamine sulfate solution (pH 5.3) was added dropwise to fraction I and stirred for 30 min at 4°C. The mixture was centrifuged at 30 000 × *g* for 30 min. The precipitate containing most of the nucleic acids and small acidic proteins was discarded, the supernatant (fraction II) contained all the enzyme activity. It was fractionated immediately with $(NH_4)_2SO_4$.

$(NH_4)_2SO_4$ fractionation. To this solution, $(NH_4)_2SO_4$ was added slowly with stirring to give 45% saturation, the pH was adjusted to 7.4. The precipitate contained less than 1% of the enzyme activity. The supernatant which contained most of the activity was brought to 90% saturation by $(NH_4)_2SO_4$. After centrifugation, the precipitate was taken up in buffer A (fraction III) and dialyzed for 24 h against three changes of 4 l buffer A and one change of 2 l buffer A/50% glycerol.

Batch hydroxyapatite treatment. Fraction III was added to 800 ml hydroxyapatite slurry, previously equilibrated with buffer 2. The mixture was stirred for 30 min and centrifuged for 15 min at 10 400 × *g*. The hydroxyapatite pellet was suspended in 2 l buffer 20. After centrifugation, the hydroxyapatite pellet was treated successively with 2 l each of buffer 30 and buffer 40. The buffer 40 eluate contained 45% of the enzyme activity. It was concentrated to 130 ml by use of an Amicon ultrafiltration apparatus. The resulting suspension (fraction IV) was dialyzed against 1 l buffer A followed by 1 l buffer A/50% glycerol. 20% of the enzyme activity was detected at 0.3 M phosphate concentration. However, the specific activity was only slightly higher than that of fraction III; therefore, it was not included in further purification procedures.

Affi-gel blue column chromatography. Dialyzed fraction IV was applied to a column (3.3 × 12 cm) containing Affi-gel blue agarose equilibrated in buffer A. After the sample had been absorbed to the column bed, the column was washed successively with 300 ml buffer A, 300 ml buffer A/0.25 M KCl, and 400 ml buffer A/0.6 M KCl. Enzyme activity was found in the 0.6 M KCl eluant fraction. This fraction was concentrated and dialyzed overnight against 1 l buffer A/50% glycerol. The final volume of fraction V was about 7 ml. This step provided a 5-fold purification with good recovery, and was completed in less than 2 h.

DNA-agarose column chromatography. Dialyzed fraction V was applied to a DNA-agarose column (2.2 × 15 cm) equilibrated in buffer B. After the sample had been absorbed, the column bed was washed with 20 ml buffer B. The column was eluted with a linear gradient of 0.05—0.5 M NaCl in buffer B. The total volume of the gradient was 200 ml. Fractions containing enzyme activity were detected in the 0.3 M NaCl region (Fig. 1), pooled as indicated, and dialyzed overnight against buffer 50/50% glycerol. The final volume of fraction VI after concentration was 2.5 ml.

Sephadex G-100 gel filtration. Fraction VI was applied to a 2.3 × 67.5 cm

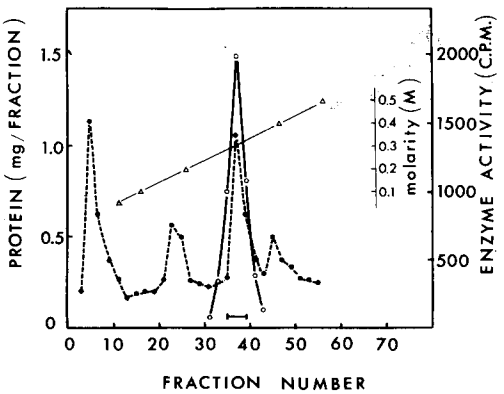

Fig. 1. Elution profile obtained from affinity chromatography of Fraction V on DNA-agarose. Enzyme activity (○———○) was measured as described in Materials and Methods under assay a, and is expressed as radioactivity in cpm. Protein (●- - - - - -●) was determined according to method of Shaffner and Weissman [23]. △———△, NaCl molarity of the eluting gradient. The solid bar indicates the fractions pooled for the next step.

column of Sephadex G-100 (superfine) gel. The column, packed by gravity, was previously equilibrated with buffer 50 and eluted with the same buffer. Histidyl-tRNA synthetase activity was eluted at about 99 ml. Tubes containing enzyme purified 2-fold or more were pooled, concentrated and dialyzed against buffer A/50% glycerol (fractionVII).

Hydroxylapatite column chromatography. Fraction VII containing 1.2 mg of protein in 3.8 ml was loaded on a 1 ml hydroxyapatite column (in a Pasteur pipette) previously equilibrated in buffer 2. The column was eluted with 3 ml buffer 15, followed by 3 ml buffer 35. Enzyme activity was found in the 0.35 M potassium phosphate fraction. This was dialyzed against 100 ml buffer A/50% glycerol and stored at −20°C (fraction VIII).

Results

Purification of the enzyme

A summary of the eight step purification procedure is presented in Table I.

TABLE I

PURIFICATON OF HISTIDYL-tRNA SYNTHETASE FROM *N. CRASSA*

Fraction		Volume (ml)	Protein (mg)	Total activity (cpm)	Specific activity (cpm/μg)	Recovery (%)	Purification factor
I	Crude extract	1302	8379.6	$9.8 \cdot 10^8$	$1.16 \cdot 10^2$	100	1
II	Protamine sulfate	1450	5308	$1.06 \cdot 10^9$	$1.99 \cdot 10^2$	100	1.7
III	$(NH_4)_2SO_4$	90	1680	$1 \cdot 10^9$	$5.95 \cdot 10^2$	100	5.1
IV	Hydroxyapatite (I)	45	252	$4.5 \cdot 10^8$	$1.78 \cdot 10^3$	45	15.3
V	Affi-gel blue	6.7	27.8	$2.5 \cdot 10^8$	$8.99 \cdot 10^3$	25	77.0
VI	DNA-agarose	2.5	6.0	$1.5 \cdot 10^7$	$2.50 \cdot 10^4$	15	214.2
VII	Sephadex G-100	3.8	1.2	$7.5 \cdot 10^7$	$6.25 \cdot 10^4$	7.5	535.5
VIII	Hydroxyapatite (II)	1	0.279	$3 \cdot 10^7$	$1.07 \cdot 10^5$	3	921.3

The specific activity of the purified histidyl-tRNA synthetase in fraction VIII was 462, measured by esterification assay b as μmol of histidyl-tRNA formed/mg of enzyme at 34°C for 1 h. This is about 921 times that of the crude extract.

Purity of the enzyme

When fraction VII was treated as described in Materials and Methods and 5 μg applied to an SDS-polyacrylamide gel, two bands were discernible. When 5 μg of fraction VIII were treated similarly only one band was discernible, thus the enzyme is considered to be essentially homogeneous. It is possible that any protein contaminant less than 0.5 μg may escape detection by this method.

Stability of the enzyme

The purified histidy-tRNA synthetase, stored in buffer A containing 50% glycerol, is stable for at least 6 months at −20°C. In all steps of the purification procedure following treatment with $(NH_4)_2SO_4$ fractionation, 50% glycerol was required to protect the enzyme. Storing the enzyme preparation at 4°C without glycerol for more than 18 h results in a loss of 80% of activity. Freezing and thawing resulted in loss of all activity. Addition of 0.1 mM phenylmethylsulfonyl fluoride in the homogenizing buffer gave higher yields of stable enzyme, presumably by inhibition of protease activity [26].

Determination of the apparent molecular weight

The M_r of the native enzyme was estimated to be 125 000 from its elution profile on a column of Sephadex G-150 using catalase, aldolase, serum albumin, ovalbumin and myoglobin as protein markers assuming compact globular structure for each protein. The M_r value of histidyl-tRNA synthetase from *N. crassa* is higher than that of the *Escherichia coli* enzyme (M_r = 85 000) [27] and of the *S. typhimurium* enzyme (M_r 100 000) [28].

Evidence for subunits

Histidyl-tRNA synthetase subjected to SDS-polyacrylamide gel electrophoresis with appropriate markers, demonstrated a single band corresponding to a M_r of 62 500, therefore it can be concluded the enzyme is a dimer composed of subunits having apparently identical mass.

Amino acid composition

The amino acid composition of the purified enzyme was determined as described in Materials and Methods and is listed in Table II, together with data on histidyl-tRNA synthetase from *E. coli* [27] and arginyl-tRNA synthetase from *N. crassa* [29]. No significant similarities could be found. It is of interest to note that the *N. crassa* enzyme has no cysteine residues.

Optimal conditions for assay

The requirements for the esterification of histidine to tRNA were studied. Using standard assay b and 1.4 ng of the purified enzyme, the assay was linear up to 5 min. The optimal ATP concentration was approx. 2 mM. The optimal Mg^{2+} concentration was found to be 20 mM. Although histidyl-tRNA synthe-

TABLE II

AMINO ACID COMPOSITION OF HISTIDYL-tRNA SYNTHETASE FROM *N. CRASSA* AND *E. COLI*, AND ARGINYL-tRNA SYNTHETASE FROM *N. CRASSA*

n.d., not determined.

Amino acid	Composition (mol/mol enzyme)		
	Histidyl-tRNA synthetase		Arginyl-tRNA synthetase * *N. crassa*
	N. crassa	*E. coli* **	
Lysine	64	35.5	73
Histidine	30	36.6	21
Arginine	74	15.7	32
Aspartic acid/asparagine	103	63.3	75
Methionine	19	—	7
Threonine	65	34.6	40
Serine	71	36.6	58
Glutamic acid/glutamine	101	82.6	90
Proline	58	22.5	27
Glycine	134	75.9	78
Alanine	124	71.5	64
Half-cystine	—	7.9	5
Alanine	96	40.3	37
Isoleucine	61	29.2	32
Leucine	100	84.2	67
Phenylalanine	44	22.0	22
Tryptophan	n.d.	1.81	21
Tryosine	26	3.5	21

* See Ref. 29.
** See Ref. 27.

tase from *E. coli* required 2-mercaptoethanol for its activity [27], a requirement for reducing agents was not established in the case of the *N. crassa* enzyme. The presence of bovine serum albumin was required in order to prevent the inactivation of the purified enzyme.

The enzyme has a narrow pH range, and displays a maximum activity at pH 6.5 in 0.1 M Hepes buffer. The Michaelis constants for histidine, ATP and $tRNA^{His}$ were measured and were $5.8 \cdot 10^{-6}$ M, $5.9 \cdot 10^{-4}$ M and $1.2 \cdot 10^{-7}$ M, respectively.

Inhibition by indoleglycerol phosphate

Fig. 2A is a double-reciprocal plot of data obtained by varying histidine concentration from 1.8 to 9.1 μM in the presence of indoleglycerol phosphate, an intermediate in the tryptophan biosynthetic pathway, at 0.75 and 2.2 mM. The K_i value for indoleglycerol phosphate is $1.2 \cdot 10^{-3}$ M. The pattern of inhibition is non-competitive.

Effect of imidazoleglycerol phosphate

Imidazoleglycerol phosphate, an intermediate in the histidine biosynthetic pathway has been shown to inhibit the charging of $tRNA^{His}$ in vivo [17] and to inhibit the activity of a crude preparation of histidyl-tRNA synthetase of *N. crassa*. By varying the histidine concentration from 1.8 to 9.2 μM in the pres-

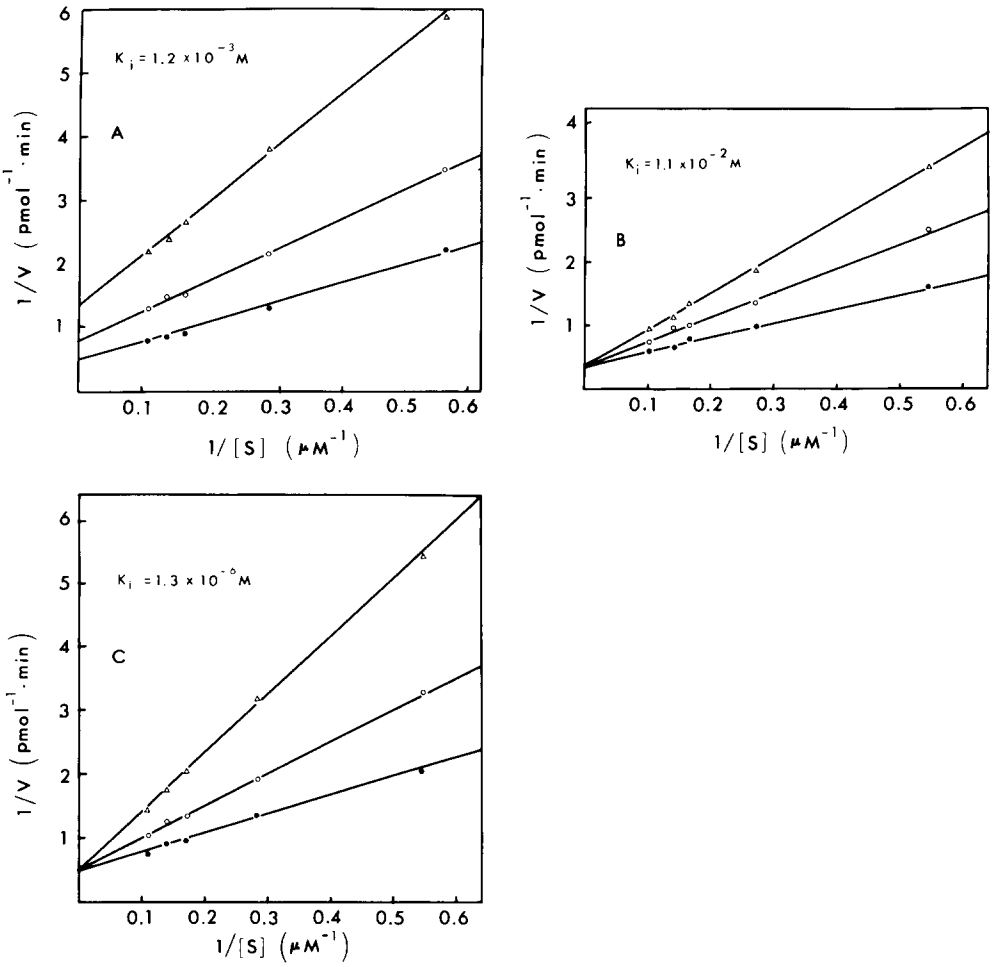

Fig. 2. Determination of K_i values for indoleglycerol phosphate (A), imidazoleglycerol phosphate (B) and L-histidinol (C). The assay conditions are described in Materials and Methods under assay b. The concentration of histidine was varied as indicated. In (A) the concentrations of indoleglycerol phosphate were: △, 2.2 mM; ○, 0.75 mM, and ●, 0 mM. In (B) the concentrations of imidazoleglycerol phosphate were: △, 20 mM; ○, 10 mM, and ●, 0 mM. In (C), the concentrations of histidinol were: △, 3 μM; ○, 1 μM, and ●, 0 μM. The K_i values for the intermediates were determined by linear regression analysis.

ence of imidazoleglycerol phosphate at 10 mM and 20 mM, the K_i for imidazoleglycerol phosphate is $1.1 \cdot 10^{-2}$ M and the pattern of inhibition was competitive (Fig. 2B). This is expected, since imidazoleglycerol phosphate resembles histidine structurally while indoleglycerol phosphate does not.

Inhibition by histidinol

Histidinol, an intermediate in the histidine biosynthetic pathway, is an analogue of histidine and a well-known inhibitor of several procaryote histidyl-tRNA synthetases [30,31]. It is found to be a competitive inhibitor with a K_i of $1.3 \cdot 10^{-6}$ M (Fig. 2C). Therefore histidinol is a 10 000-times more potent inhibitor than imidazoleglycerol phosphate, and a 1000-times more potent inhibitor than indoleglycerol phosphate.

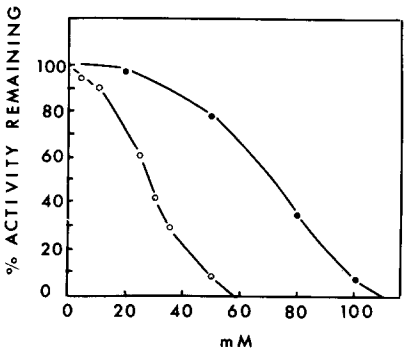

Fig. 3. Effect of varying concentrations of L-argininosuccinate and L-ornithine on the rate of histidyl-tRNA formation. The rate of uninhibited reaction (3 pmol/min, with a concentration of 24.5 μM histidine) was considered to be 100%. Values at the various concentrations of L-argininosuccinate (●) and L-ornithine (○) were plotted as the percentage of the rate of the uninhibited reaction. The assay conditions were the same as those described in Materials and Methods under assay b.

Effect of ornithine and argininosuccinic acid

According to Lester's polyrepressor hypothesis [16], tryptophan, histidine, and arginine biosynthetic enzymes are under the same regulatory control, i.e. aminoacyl-tRNAs, therefore it was of interest to see whether intermediates of the arginine biosynthetic pathway had any effect on the purified histidyl-tRNA synthetase. Fig. 3 shows that ornithine at 28 mM inhibited 50% of the enzyme activity, while 72 mM argininosuccinic acid was required to demonstrate a 50% inhibition of the enzyme activity. They are both 'poor' inhibitors compared to histidinol and indoleglycerol phosphate.

Discussion

Histidyl-tRNA synthetase has been purified from *S. typhimurium* [28], *E. coli* [27] and partially purified from yeast [18]. To our knowledge this is the first report on the isolation in pure form and characterization of this enzyme from eucaryotic cells.

Aminoacyl-tRNA synthetases are often difficult enzymes to purify. The procedures adapted may be laborious with low overall yield. Most synthetases are unstable at temperatures higher than 4°C and may require special agents such as glycerol to protect activity during the purification. This also proved to be the case for histidyl-tRNA synthetase from *N. crassa*. The enzyme preparation was unstable at 4°C after the $(NH_4)_2SO_4$ fractionation, unless glycerol was added to a final concentration of 50%. Eight steps of purification were required to obtain the enzyme in pure form and the overall yield was 3%. The recovery may have been increased by utilizing a broader cut in the batch hydroxyapatite treatment; however, the same degree of final purification might not have been achieved. The crude enzyme preparation was not retained by CM-cellulose. In contrast to the *E. coli* enzyme and the *S. typhimurium* enzyme, it was not retained by phosphocellulose. When the crude extract was applied to a DEAE-cellulose column, the enzyme activity was eluted with 0.1 M KCl and an 8-fold increase in the specific activity was achieved, however, 80% of the activity was

lost. This was also found to be the case during the purification of the yeast enzyme [18] and the *E. coli* enzyme [32]. Addition of 10% glycerol during DEAE-cellulose column chromatography did not protect the enzyme from inactivation, and higher amounts of glycerol in the system greatly decreased flow rate, making this procedure impractical.

Single-stranded DNA-agarose has been used successfully as an affinity chromatographic material for the purification of DNA- and RNA-binding enzymes [20,33—35]. Histidyl-tRNA synthetase from *N. crassa* was retained by the DNA-agarose column and gradient elution yielded enzyme activity at 0.3 M NaCl concentration. A 3-fold purification with 60% yield was accomplished by the step. This may prove to be a useful procedure in purification of other aminoacyl-tRNA synthetases.

Chromatographic and electrophoretic studies indicated that the histidyl-tRNA synthetase has an apparent M_r of 125 000 and consists of two apparently identical subunits. The *E. coli* K12 histidyl-tRNA synthetase isolated by Kalousek and Konigsberg [27] has an M_r of 85 000 and consists of identical monomers. The *S. typhimurium* enzyme is also a dimer and has an M_r of 100 000 [28]. It seems likely that the dimeric structure of histidyl-tRNA synthetase has been preserved during evolution with an increase in molecular weight. This is also the case for other aminoacyl-tRNA synthetases [1].

Although mitochondrial aminoacyl-tRNA synthetases have been identified in *N. crassa* [36,37], the results from SDS gel electrophoresis indicates only a single species of histidyl-tRNA synthetase in the enzyme preparation described.

Spurgeon and Matchett [17] investigated the inhibition of histidyl-tRNA synthetase by various intermediates of amino acid biosynthetic pathways in a relatively crude preparation containing all of the aminoacyl synthetases obtained by protamine sulfate precipitation and back extraction with $(NH_4)_2SO_4$. 4 mM indoleglycerol phosphate was reported to inhibit the charging activity of histidyl-tRNA synthetase by 50%. Imidazoleglycerol phosphate at 3 mM was reported to produce 20% inhibition of histidyl-tRNA charging activity, and at 6 mM, only 5% more inhibition was produced. Argininosuccinate (concentration unspecified), an intermediate in the arginine biosynthetic pathway, was reported to have no effect on the charging of histidine. The K_i values for indoleglycerol phosphate, imidazoleglycerol phosphate, L-histidinol, and concentrations required for 50% inhibition by L-ornithine and L-argininosuccinate on the purified histidyl-tRNA are 1.2 mM, 11.0 mM, 1.3 μM, 28 mM, and 72 mM, respectively.

It would appear that the inhibition of the enzyme by these intermediates (with the exception of L-histidinol) is not physiologically significant under normal metabolic conditions. However, under conditions of arginine limitation or in the presence of citrulline, the *arg-10* mutant of *N. crassa* accumulates argininosuccinic acid up to 40 mM, that is, five times higher than its K_i value (8 mM) [29] for arginyl-tRNA synthetase. It is possible that *N. crassa* amino acid auxotrophs, or perhaps wild-type cells, may accumulate high levels of certain intermediates under varying physiological conditions, and therefore these may display some regulatory functions in vivo.

It is also possible that various intermediates are not uniformly distributed in the cell, local concentrations may reach a high level (in the mM range) thus

enabling them to exert a regulatory role. Since most eucaryotes are large in volume than procaryotes, this type of compartmentation might be advantageous.

Acknowledgements

This work was supported by the Biomedical Research Support Grant RR 7059 and the Busch Memorial Fund.

References

1 Ofengand, J. (1977) in Molecular Mechanisms of Protein Biosynthesis (Weissbach, H. and Pestka, S., eds.), pp. 7—79, Academic Press, New York
2 Söll, D. and Schimmel, P. (1974) in The Enzymes (Boyer, O., ed.), 3rd edn., Vol. X, pp. 489—538, Academic Press, New York
3 Kisselev, L.L. and Favorova, O.O. (1974) Adv. Enzymol. 40, 141—238
4 Littauer, U.Z. and Inouye, H. (1973) Annu. Rev. Biochem. 42, 439—470
5 Neidhadt, F.C., Parker, J. and Mckeever, W.G. (1975) Annu. Rev. Microbiol. 29, 215—250
6 Brenchley, J.E. and Williams, L.S. (1975) Annu. Rev. Microbiol. 29, 251—274
7 Stephens, J.C., Artz, S.W. and Ames, B.N. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4380—4393
8 Moore, P.A., Jayme, D.W. and Oxender, D.L. (1977) J. Biol. Chem. 252, 7427—7430
9 Brenner, M. and Ames, B.N. (1971) Metab. Pathways 5, 349—387
10 Wyche, J.H., Ely, B., Cebula, T.A., Snead, M.C. and Hartman, P.E. (1974) J. Bacteriol. 117, 708—716
11 Carsiotis, M., Jones, R.F. and Wesseling, A.C. (1974) J. Bacteriol. 119, 893—898
12 Carsiotis, M. and Jones, R.F. (1974) J. Bacteriol. 119, 889—892
13 Guerzoni, M.E. (1972) Arch. Mikrobiol. 86, 57—68
14 Schürch, A.E., Miozzari, O.J. and Hutter, R. (1974) J. Bacteriol. 117, 1131—1140
15 Lara, J.C. and Mills, S.E. (1973) Abstr. Annu. Meet. Am. Soc. Microbiol. P5, 141
16 Lester, G. (1971) J. Bacteriol. 107, 193—202
17 Spurgeon, S.L. and Matchett, W.H. (1977) J. Bacteriol. 129, 1303—1312
18 Von Tigerstrom, M. and Tener, G.M. (1967) Can. J. Biochem. 45, 1067—1074
19 Vogel, H.J. and Bonner, D.M. (1956) Microbiol. Genet. Bull. 13, 42
20 Schaller, H., Nüsslein, C., Bonhoeffer, F.J., Kurz, C. and Nietzschmann, I. (1972) Eur. J. Biochem. 29, 474—481
21 Perry, R.P., LaTorre, J., Kelley, D.E. and Greenberg, J.R. (1972) Biochim. Biophys. Acta 262, 220—226
22 Mosteller, R.D., Culp, W.J. and Hardesty, B. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 1817—1824
23 Shaffner, W. and Weissman, C. (1973) Anal. Biochem. 56, 502—514
24 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
25 Stein, S., Bohlen, P., Stone, J., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 202—212
26 Fahrney, D.E. and Gold, A.M. (1963) J. Am. Chem. Soc. 85, 997—1000
27 Kalousek, F. and Konigsberg, W.H. (1974) Biochemistry 13, 999—1006
28 DeLorenzo, F. and Ames, B.N. (1970) J. Biol. Chem. 245, 1710—1716
29 Nazario, M. and Evans, J.A. (1974) J. Biol. Chem. 249, 4934—4942
30 Hansen, B.S., Vaughan, M.H. and Wang, L.J. (1972) J. Biol. Chem. 247, 3854—3857
31 Lepore, G.C., Di Natale, P., Guarini, L. and DeLorenzo, F. (1975) Eur. J. Biochem. 56, 369—374
32 McCorquodale, D.J. (1964) Biochim. Biophys. Acta 91, 541—548
33 Bolton, E.T. and McCarthy, B.J. (1962) Proc. Natl. Acad. Sci. U.S.A. 48 , 1390—1397
34 Bendich, A.J. and Bolton, E.T. (1968) Methods Enzymol. 12B, 635—640
35 Germershausen, J. (1978) Ph.D. Thesis, Rutgers University, Newark, NJ
36 Weeks, C.O. and Gross, S.R. (1971) Biochem. Genet. 5, 505—514
37 Beuchamp, P.M. and Gross, S.R. (1976) Nature 261, 338—340