The results confirm that a conformational change accompanies the binding of substrate to the galatose-binding protein. This changes does not involve a major refolding of the peptide backbone but is rather confined to small alterations that seem to involve only the active site region of the molecule. The substrate-induced fluorescence increase and ultraviolet difference spectra are for the most part a result of a direct interaction between the substrate and a tryptophan residue present in the active site. At least one other tryptophan is involved, however, and this residue appears to lie in the immediate vicinity of an amino acid(s) which develops a more negative charge concomitant with substrate binding. The role of this conformational change in the normal physiological functioning of the galactose-binding protein remains to be established.

Acknowledgments

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Purification and Characterization of Histidyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*†

Frantisek Kalousek and William H. Konigsberg*

ABSTRACT: Histidyl-tRNA synthetase from *Escherichia coli* K12 was purified 820-fold to apparent homogeneity as shown by electrophoresis in polyacrylamide gels at pH 8.5 and in polyacrylamide gels containing sodium dodecyl sulfate. No other aminoacyl-tRNA synthetase activity could be detected. The enzyme has a mol wt of ~85,000 and is a dimer consisting of apparently identical 42,500-dalton subunits. The enzyme has the following characteristics: (a) two molecules of ATP and histidine are bound per molecule of enzyme; (b) the K_m values for ATP and histidine are similar to those found for the substrates of other aminoacyl-tRNA synthetases; (c) histidinol is an effective inhibitor; (d) two sulfhydryl groups per molecule of the histidyl-tRNA synthetase reacted with 5,5′-dithiobis(2-nitrobenzoic acid) and the SH groups are essential for enzymatic activity; (e) a low concentration of trypsin destroys the enzyme activity.

The proper attachment of amino acids to their cognate tRNAs by aminoacyl-tRNA synthetases is one of the crucial steps for ensuring fidelity in the translation of the genetic code. This fidelity depends on the correct recognition of both the amino acid and the corresponding tRNA by the aminoacyl-tRNA synthetase. There seems to be only one such enzyme for each of the 20 amino acids (Lengyel and Söll, 1969). Many of them have been purified and characterized from procaryotic and eucaryotic organisms.

These enzymes have been classified into three groups based on their quaternary structure (Muench and Joseph, 1971). The apparent diversity in quaternary structure among these enzymes has prompted speculation that some of them are involved in other functions of the cell such as regulation of certain biosynthetic enzymes (Calvo and Fink, 1971). The histidyl-tRNA synthetase might be involved in this way since mutants having a lesion in the structural gene for this enzyme show elevated

† From the Department of Molecular Biophysics and Biochemistry, Yale University Medical Center, New Haven, Connecticut 06510. *Received June 25, 1973.* Supported by a research grant from the U. S. Public Health Service (GM 12607).

levels of the enzymes required for histidine biosynthesis (Roth *et al.*, 1966). The His-tRNA synthetase[1] from *Salmonella typhimurium* has been purified 800-fold and characterized with respect to molecular weight, and various kinetic parameters exhibited by this enzyme have been determined (De Lorenzo and Ames, 1970).

Because of the high levels and apparent stability of His-tRNA synthetase[1] in crude extracts of *Escherichia coli* and because of its unusual properties, *e.g.*, it is one of the few synthetases that can be retained by phosphocellulose, we decided to purify and characterize the enzyme with a view toward using it for crystallization and for comparison with other synthetases prepared in our laboratory (Katze and Konigsberg, 1970). In particular we are attempting to correlate the quaternary structure of several synthetases with some of their functional properties (Waterson *et al.*, 1973; Waterson and Konigsberg, 1973).

Here we describe the purification of His-tRNA synthetase from *E. coli* K12. Some properties of the purified enzyme including its stability, substrate specificity, amino acid composition, molecular weight, and the number of subunits are presented. Studies on the binding of histidine and ATP, titration of sulfhydryl groups, and dissociation of the enzyme into monomers of equal mass support the notion that histidyl-tRNA synthetase from *E. coli* is a dimer made up of identical subunits.

Materials and Methods

E. coli K12 (CA 244) was a generous gift of D. Söll. *E. coli* tRNA was obtained from Schwarz/Mann. L-[3-^{3}H]Histidine, uniformly labeled [^{3}H]ATP, and sodium [^{32}P]pyrophosphate were purchased from New England Nuclear. Polyethylenimine was purchased from Brinkmann Instruments. DEAE-Cellulose, type DE-52, and phosphocellulose were obtained from Whatman. Hydroxylapatite was prepared according to the method of Main *et al.* (1959). All other chemicals were of analytical grade. Seryl-tRNA synthetase, prepared by the method of Katze and Konigsberg (1970), was a gift of S. Clarke.

Protein Assay. The protein concentration was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard or by using the 280/260 absorbancy ratio as described by Warburg and Christian (1942). Column effluents were monitored using the absorbance at 280 nm.

Assay of Histidyl-tRNA Synthetase. (a) ATP–PP$_i$ EXCHANGE ASSAY. The activity of the enzyme as measured by histidine-dependent exchange of PP$_i$ into ATP with [^{32}P]-pyrophosphate was determined as described by Calendar and Berg (1966). The assay was modified by reducing the final volume to 0.1 ml and using 50 mM Tris-HCl (pH 8.0) as the buffer.

(b) AMINOACYLATION ASSAY. The activity was measured by determining the rate of histidyl-tRNA formation at 37°. The reaction mixture (0.05 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM magnesium acetate, 4 mM 2-mercaptoethanol, 2 mM ATP, 20 μM L-[^{3}H]histidine, 50 μg of tRNA, and enzyme at various concentrations. After incubation at 37° for 10 min, 40-μl aliquots were pipeted onto 2.3-cm Whatman No. 3MM paper disks which were washed, dried, and counted as described by Rubin *et al.* (1967). One unit of the enzyme activity was defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of histidine into histidyl-tRNA in 10 min.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman Model 120B amino acid analyzer. Samples were hydrolyzed in 3 ml of redistilled 6 N HCl for the various times. Half-cystine was determined by performic acid oxidation of the enzyme followed by HCl hydrolysis (Moore, 1963). Tryptophan was determined after hydrolyzing the sample in the presence of 5% thioglycolic acid (Matsubara and Sasaki, 1969).

Estimation of Labeled Nucleotides. Electrophoresis on thin layer plates of cellulose was carried out for the separation of labeled ATP from degraded products using 0.1 M sodium citrate buffer (pH 4.5). The radioactive spots were scraped into vials and counted.

Analytical Polyacrylamide Gel Electrophoresis. Discontinuous polyacrylamide gel electrophoresis was carried out in 7.5% gels (pH 8.9) (Davis, 1964). Sodium dodecyl sulfate–acrylamide gel electrophoresis in 5% gels was performed according to the method of Weber and Osborn (1969). Potassium ions were removed from the sample by dialysis against 0.05 M sodium phosphate buffer (pH 7.1), 10% glycerol, and 20 mM 2-mercaptoethanol. Samples were incubated overnight at 37° with 1% 2-mercaptoethanol and 1% sodium dodecyl sulfate.

Preparation of Antiserum. Rabbit antiserum was prepared by injection of 300 μg (0.2 ml) of the purified enzyme mixed with 4 vol of complete Freund's adjuvant. One-third of the suspension was injected into the footpads and the rest subcutaneously. Similar injections were administered 3 weeks later. Eight weeks after the first injection, blood was collected by heart puncture and the serum separated, centrifuged, and stored at −20°. Bleeding was repeated 3 times at intervals of 3 weeks.

Equilibrium Dialysis. These studies were performed using dialysis cells similar to those described by Myer and Schellman (1962) but with the volume reduced to 0.1 ml per cell compartment. All volume measurements were made using a Hamilton syringe. Enzyme solution, buffer, and substrate were added to give a final volume of 0.095 ml in each compartment. The final concentrations of the components were: enzyme, 1.1 mg/ml; histidine, 1–50 μM; and ATP, 10–500 μM. The membrane separating the compartments was cut from 23/32 Visking dialysis tubing, boiled in 0.25 M sodium bicarbonate containing 0.1 mM EDTA, and then thoroughly washed with distilled water. Before use they were soaked for at least 24 hr in the buffer used for the experiment (0.1 M potassium phosphate (pH 7.0)–4 mM 2-mercaptoethanol–10 mM magnesium acetate). The contents of the cell compartments were stirred at 4° until equilibrium was reached (20 hr for histidine and 45 hr for ATP). Three samples of 20 μl each were removed from each compartment and counted in 5 ml of Bray's scintillation fluid.

Titration of Sulfhydryl Groups. Sulfhydryl group determination was carried out using Nbs_2 at pH 7.4 (Ellman, 1959).

Determination of Molecular Weight by Equilibrium Centrifugation. Sedimentation equilibrium studies were carried out in a Spinco Model E ultracentrifuge by the method of Yphantis (1964) using the standard double interference cell. The ultracentrifuge was equipped with Rayleigh interference optics. The temperatures of the runs were 8 and 25°, respectively, and all solutions contained 50 mM Tris (pH 7.4) and 4 mM dithiothreitol. A value of 0.73 ml/g was used for the

[1] Abbreviations used are: His-tRNA synthetase, histidyl transfer ribonucleic acid synthetase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); *p*-HgBzO, *p*-mercuribenzoate; MalNEt, *N*-ethylmaleimide; PP$_i$, inorganic pyrophosphate.

TABLE I: Purification of Histidyl-tRNA Synthetase.

Fraction	Vol (ml)	Protein (mg)	Act. (Units × 10^{-5})	Sp Act. (Units/mg)	Recovery (%)	Purification Factor
I, crude extract	4000	112,000	167	149	100	1
II, polyethylenimine	4000	59,000	153	260	91	1.75
III, DEAE-cellulose	1500	14,000	76.5	546	46	3.7
IV, phosphocellulose	48	111	67.5	60,700	40	406
Va, preparative gel electrophoresis	7.1[a]	21.7	25.1	116,000	15.1	780
Vb, hydroxylapatite	16[a]	28.0	36.7	121,800	22	830

[a] Calculated to the total amount of fraction IV.

partial specific volume, based upon the amino acid composition (McMeekin and Marshall, 1952).

Results

Purification of the Enzyme. All steps in the purification were performed at 0–4° and the results are summarized in Table I.

Crude Extract. Frozen *E. coli* paste (1100 g) was suspended in 4500 ml of 50 mM potassium phosphate (pH 7.0) containing 2 mM 2-mercaptoethanol. The cells were ruptured in a Sorvall-Ribi refrigerated cell fractionator at a pressure of 20,000 psi. The lysate was centrifuged for 30 min at 16,000*g* and the resulting supernatant (fraction I) (4000 ml) was used in the subsequent steps.

Polyethylenimine Precipitation.[2] To fraction I which had a 280/260 ratio of 0.55 and a protein concentration of 28 mg/ml, 550 ml of 5% polyethylenimine was added dropwise with continuous stirring. The pH of the polyethylenimine was adjusted beforehand to 7.0 with solid Tris. The solution was then stirred for an additional 30 min followed by 30 min of centrifugation at 10,000*g*. The clear supernatant was essentially free of nucleic acids and ribosomes which precipitate under these conditions. All histidyl-tRNA synthetase activity was in the supernatant (fraction II, 4000 ml), which had a 280/260 ratio of 0.90.

DEAE-Cellulose Fractionation. Glycerol was added to fraction II to give a final concentration of 10%. The sample was then applied to a column of DEAE-cellulose (7.5 × 30 cm) which had been previously equilibrated with a buffer composed of 50 mM potassium phosphate (pH 7.0), 2 mM 2-mercaptoethanol, and 10% glycerol. The flow rate during adsorption of the sample was kept at 180 ml/hr. After adsorption the column was washed with 3500 ml of the same buffer which had, in addition, 50 mM potassium chloride. Elution was carried out with a linear concentration gradient of potassium chloride (50–150 mM) contained in the phosphate buffer. The total volume of the gradient was 12 l. The flow rate during washing and elution was increased to 360 ml/hr and 26-ml fractions were collected. Tubes containing enzyme activity of at least twice the specific activity of fraction II were pooled and concentrated against 30% polyethylene glycol–2 mM 2-mercaptoethanol to give a final volume of 1500 ml (fraction III).

Phosphocellulose Chromatography. Fraction III was applied to a column of phosphocellulose (7.5 × 27 cm) equilibrated with a buffer containing 20 mM potassium phosphate (pH 7.0), 2 mM 2-mercaptoethanol, and 10% glycerol. After adsorption of the sample, the column was washed with 1500 ml of the same buffer until the optical density of the effluent approached zero. The enzyme was eluted with a linear gradient made by mixing 2000 ml of 20 mM and 2000 ml of 250 mM potassium phosphate (pH 7.0), containing 2 mM mercaptoethanol and 10% glycerol (Figure 1). The flow rates during adsorption, washing, and elution were the same as those used for DEAE-cellulose chromatography. Fractions containing the enzyme activity were pooled and concentrated as before to 48 ml (fraction IV).

For the final purification, either preparative gel electrophoresis or fractionation on hydroxylapatite was used.

Preparative Gel Electrophoresis. Glycerol was added to 15 ml of fraction IV to give a final concentration of 25%. This solution was then carefully layered under the upper electrophoresis buffer and subjected to electrophoresis in a Büchler preparative gel apparatus using a 7.5% polyacrylamide gel. The height of the resolving gel was 3 cm, and the volume was 60 ml. The stacking gel was omitted. The upper buffer contained 20 mM Tris (pH 8.5), 15 mM glycine, 5 mM cysteine, and 20 mM 2-mercaptoethanol. The lower buffer consisted of 120 mM Tris-HCl (pH 8.1), 20 mM 2-mercaptoethanol, and 10% glycerol. The gel was prerun for 30 min at 30 mA before the sample was applied and during the run the current was kept at 50 mA. The peak of the activity (which usually appeared after 12 hr) was pooled, dialyzed against 30% polyethylene glycol containing 50 mM potassium phosphate (pH 7.0) and 5 mM 2-mercaptoethanol, and then stored in 50% glycerol–5 mM 2-mercaptoethanol at −20° (fraction Va).

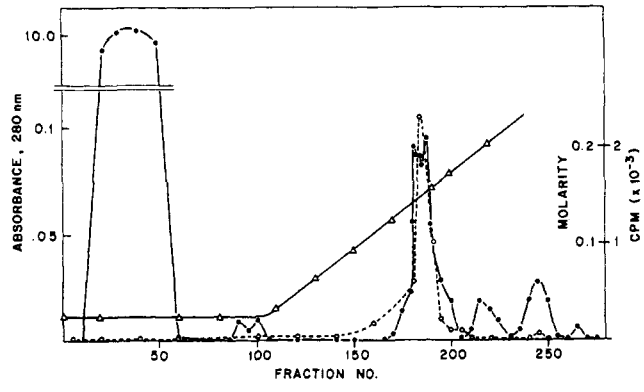

FIGURE 1: Elution profile of histidyl-tRNA synthetase from a phosphocellulose column. The concentration of phosphate was calculated on the basis of the molarity of phosphate anions. Other details are described in the text: (●) absorbance; (○) activity; (Δ) molarity.

[2] Personal communication, G. Weimann, Boehringer Mannheim Corp.

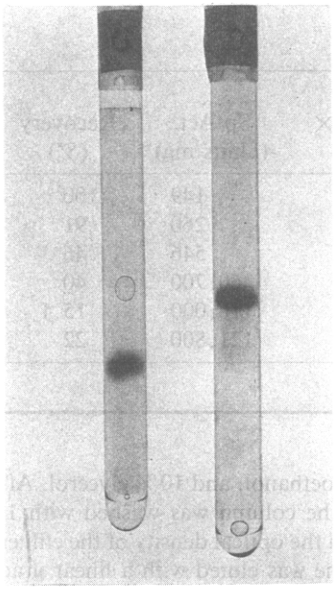

FIGURE 2: Polyacrylamide gel electrophoresis of purified histidyl-tRNA synthetase: (a) 15 μg of fraction VIb at pH 8.5; (b) 30 μg in the presence of sodium dodecyl sulfate.

Chromatography on the Hydroxylapatite. The remaining part of fraction IV (33 ml) was applied to a column of hydroxylapatite (0.9 × 22 cm) which had previously been equilibrated with 20 mM potassium phosphate (pH 8.0), 2 mM 2-mercaptoethanol, and 10% glycerol. The flow rate of the column was adjusted to 18 ml/hr. Before application to the column the sample was diluted with 10% glycerol to give a conductivity equal to that of the initial buffer. The column was washed with 100 ml of the same buffer and then with 300 ml of 50 mM potassium phosphate buffer (pH 8.0). Histidyl-tRNA synthetase was eluted by a linear concentration gradient from 50 to 250 mM potassium phosphate (pH 8.0), total volume 400 ml. Fractions of 9 ml were collected, and the major portion of the enzyme was pooled, concentrated against 30% polyethylene glycol containing 5 mM 2-mercaptoethanol, and stored at 20° (fraction Vb).

General Remarks on the Purification Procedure. In some cases the cells were ruptured by sonication. Although this procedure required more time (about 6 hr for 1 kg of *E. coli*), fraction I prepared in this way had a higher specific activity, compared to fraction I prepared by the Sorvall-Ribi fractionator.

Histidyl-tRNA synthetase is very sensitive to oxidation. Since incubation of the enzyme at 20° for 30 min in the absence of reducing agents caused 90% inactivation, 2-mercaptoethanol or dithiothreitol was added to all buffers. It has been shown that glycerol has a stabilizing effect on aminoacyl-tRNA synthetases (Katze and Konigsberg, 1970; Joseph and Muench, 1971) and in some cases even increased the yield of the enzyme in the first steps of the purification (Muench and Berg, 1966). However, we did not find any stabilizing effect of glycerol during the first steps in the purification of the histidyl-tRNA synthetase.

The removal of nucleic acids and ribosomes from fraction I by precipitation with polyethylenimine appeared to be a more efficient and faster method than autolysis which can damage the aminoacyl-tRNA synthetases (Lemoine *et al.*, 1968). It has an advantage over ammonium sulfate precipitation or liquid polymer extraction where large losses of enzyme activity often occur (Berthelot and Yaniv, 1970; Kalousek and Rychlik, 1965). The use of polyethylenimine as a general reagent for partial fractionation of proteins and nucleic acids will be described in detail elsewhere.

TABLE II: Amino Acid Dependent ATP-$^{32}PP_i$ Exchange by Purified Histidyl-tRNA Synthetase (Fractions Va and Vb).[a]

Addition to Standard Reaction Mixture	ATP-$^{32}PP_i$ Exchange (cpm)
L-Histidine	16,200
19 amino acids plus L-histidine	9,287
19 amino acids minus L-histidine	710
19 amino acids minus L-histidine plus crude tRNA	815

[a] Assay conditions were the same as described under Methods. The concentration of all amino acids used was 2×10^{-4} M. tRNA was used at a concentration of 0.4 mg/ml of incubation mixture.

His-tRNA synthetase is accompanied by three other proteins with slower electrophoretic mobility in the effluent during elution from phosphocellulose. This enabled us to obtain good separation on polyacrylamide gel electrophoresis. Only Whatman phosphocellulose from two types that were tested (Whatman and MN 2100 P) had the ability to bind histidyl-tRNA synthetase under our conditions. NM 2100 P did not bind any His-tRNA synthetase, even if the ionic strength of potassium phosphate buffer was lowered to 5 mM.

Criteria of Purity. The degree of purity of the His-tRNA synthetase preparation was estimated from the results of analytical polyacrylamide gel electrophoresis run both under standard conditions (Tris-glycine buffer (pH 8.5)) and in the presence of sodium dodecyl sulfate (Figure 2). In both instances only one band appeared, even when more than 40 μg of protein was applied to the gels. To test for other amino acid activating enzymes which might have been present in the His-tRNA synthetase preparation at levels too low to detect by protein staining, the ATP-$^{32}PP_i$ exchange reaction was measured using a mixture of 19 amino acids with the omission of histidine. As can be seen from the results in Table II, the amount of ATP-$^{32}PP_i$ exchange stimulated by the amino acid mixture was less than 2% of that stimulated by L-histidine.[3] These results indicate only a very low level of contamination of the His-tRNA synthetase preparation by other amino acid activating enzymes. In the case of glutaminyl- and argininyl-tRNA synthetases, which require $tRNA^{Gln}$ (Ravel *et al.*, 1965) and $tRNA^{Arg}$ (Mitra and Mehler, 1966) for ATP-$^{32}PP_i$ exchange to occur, an additional control was run which contained tRNA in addition to the 19 amino acids minus L-histidine and no exchange was observed (Table II). Inhibition of the exchange reaction was found (Table II) when the other 19 amino acids were included with L-histidine. A similar effect has been noticed with *E. coli* tryptophanyl-tRNA synthetase (Joseph and Muench, 1971).

Determination of Molecular Weight. The molecular weight range of the native enzyme was initially estimated as 84,000 ± 3000 by its elution position on a column of Sephadex G-150 using rabbit muscle aldolase (mol wt 149,000), *E. coli* seryl-

[3] To calculate the exact amount of contaminating aminoacyl-tRNA synthetases is nearly impossible as there is a great difference in the rate of reaction for different enzymes (Mehler, 1970).

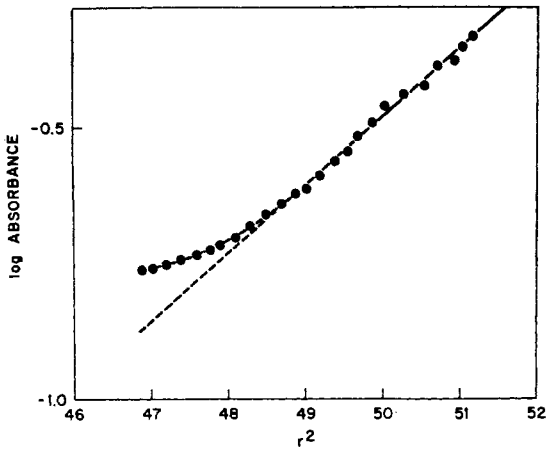

FIGURE 3: Sedimentation equilibrium centrifugation at 25° (7000 rpm for 48 hr). The concentration of protein was 0.400 A_{280} unit/ml.

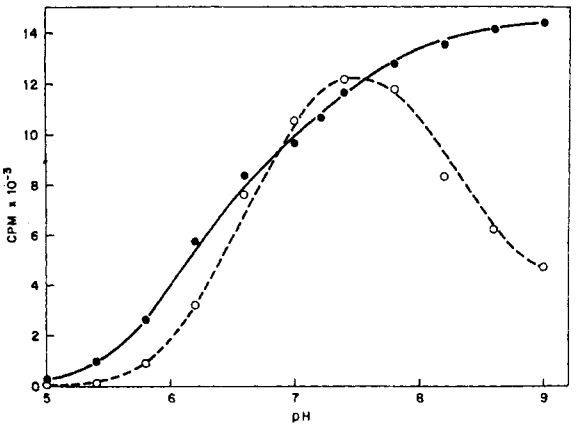

FIGURE 4: The effect of pH on the ATP-$^{32}PP_i$ exchange reaction (●) and the rate of histidyl-tRNA formation (○). Experiments were described under Methods except that the pH was varied. Sodium cacodylate (pH 5.0–7.0) and Tris-HCl (pH 7.0–9.0) were used.

tRNA synthetase (mol wt 98,000), ovalbumin (mol wt 43,000) and lysozyme (mol wt 17,200) as protein markers assuming compact globular structures for each protein. A more precise value was obtained by sedimentation equilibrium centrifugation. A linear plot of ln $(y_r - y_0)$ against r^2 was obtained indicating homogeneity of the sample and lack of dissociation or aggregation under the conditions used. The molecular weight determined under these conditions gives a value of 86,000 ± 1500. However, when higher temperatures (25°) were used during the run, the native enzyme tended to aggregate giving a mol wt of 90,000 at the meniscus and 175,000 at the bottom of the cells (Figure 3). More than 80% of activity was lost in this case.

Evidence for Subunits. When the histidyl-tRNA synthetase was run in sodium dodecyl sulfate–polyacrylamide gels with appropriate markers, under the conditions described by Weber and Osborn (1969), a single band corresponding to the mol wt of 41,000–42,000 daltons was observed indicating that the enzyme is a dimer composed of subunits having apparently identical mass.

Histidine Analogs. The most potent inhibitors of histidine-dependent ATP-$^{32}PP_i$ exchange were L-histidinol and L-histidine hydroxamate. However, none of the compounds listed in Table III promote the ATP-$^{32}PP_i$ exchange. The small amount of stimulation observed with L-histidine hydroxamate was probably due to partial breakdown of the hydroxamate to free histidine.

Optimal Assay Conditions. The effect of pH on both the exchange reaction and aminoacylation of tRNA is shown in Figure 4. The optimal pH for histidyl-tRNA formation was 7.4 and for histidine-dependent ATP-$^{32}PP_i$ exchange it was between pH 8.0 and 9.0. Inorganic pyrophosphate which inhibits the charging reaction catalyzed by seryl-tRNA synthetase (Katze and Konigsberg, 1970) and arginyl-tRNA synthetase (Mitra and Mehler, 1967) did not have any effect on the charging of $tRNA^{His}$ at concentrations up to 4×10^{-5} M, although a small amount of inhibition was observed at concentrations of 2.5×10^{-4} M. The optimal concentration of PP_i for exchange was 2 mM but concentrations even five times higher did not influence the rate of exchange by the enzyme. The optimal concentration of Mg^{2+} was between 5 and 10 mM; a concentration of 15 mM decreased the rate of aminoacylation by 44%. The apparent K_m values for histidine (Lineweaver and Burk, 1934) in the exchange and charging reactions and for ATP in the exchange reaction were determined (Table IV). The values are comparable to those published for other aminoacyl-tRNA synthetases (Katze and Konigsberg, 1970; Joseph and Muench, 1971). The differences in apparent K_m values in two different reactions, *e.g.*, exchange and acylation, were also observed for the other enzyme (Katze and Konigsberg, 1970).

TABLE III: Effect of Analogs of Histidine on Histidyl-tRNA Synthetase Activity.

Analogs	ATP-$^{32}PP_i$ Exchange in the Presence of L-Histidine (%)	ATP-$^{32}PP_i$ Exchange in the Absence of L-Histidine (%)	L-[^{3}H]-Histidine Incorpd into tRNA (%)
None	100	0	100
D-Histidine	63	0	85
N-Acetylhistidine	86	0	61
1,2,4-Triazole-3-alanine	51	0	75
L-Histidinol	12.5	0	34
L-Histidine hydroxamate	10.1	20	81

[a] The details are described under Methods. The concentration of analogs was 20 mM for histidine-dependent ATP-$^{32}PP_i$ exchange, 10 mM when tested as a substrate for the exchange, and 0.02 mM in the aminoacylation reaction.

TABLE IV: Apparent K_m Values for Substrates of Histidyl-tRNA Synthetase.[a]

Substrate	K_m for ATP-$^{32}PP_i$ Exchange	K_m for Aminoacylation of tRNA
L-Histidine	1.0×10^{-4}	6×10^{-6}
ATP	3.2×10^{-4} (pH 7.4)	
	1.5×10^{-4} (pH 8.0)	

[a] Experiments were described under Methods except that the substrate concentration was varied. The K_m values were determined from the Lineweaver–Burk plot (1934).

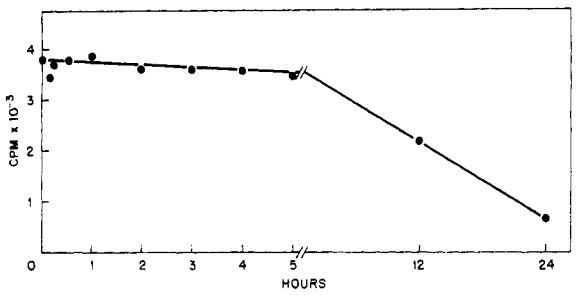

FIGURE 5: Stability of the histidyl-tRNA synthetase at 37°. Fraction VIb (200 μl) diluted to the concentration 0.5 mg/ml was used. At specified intervals, 10 μl of the enzyme was pipeted and the charging activity was measured under the conditions given under Methods.

Stability of the Enzyme. Histidyl-tRNA synthetase retains its initial activity for more than 4 months when kept at −20° in 50% glycerol containing 5 mM 2-mercaptoethanol. When 2-mercaptoethanol is omitted, the enzyme loses 80% of its activity in 48 hr. The activity is only partly restored by re-addition of 2-mercaptoethanol. When the enzyme was incubated at 37°, the activity was essentially unchanged for at least 5 hr. Even after 12 hr of incubation only 40% of the enzymatic activity is lost (Figure 5). Glycerol does not have any stabilizing effect when the enzyme is incubated at 37°. On the other hand the absence of 2-mercaptoethanol caused a total loss of activity in a few minutes.

Amino Acid Composition. The amino acid composition of the purified enzyme, expressed in numbers of residues per 84,000 daltons dimer, is shown in Table V. Except for tyrosine and tryptophan the composition does not differ markedly from the composition of other aminoacyl-tRNA synthetases (Stern and Peterkofsky, 1967; Yaniv and Gros, 1969; Helene *et al.*, 1971).

TABLE V: Amino Acid Composition of Histidyl-tRNA Synthetase.

Amino Acid	Residues/84,000 g of Enzyme[a]			
	A	B	C	Av
Lys	34.4	36.7	*b*	35.5
Arg	14.7	16.6	*b*	15.7
His	35.7	37.5	*b*	36.6
Asp	61.0	61.0	67.8	63.3
Thr	38.4	33.0	32.3	34.6
Ser	37.8	36.8	35.0	36.6
Glu	84.0	78.5	85.2	82.6
Pro	22.2	21.4	23.4	22.5
Gly	76.4	77.0	74.3	75.9
Ala	70.5	71.7	71.5	71.5
Half-Cys			7.9[c]	7.9
Val	40.7	40.3	37.4	40.3[e]
Met	9.1	9.7	10.4[d]	
Ile	30.6	29.2	26.3	29.2[e]
Leu	83.5	87.1	82.0	84.2
Tyr	3.5			3.5
Phe	22.6	21.4		22.0
Trp	1.82			1.82

[a] A, 24 hr with dithioglycol; B, 72 hr; C, performic acid. [b] Not estimated. [c] Determined as cysteic acid. [d] Determined as methionine sulfone. [e] 72-hr value used.

TABLE VI: Effect of Sulfhydryl Reagents on Histidyl-tRNA Synthetase.[a]

Additions	ATP-$^{32}PP_i$ Exchange	His-tRNA Formation
None	100	100
p-HgBzO, 1×10^{-5} M	31	5
p-HgBzO and 2-mercaptoethanol, 1×10^{-3} M	100	98
p-HgBzO, ATP, and Mg^{2+}	28	*b*
p-HgBzO and L-histidine	21	*b*
p-HgBzO, ATP, Mg^{2+}, and L-histidine	106	*b*
MalNEt, 1×10^{-4} M	72	64
MalNEt, 1×10^{-3} M	45	<1
Iodoacetamide, 1×10^{-3} M	102	95
Iodoacetic acid, 1×10^{-3} M	94	87

[a] Histidyl-tRNA synthetase free of 2-mercaptoethanol but with 10^{-6} M dithithreitol present was incubated with various sulfhydryl reagents in Tris-HCl buffer (pH 7.4) for 30 min at 37° following additional 15-min incubation at the same temperature with substrates. Concentrations used were 0.1 mM histidine, 1 mM ATP, and 5 mM magnesium acetate. After the treatment His-tRNA synthetase was assayed as described under Materials and Methods. [b] Not estimated.

Sulfhydryl Group Determination. The effect of various reagents that attack sulfhydryl groups is given in Table VI. In agreement with an earlier observation made with crude extract (Stern and Peterkofsky, 1967), treatment of histidyl-tRNA synthetase with *p*-chloromercuribenzoate destroys 70% of the ATP-$^{32}PP_i$ exchange activity and nearly all charging activity. In the presence of L-histidine, ATP, and Mg^{2+} the enzyme is completely protected from inactivation by *p*-HgBzO but the presence of both substrates is essential. Neither iodoacetic acid nor iodoacetamide affected the activity and no carboxymethylation of the enzyme was detected by using [^{14}C]iodoacetic acid during 60 min of incubation with the reagent. *N*-Ethylamaleimide, however, inhibited both charging and exchange reactions. When the native enzyme was titrated with Nbs_2, 1.9 reactive sulfhydryl groups per molecule of native enzyme (dimer) were detected (Figure 6). Since the dialysis in the absence of dithiothreitol or 2-mercaptoethanol resulted in the loss of His-tRNA synthetase activity the enzyme had to be titrated in the presence of small quantities of dithiothreitol. Although this compound reacts with Nbs_2 instantaneously it does not interfere with the titration of sulfhydryl groups in the enzyme since the dithiothreitol and Nbs_2 blank can be subtracted. The titration is accompanied by the total loss of both enzymatic activities. As in the case of *p*-chloromercuribenzoate, the presence of histidine and ATP markedly slows the reaction of sulfhydryl groups and the activity of the enzyme is only slightly affected (Figure 6). The enzyme, denatured by 6 N guanidinium chloride, contained 6.8 mol of sulfhydryl groups per 85,000 daltons.

Immunological Titration. A rabbit antiserum prepared against pure histidyl-tRNA synthetase (fractions Va and Vb, Table I) eliminates the aminoacylation activity of both the purified enzyme and the enzyme present in fresh cell extracts of *E. coli* (Figure 7). The same amount of the serum is required in both cases. The neutralization of 0.6 unit of pure enzyme requires 0.5 μl of antiserum. In contrast, ATP-$^{32}PP_i$ exchange

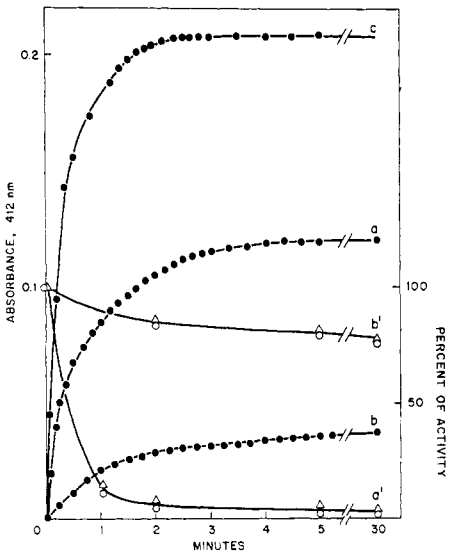

FIGURE 6: Titration of sulfhydryl groups with Nbs_2. Histidyl-tRNA synthetase was dialyzed before titration against 0.05 M Tris-HCl (pH 7.4) in the presence of dithiothreitol (1.5 μg/ml). Dialyzed enzyme (0.4 mg) was titrated with Nbs_2 (final concentration 1×10^{-4} M) in a volume of 1 ml. The production of 5-thio(2-nitrobenzoic) anion was measured by the increase of absorbance at 412 nm. A molar absorbance coefficient of 13,600 was used for the calculations. From a parallel experiment with native enzyme 25-μl aliquots of sample were taken (at indicated intervals), diluted, and assayed for enzymatic activity: (●) absorption at 412 nm; (a) native enzyme; (b) native enzyme plus 10^{-3} M ATP; 10^{-3} M Mg^{2+} and 10^{-4} M L-histidine; (c) enzyme with 5 M guanidinium chloride. In experiment c only half the amount of the enzyme was used compared to a and b: (a',b') activity as expressed by acylation of tRNA (○) and by ATP-$^{32}PP_i$ exchange reaction (Δ).

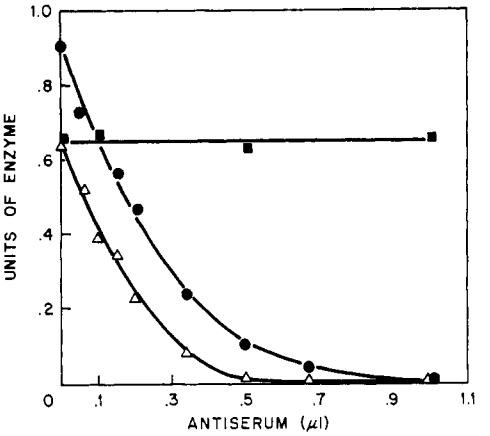

FIGURE 7: Titration of histidyl-tRNA synthetase with rabbit antiserum. Enzyme and antiserum were incubated for 20 min at 37° and then assayed for activity: (●) crude extract; (Δ) purified histidyl-tRNA synthetase; (■) control. As a control, antiserum prepared against pure *E. coli* seryl-tRNA synthetase was used.

was not affected by a concentration of antiserum 50 times higher than that required to block the aminoacylation.

Binding of L-*Histidine and ATP.* The binding of L-histidine and ATP was studied by equilibrium dialysis. Extrapolation of the binding curve in the Scatchard plots (Scatchard, 1949) to saturating concentrations gave the value of 2 mol of L-histidine and 2 mol of ATP bound per mol of enzyme. The affinity constant for L-histidine was 3.6×10^4 M^{-1}, indicating a K_s value of 2.8×10^{-5} M, and that for ATP was 2.2×10^3 M^{-1}, indicating a K_s of 4.5×10^{-4} M, which is close to the K_m value for histidine and ATP as determined from the kinetics of the ATP-PP_i exchange reaction. Because lysyl-tRNA synthetase from *E. coli* and yeast contained traces of ATPase activity (Rymo *et al.*, 1972) which required the correction for the binding of ATP we also checked this activity in our enzyme. Even in prolonged incubation at 4° no substantial degradation of ATP was observed.

Discussion

The purification of histidyl-tRNA synthetase results in an enzyme that appears to be homogeneous as indicated by the single bands observed during gel electrophoresis under different conditions. The enzyme has been purified 820-fold with a yield of 22%. Sedimentation, chromatographic, and electrophoretic studies suggest that the enzyme has an apparent mol wt of 85,000 and consists of two apparently identical subunits. Histidyl-tRNA synthetases from two other organisms have been described previously—one partially purified from yeast (von Tigerstrom and Tenner, 1967) and one from *S. typhimurium* (De Lorenzo and Ames, 1970) which was purified to the apparent homogeneity. The histidyl-tRNA synthetases from *E. coli* K12 and *S. typhimurium* seem to be very similar. Both enzymes have the same molecular weight and similar elution profiles when chromatographed on hydroxylapatite and on phosphocellulose.

Of the seven–eight cysteine residues in His-tRNA synthetase (85,000 daltons) only two reacted rapidly with Nbs_2 resulting in loss of both charging and exchange activities. As expected, the activity could be partially restored by reducing agents. Neither histidine nor ATP alone can protect the enzyme against reaction with Nbs_2, which suggests that the histidyl–AMP–enzyme complex is required for protection. The formation of such a complex may result in conformational changes in the enzyme where normally reactive sulfhydryl groups are now relatively inaccessible. The protective effect of histidyladenylate against sulfhydryl reagent is similar to that observed for isoleucyl-tRNA synthetase (Iaccarino and Berg, 1969) and for methionyl-tRNA synthetase (Bruton and Hartley, 1970). The differential reactivity observed with iodoacetamide and iodoacetic acid *vs.* that found with other sulfhydryl reagents such as *N*-ethylmaleimide may be explained by the geometry of the residues around the reactive sulfhydryl groups which might inhibit the displacement of the iodine atom. A rabbit antiserum directed against purified enzyme neutralized all histidyl-tRNA synthetase activity from crude extracts as measured by attachment of histidine to tRNA. The observation that the ATP-$^{32}PP_i$ exchange reaction was not affected at all by antiserum may be explained by the difference in the size of the substrates. The antibody–enzyme complex might block the binding of tRNA but not of histidine and ATP. A similar effect was observed in our laboratory in the case of seryl-tRNA synthetase (Katze and Konigsberg, 1970). On the other hand it should be noted that several reports concerning the loss of ATP-$^{32}PP_i$ exchange reaction during titration with enzyme-specific antisera have been reported (Calendar and Berg, 1966; Baldwin and Berg, 1966). The inhibition of the enzyme in crude extracts by His-tRNA synthetase specific antiserum and the presence of one peak of activity under different chromatographic conditions indicate that only a single species of histidyl-tRNA synthetase exists in *E. coli*. It was reported recently that primary structures of $tRNA^{His}$ from *S. typhimurium* and *E. coli* K12 (Singer and Smith, 1972; Harada *et al.*, 1972) are identical and it would be interesting to see if both histidyl-tRNA synthetases are serologically related.

One of the most potent inhibitors among the analogs of histidine was L-histidinol. The same compound has been also found to be a reversible inhibitor of protein biosynthesis in cultured human cells where its presence probably decreases the amount of activated histidine (Hansen *et al.*, 1972) because the histidine-dependent ATP–$^{32}PP_i$ exchange reaction is inhibited. Competitive inhibition by histidinol has been also described in the case of histidyl-tRNA synthetase from *S. typhimurium* (Ames and Hartman, 1971).

Recently Cassio and Waller (1971) were able to achieve limited proteolysis of methionyl-tRNA synthetase without loss of enzymatic activity. Limited proteolytic cleavage of leucyl-tRNA synthetase affected only aminoacylation but not ATP–$^{32}PP_i$ exchange (Rouget and Chapeville, 1971). It was of interest to see if similar results could be obtained with histidyl-tRNA synthetase. Our preliminary results (Kalousek and Konigsberg, 1973) show that histidyl-tRNA synthetase as well as other aminoacyl-tRNA synthetases with dimeric structures (with the exception of methionyl-tRNA synthetase) lose both ATP–$^{32}PP_i$ exchange and charging activity under conditions of limited proteolysis, whereas aminoacyl-tRNA synthetases having single chains are unaffected by this treatment.

The single band observed in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate provides evidence that the subunits have identical molecular weights. Evidence concerning the functional identity of the subunits comes from the observation that the native enzyme can bind two molecules of histidine and ATP. The molecular weights of most native aminoacyl-tRNA synthetases from *E. coli*, regardless of their quaternary structure, are in the range of 100,000 daltons. The enzymes which are dimers bind two molecules of substrate (Waterson *et al.*, 1973; Rymo *et al.*, 1972) whereas those composed of a single chain such as valyl-tRNA synthetase from yeast and *E. coli* and also isoleucyl-tRNA synthetase from *E. coli* have one binding site for the substrate per native molecule (Berthelot and Yaniv, 1970; Rymo *et al.*, 1972). The possible origin and function of these two classes of aminoacyl-tRNA synthetases are currently being investigated (Waterson and Konigsberg, 1973).

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