Chemical and Physical Studies on the Structure of the Histidyl Transfer Ribonucleic Acid Synthetase from Salmonella typhimurium*

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SUMMARY

The histidyl-tRNA synthetase from Salmonella typhimurium was purified to homogeneity by methods described previously. The molecular weight of the native enzyme was determined as 78,000 by high speed equilibrium centrifugation and as 92,000 by gel filtration; a subunit molecular weight of about 40,000 was found by centrifugation in 6 M guanidine HCl, and by sodium dodecyl sulfate gel electrophoresis. The isoelectric point was found to be 5.9 by focusing in polyacrylamide gels; the extinction coefficient at 280 nm was determined to be 80,000 M⁻¹ cm⁻¹.

The amino acid composition of the enzyme was determined. In particular, 8 cysteine, 34 lysine, 53 arginine, and 13 histidine residues were found for each molecule, calculated on the basis of an 80,000 molecular weight. Thirteen tryptophan residues were found by absorption spectroscopy in 6 M guanidine HCl; a tyrosine content of 20 residues per 80,000 was estimated. Titration with 5,5'-dithiobis(2-nitrobenzoic acid) reagent showed that 2 cysteine residues were free; titration with 5,5'-dithiobis(2-nitrobenzoic acid) reagent in 8 M urea demonstrated 2 additional cysteine residues.

A peptide map of a tryptic digest showed the presence of about 33 ninhydrin staining spots; no NH₂-terminal amino acid could be detected. It is inferred that the histidyl-tRNA synthetase in its native state is a dimer, probably composed of identical subunits.

The enzyme is stable to storage in 50% glycerol at -20°; at 4° in dilute solution the enzyme slowly loses activity. Gel chromatography shows the appearance of a high molecular weight, inactive form during storage at 4°. This material can be reactivated by incubation with reducing agents.

EXPERIMENTAL PROCEDURE

Materials

L-[³⁵S]Histidine (51 Ci per mmole) in 2% ethanol, was obtained from Amersham and stored at 4°. The crystalline sodium salt of ATP and histidine were obtained from Sigma; bovine serum albumin from BDH; alcohol dehydrogenase, ovalbumin, and lysozyme from Worthington; chymotrypsinogen, pepsin and catalase from Boehringer; sodium dodecyl sulfate from Matheson Coleman; guanidine HCl from Carlo Erba; Sephadex from Pharmacia; and Ampholine from LKB. Glass fiber filters (type A,
and stored at -20 °C.

2 mM 2-mercaptoethanol; and 0.5 mM EDTA.

mal salt medium (7) containing 0.5% glucose. The cells (in late exponential phase of growth) were harvested by centrifugation and stored at -20 °C.

Homogeneous histidyl-tRNA synthetase was prepared from LT-2 cells as described previously (5). However, for a large scale preparation of enzyme from 9 kg of Salmonella cells, it was found convenient to introduce an ammonium sulfate fractionation after the lysis of the cells. The enzyme is contained in the 30 to 65% ammonium sulfate cut. The histidyl-tRNA synthetase prepared by these methods is at least 95% pure by disc gel electrophoresis and by gel filtration. A minor inactive component of lower molecular weight was found in some preparations but could be removed on a G-150 column.

Methods

Assay of Histidyl-tRNA Synthetase—Enzyme activity was determined by measuring the esterification of L-[H]histidine to tRNA as described earlier (5).

Dodecyl Sulfate Gel Electrophoresis—Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn (8).

Equilibrium Sedimentation—Equilibrium sedimentation experiments were carried out in a Spinco model E ultracentrifuge equipped with interference optics according to the method of Yphantis (9). Samples of enzyme of 0.150 ml were placed in a double-sector standard cell in an ANH rotor and centrifuged to equilibrium over 1 to 2 days at 20 °C. Photographs were analyzed in a Nikon comparator. Values of $f = 0.74$ (calculated from the amino acid composition) and $\rho - 1.0$ were used in calculation of molecular weights. For 6 M guanidine hydrochloride $\rho = 1.1415$ was used.

Preparation of Peptide Maps—Peptide maps were prepared by the method of Katz et al. (10). After reduction and alkylation, the enzyme was dissolved in 0.05 M ammonium bicarbonate buffer, pH 8.1, at a concentration of 5 mg per ml. It was digested at 37 °C for 3 hours with 1.0% (by weight) trypsin which had been treated to abolish residual chymotryptic activity. The reaction mixture was then lyophilized and dissolved in 50% aqueous pyridine. The solution was applied to a sheet of Whatman No. 3MM filter paper and chromatographed in the upper phase of butanol-acetic acid-water (4:1:5). After drying, the paper was subjected to electrophoresis in the second dimension in pyridinium acetate buffer, pH 3.6, for 60 min at 2500 volts in a Gilson model D electrophoresis apparatus. After drying, the paper was stained by dipping the paper in 0.25% ninhydrin solution in ethanol or a cadmium-ninhydrin solution.

Amino Acid Analyses—Samples were hydrolyzed in constant boiling HCl in vacuo at 110 °C. Amino acid determinations (11) were made on four different preparations of the enzyme. Most hydrolysates were done for 24 hours; several were timed hydrolysates (24, 48, and 72 hours) and values of each residue were extrapolated to 24-hour values for comparisons. Analyses were also performed on performic acid-oxidized samples and cysteine was determined as cysteic acid. Results were calculated as residue weight for each amino acid per 100 g of protein, averaged, and expressed per 80,000 molecular weight. A variation of about 10 to 15% was found in these values among different samples.

Determination of Tryptophan and Tyrosine—The content of tryptophan and tyrosine was determined according to Edelhoch (12).

Determination of Cysteine Residues—The content of cysteine residues was determined according to the method of Ellman (13) with 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm. For the cysteine residue determinations the protein samples were subjected to gel filtration through a column of Sephadex G-25 immediately before reaction with 5,5'-dithiobis(2-nitrobenzoic acid). The content of cysteine residues also was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid) in 8 M urea.

The total content of accessible half-cystine residues was also studied by alkylation of the enzyme with the [14C]iodoacetic acid in 8 M urea. Samples of the enzyme (1 to 2 mg) in 0.8 ml of 10 mM Tris (pH 7.8) containing 10 mM NaNCl and 8 M urea were treated with 1 mM 2-mercaptoethanol at 37 °C for 2 hours. To this mixture were added 1.5 mg of [14C]iodoacetic acid dissolved in 0.1 ml of 10 mM Tris-HCl (pH 8.5). The mixture was incubated for 10 min at room temperature and 3 μl (40 μmoles) of 2-mercaptoethanol were added to stop the reaction. Removal of excess reagents was carried out by filtration on a Sephadex G-25 (fine) column (1 X 10 cm).

Isolelectric Focusing—The isoelectric focusing of the enzyme was done in polyacrylamide gels by the method of Dale and Latner (14). Enzyme, 25 or 50 μg, was mixed with a 7% acrylamide gel solution which contained 1% of a pH 5 to 8 or pH 3 to 10 Ampholine (LKB Products-AB, Stockholm, Sweden) solution.

The gels were polymerized in tubes (12 X 0.4 cm) by exposure to ultraviolet light and electrofocusing was done, with the cathodic end at the top, at 4 °C for 22 hours at 150 volts. The gel was treated with 10% trichloroacetic acid and then stained with 0.2% bromphenol blue in ethanol-water-acetic acid (50:45:5) for 1 hour and destained with ethanol-water-acetic acid (30:65:5). A control gel was cut into 4-mm sections and each piece was equilibrated with 1 ml of water and the pH then was determined.

Determination of Absorption Coefficient—For determination of the absorption coefficient, protein solutions were thoroughly dialyzed against 10 mM potassium phosphate, pH 7.0, and then centrifuged at 20,000 rpm for 1 hour. Absorbance then was measured on a Zeiss PMQ-11 spectrophotometer and triplicate aliquots were dried to constant weight in a drying pistol and measured with a Cahn Micro-gram Electrobalance. Parallel determination was carried out for samples containing only 10 mM potassium phosphate buffer and these values were subtracted from those of the enzyme samples.

Spectroscopy—Absorption spectra were recorded on a Cary 15 spectrophotometer at 25 °C. Fluorescence emission spectra were recorded on a Hitachi-Perkin-Elmer spectrofluorometer, model SP-2, with 3-cm quartz cells, 278-nm excitation, and 5-μm emission slits, at 25 °C. Excitation spectra were recorded similarly using 335-nm emission with 3-μm excitation slits. Excitation spectra were corrected with numerical parameters calculated by normalizing absorption and excitation spectra of an L-tryptophan standard. Emission spectra were corrected by normalizing the standard to published corrected spectra (15).

RESULTS

Molecular Weight Determinations—Chromatography of the histidyl-tRNA synthetase on a column of Sephadex G-150 showed that over 90% of the protein moved as a single symmetric peak.
The molecular weight of this protein as determined in comparison with other protein standards (Fig. 1) was 92,000.

High speed equilibrium sedimentation of two homogenous preparations of the histidyl-tRNA synthetase gave values for the molecular weight of 78,000. A typical plot of log y versus r² is shown in Fig. 2. A significant curvature in this plot was found in several enzyme preparations but was not present after the enzyme had been passed through a Sephadex G-150 column before centrifugation. The origin of the low molecular weight component is not clear; it may represent small amounts of other protein species or a reversible association-dissociation equilibrium. These values of molecular weight are about 10 to 20% lower than we previously reported (5). However, as discussed below, they are more consistent than the previous value with the information about the subunit molecular weight.

**Subunit Molecular Weight Determinations**—The high speed equilibrium method in 6 M guanidine-HCl gave a single slope of the log y versus r² plot (Fig. 3); the molecular weight calculated from the slope is 41,000.

Since there are many theoretical problems with the determination of molecular weight in such systems, an alternate determination of subunit molecular weight was carried out by electrophoresis of the enzyme in polyacrylamide gel containing sodium dodecyl sulfate (Fig. 4). A value of 40,600 was found. In the absence of 2-mercaptoethanol the estimated value was 44,700. These values are probably not significantly different from each other in view of the uncertainty in measuring mobility values.

We conclude from these studies that the enzyme whose molecular weight is between 80,000 and 90,000 is made up of two subunits of equal mass. Dissociation occurs in sodium dodecyl sulfate without reducing agents; therefore it is unlikely that the subunits are held together by disulfide bonds.

**Extinction Coefficient and Spectra**—The absorption coefficient A₅₅₀₅ at 278 nm determined as described under “Methods” is 9.97. ε = 8.0 × 10⁴ M⁻¹ cm⁻¹ on the basis of a molecular mass of 80,000 daltons.

The absorption spectrum (Fig. 5) is typical of a protein without bound or extrinsic chromophores, with a maximum at 278 nm and a 280:260 nm ratio of 1.65.

The fluorescence emission spectrum (Fig. 5) shows primarily contributions from tryptophan residues. The maximum is at 330 nm, indicating that the average environment of these residues is quite hydrophobic. Some contribution of tyrosine residue emission can be detected in the relative convexity of the curve between 300 and 320 nm. The fluorescence excitation spectrum is, as expected for a pure protein, coincident with the absorption spectrum in the near ultraviolet region. These properties have been found useful for studying the interactions of the enzyme with its ligands, as will be reported later.

1 T. Cebula (Ph.D. thesis, Johns Hopkins University, June 1973) has obtained a molecular weight of 80,000 by gel filtration and one of 40,000 for the subunit by sodium dodecyl sulfate polyacrylamide electrophoresis.
FIG. 4. Estimation of the molecular weight of histidyl-tRNA synthetase in sodium dodecyl sulfate by polyacrylamide gel electrophoresis (8). The molecular weight of the protein markers are plotted, on a logarithmic scale, against their mobilities on acrylamide gels, during electrophoresis in the presence of sodium dodecyl sulfate, with and without 2-mercaptoethanol. The proteins are: 1, lysozyme (mol wt 14,000); 2, pepsin (mol wt 34,000); 3, ovalbumin (mol wt 43,000); and 4, catalase (mol wt 60,000). Solid line, sodium dodecyl sulfate with 2-mercaptoethanol; dotted line, sodium dodecyl sulfate without 2-mercaptoethanol.

No change of the ultraviolet spectrum of the histidyl-tRNA synthetase was observed when it was compared in the following buffers: (a) Tris-HCl (50 mM, pH 7.0), NaCl (40 mM), and 2-mercaptoethanol (2 mM); (b) sodium cacodylate (10 mM, pH 7.0), NaCl (100 mM), and 2-mercaptoethanol (2 mM); (c) potassium phosphate (10 mM, pH 7.5) and 2-mercaptoethanol (2 mM).

Tyrosine and Tryptophan Content—The absorption spectrum of the enzyme was determined in 0.02 M phosphate buffer, pH 8.5, containing 6 M guanidine HCl by the method of Edelhoch (12). From the absorption at 280 and 288 nm it was calculated that there are 13 tryptophanyl and 28 tyrosyl residues per 80,000 molecular weight. On the basis of the change in optical density at 300 nm, upon the addition of NaOH to pH 11, we compute that the enzyme contains 19 tyrosyl residues. This method of estimation of tyrosine content is more accurate, especially when cysteine residues are present.

Isoelectric Focusing—When the histidyl-tRNA synthetase was studied by isoelectric focusing in gels with pH gradients from 3 to 10, an isoelectric point of about 6.3 was found. Repeated examination in gels with pH gradients from 5 to 8 gave a value of 5.9 as the isoelectric point.

Peptide Map—Fig. 6 is a photograph of a two-dimensional map of the tryptic peptides of the histidyl tRNA synthetase. There were about 21 clearly defined spots and another 12 lighter spots detected; in addition, a number of diffuse staining regions were noted. These results were obtained in several studies using both ninhydrin and cadmium-ninhydrin staining. Since there are about 87 lysine and arginine residues (see below), these results of somewhat fewer than half the expected number of spots suggest that the two subunits are identical. They are not conclusive, however.

Several attempts were made to detect one or more NH2-terminal residues by the method of Gray (16). No detectable spot was seen under conditions in which other proteins gave clear results.

Amino Acid Composition—The amino acid composition of purified histidyl-tRNA synthetase, expressed as residues per molecule of 80,000 molecular weight, is summarized in Table I. This composition is in general similar to the composition of other aminocacyl-tRNA synthetases (17). The most unusual features of the amino acid composition are the low content of half-cystine residues and the high content of arginine residues. The high
number of glutamic and aspartic residues is consistent with the behavior of the synthetase as an acidic protein in the isoelectric focusing studies.

Titration of Cysteine Residues—It was found that 2.2 cysteine residues per mole of enzyme could be titrated with 5,5′-dithiobis(2-nitrobenzoic acid) in the native state. Titration with this reagent in 8 M urea gave a value of 4.2 moles of cysteine per mole of enzyme. The addition of 5 mM β-mercaptoethanol changed this value only by a small fraction of a residue per 80,000 molecular weight. By alkylation with [14C]iodoacetate in 8 M urea and 1 mM β-mercaptoethanol, the extent of labeling based on radioactivity measurements was between 3 and 5 moles of [14C]iodoacetate per mole of protein. The 8.0 half-cystine residues present as cysteic acid per 80,000 molecular weight protein (see Table I) suggest that about 4 half-cystine residues are not titratable with iodoacetate even in the presence of urea and reducing agent.

Stability of Histidyl-tRNA Synthetase—The enzyme is stable when stored in 90% glycerol with 2 mM β-mercaptoethanol at −20° for periods of months and it is stable for a few days in dilute aqueous solutions with 2 mM β-mercaptoethanol at 4°. However, after several days at 4°, enzymatic activity significantly decreases; we have found that this loss of activity is correlated with the appearance of an inactive high molecular weight form of the enzyme. This material can be separated from the active form of the enzyme by gel chromatography. It cross-reacts with antibodies to the enzyme as measured by double diffusion and migrates in sodium dodecyl sulfate gel electrophoresis as the native enzyme only when 2-mercaptoethanol is added. It can be partially reactivated by treatment with 2 mM β-mercaptoethanol or 2 mM dithiothreitol; as much as 50% of the native activity is obtained after 24 to 48 hours exposure to these reducing agents at 4°. However, subsequently enzymatic activity again decreases upon prolonged standing of the enzyme at 4°. Therefore instability is due, at least in part, to formation of disulfide bonds and perhaps also to formation of mixed disulfide bonds with mercaptoethanol.

Discussion

Our findings of equilibrium sedimentation and of gel filtration show that histidyl-tRNA synthetase from S. typhimurium has a molecular weight of about 80,000. When equilibrium sedimentation of the enzyme is carried out in the presence of 6 M guanidine hydrochloride, a linear plot of log y versus r² is obtained (Fig. 3) and a molecular weight of 41,000 is determined; a similar value was obtained by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate, with or without reducing agents. It is therefore concluded that the enzyme is composed of two protomers of equal mass. These findings show that the histidyl-tRNA synthetase belongs to the large group of dimeric or tetrameric aminoacyl-tRNA synthetases which have subunits of molecular weight around 40,000 (18).

The enzyme has an extinction coefficient of 80,000 M⁻¹ cm⁻¹ and the absorption spectrum shows no extrinsic chromophores. The isoelectric point was found to be 5.9 by focusing in polyacrylamide gels.

Most of the synthetase molecules reported which are dimers of about 80,000 molecular weight have identical subunits. This is likely to be true for the histidyl-tRNA synthetase of Salmonella on the basis of the number of spots on our peptide maps. However, we have not been able to confirm this by NH₄OH terminal amino acid determination (a similar result for the seryl-tRNA synthetase (19) has been reported) and a definitive conclusion awaits the results of amino acid sequence determination procedures.

The amino acid composition shown in Table I is calculated on the basis of a molecular weight of 80,000. These results are meant only to characterize this protein in comparison to others being studied. The tryptophan content was estimated at 13 residues per 80,000 molecular weight on the basis of the guanidine hydrochloride spectral method of Edelhoch; the tyrosyl content was calculated at 28 by that method and at 19 by the alkali titration. The latter value is in agreement with the amino acid analysis and is probably correct.

It will be noted that this histidyl-tRNA synthetase has a significant content of histidine residues and about 8 half-cystine residues. Two residues per 80,000 molecular weight are subject to 5,5′-dithiobis(2-nitrobenzonic acid) titration. In 8 M urea, 4 cysteine residues were free to react with 5,5′-dithiobis(2-nitrobenzonic acid). Carboxymethylation in concentrated urea and 1 mM β-mercaptoethanol with [14C]iodoacetate showed about 4 reactive cysteine residues. It is not certain if the unreactive residues are unusually stable to urea denaturation and/or reducing agents or even if cysteine bridges ordinarily exist in the molecule. The absence of an effect of mercaptoethanol on the apparent subunit molecular weight values, however, suggests that there are no cysteine bridges between protomers. It should be noted, however, that these results are all consistent with the subunits being identical.

The inactivation of the enzyme at 4° is associated with aggregate formation and can be reversed with reducing agents. It is presumably related to various reactions of the cysteine residues. The enzyme is, however, sufficiently stable to allow physicochemical studies of its properties. Subsequent papers will describe the interaction of the enzyme with its several ligands.

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<tr>
<th>Amino acid</th>
<th>Residues per 80,000 mol wt</th>
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<tr>
<td>Lysine</td>
<td>34</td>
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<tr>
<td>Histidine</td>
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<tr>
<td>Arginine</td>
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<td>Cysteic acid</td>
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<td>Aspartic acid</td>
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<tr>
<td>Glutamic acid</td>
<td>91</td>
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<td>Phenylalanine</td>
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TABLE I
Amino acid composition of histidyl-tRNA synthetase

Details are given in the text under "Methods."

Our findings of equilibrium sedimentation and of gel filtration show that histidyl-tRNA synthetase from S. typhimurium has a molecular weight of about 80,000. When equilibrium sedimentation of the enzyme is carried out in the presence of 6 M guanidine hydrochloride, a linear plot of log y versus r² is obtained (Fig. 3) and a molecular weight of 41,000 is determined; a similar value was obtained by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate, with or without reducing agents. It is therefore concluded that the enzyme is composed of two protomers of equal mass. These findings show that the histidyl-tRNA synthetase belongs to the large group of dimeric or tetrameric aminoacyl-tRNA synthetases which have subunits of molecular weight around 40,000 (18).

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discussions, to Dr. A. Parente for the determination of absorption coefficient, and to Mr. E. Tufano for his assistance in performing the ultracentrifugation studies.

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