Purification of Mammalian Histidyl-tRNA Synthetase and Its Interaction with Myositis-Specific Anti-Jo-1 Antibodies†

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ABSTRACT: Histidyl-tRNA synthetase is purified to near homogeneity from rat liver. The subunit molecular weight of histidyl-tRNA synthetase is 50 000, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The Stokes radius and the sedimentation coefficient of histidyl-tRNA synthetase are 38 Å and 6.0 S, respectively. The native molecular weight of histidyl-tRNA synthetase is calculated to be 96 000 on the basis of its hydrodynamic properties. The purified histidyl-tRNA synthetase reacts with the myositis-specific anti-Jo-1 antibodies. Anti-Jo-1 immunoglobulin G reacts with the native form of histidyl-tRNA synthetase and does not react or only weakly reacts with the denatured form. The anti-Jo-1 antibodies exhibit stronger inhibition toward histidyl-tRNA synthetase that has been preincubated with tRNA than that without preincubation. Anti-Jo-1 antibodies behave as a noncompetitive inhibitor with respect to tRNA in the aminoacylation reaction catalyzed by histidyl-tRNA synthetase. The structural features of the antigen of the anti-Jo-1 antibodies in light of these results are discussed.

Histidyl-tRNA synthetase catalyzes the aminoacylation of $tRNA^{His}$ in the initial step of protein biosynthesis. The aminoacylation of tRNA-like structures has also been observed in plant and mammalian viral RNA. While plant viral RNA can be aminoacylated with histidine, valine, and tyrosine (Haenni et al., 1982), it was found that mengovirus RNA, which is a mammalian virus, could be specifically aminoacylated with histidine by mouse liver histidyl-tRNA synthetase (Solomon & Littauer, 1974). Histidyl-tRNA synthetase may also play an important role in the regulation of protein degradation as well as protein biosynthesis. Studies in Chinese hamster ovary cells show that the cells have retained their ability to react directly to amino acid starvation by a decrease in protein synthesis and an increase in protein degradation. The rate of protein degradation in at least one of the protein degradation systems correlates with the steady-state level of $tRNA^{His}$ (Scornik et al., 1980; Scornik, 1984). Recent studies have shown the involvement of $tRNA^{His}$ (Ciechanover et al., 1985) and possibly histidyl-tRNA synthetase in a nonlysosomal ubiquitin- and ATP-dependent protein degradation pathway.

The involvement of histidyl-tRNA synthetase in autoimmune diseases is another interesting feature of this enzyme. Polymyositis, a devastating inflammatory muscle disease, is associated with the myositis-specific anti-Jo-1 autoantibody, found in 30% of such patients (Arnett et al., 1981). The anti-Jo-1 antibodies are found to precipitate $tRNA^{His}$ (Rosa et al., 1983) and a phosphoprotein with a molecular weight of 50 000, identified as histidyl-tRNA synthetase (Mathews & Bernstein, 1983). Phosphorylation of histidyl-tRNA synthetase, as demonstrated with anti-Jo-1 antibodies, suggests possible regulation of aminoacyl-tRNA synthetases by reversible phosphorylation (Gerken et al., 1986).

The involvement of histidyl-tRNA synthetase in autoimmune diseases, ubiquitin-dependent protein degradation, aminoacylation of viral RNA, and reversible phosphorylation makes histidyl-tRNA synthetase of mammalian sources a particularly interesting enzyme. Anti-Jo-1 antibodies may be useful for studies of histidyl-tRNA synthetase and for unraveling possible relationships of the above-mentioned phenomena. However, the mammalian histidyl-tRNA synthetase has not been well characterized, and its interaction with anti-Jo-1 antibodies is not well understood. Studies of the interaction of histidyl-tRNA synthetase and the anti-Jo-1 antibodies may provide a model for the understanding of the interactions of a number of the autoantibodies with the protein moiety of their respective antigens, such as Sm (Lerner & Steitz, 1979), La (Rinke & Steitz, 1982), RNP (Lerner et al., 1981; White et al., 1981), and Ro (Hendrick et al., 1981). Histidyl-tRNA synthetase and anti-Jo-1 antibodies may be a particularly favorable system, since the antigen has easily assayable enzymatic and tRNA activities. In this paper, we report the purification and characterization of liver histidyl-tRNA synthetase and characteristics of its interactions with anti-Jo-1 antibodies. A preliminary report on the purification of histidyl-tRNA synthetase has appeared (Yang et al., 1984).

Materials and Methods

The materials and the general analytical methods have been described (Wahab & Yang, 1986). Buffer T contained 20 mM Tris-HCl[1] (pH 7.2), 2 mM DTE, 3 mM $MgCl_2$, and 10% glycerol. Buffer P contained 25 mM potassium phosphate (pH 7.2), 2 mM DTE, 3 mM $MgCl_2$, and 10% glycerol. *Escherichia coli* unfractionated aminoacyl-tRNA synthetases were isolated by the method of Muench and Berg (1966). Histidyl-tRNA synthetase was assayed by the aminoacylation of unfractionated yeast or calf liver tRNA (Boehringer Mann-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GAH, goat anti-human immunoglobulin; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; Ig, immunoglobulin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TBS, Tris-buffered saline.

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heim). Yeast and calf liver tRNA are aminoacylated equally well by liver histidyl-tRNA synthetase. Yeast tRNA was used during the purification, and calf liver tRNA was used for characterization as specified. Anti-Jo-1 sera were obtained from myositis patients (by F. C. Arnett) as previously described (Arnett et al., 1981). Anti-Jo-1 IgG was purified from serum by ammonium sulfate precipitation and DEAE-cellulose chromatography (Mathews & Bernstein, 1983). Ouchterlony double diffusion precipitation was performed as in Dang et al. (1983). Fab fragments of anti-Jo-1 antibodies were prepared by mercuripapain digestion (William & Chase, 1967). The reduction of the IgG to the Fab fragments was confirmed by SDS gel electrophoresis.

Purification of Histidyl-tRNA Synthetase

Histidyl-tRNA synthetase was purified from rat liver according to the method of Kane et al. (1978) with modifications. All procedures were carried out at 0–4 °C. Fresh phenylmethanesulfonyl fluoride (PMSF) was added to all buffers immediately prior to using them at a concentration of 2 mM except in the homogenization buffer where the concentration was 5 mM.

Twelve male rats (100–150 g) were fed overnight and sacrificed. Liver was rinsed and suspended in 2 volumes of buffer T with 5 mM PMSF and then minced in a Waring blender for 5 s. The blended liver was then homogenized by using a Polytron homogenizer (Type PT 1000, Brinckman Instruments) at setting 5 for 3 × 25 s with intermittent cooling for 1 min at 0 °C. The crude extract was then centrifuged at 10000 rpm for 40 min in a Sorval GSA rotor. The pellet was discarded, and the supernatant was centrifuged at 30000 rpm for 30 min in a Type 42.1 rotor in a Beckman Model L5-75 ultracentrifuge. The pellet was discarded. Inclusion of a high concentration of salt in these initial steps of purification did not appreciably enhance the yield. Ammonium sulfate (Schwartz, enzyme grade) was added to the supernatant to 40% saturation over 30 min with constant stirring. The suspension was stirred for an additional 30 min at 4 °C and then centrifuged at 10000 rpm for 40 min. The pellet was discarded, and ammonium sulfate was added to the supernatant to 70% saturation and stirred for 30 min at 4 °C. The suspension was centrifuged at 30000 rpm for 30 min. The final pellet was redissolved in buffer T by use of an ice-cold Teflon pestle. Ammonium sulfate fractionation of the homogenate gave higher yield and higher specific activity than fractionation with poly(ethylene glycol). The pellet was dialyzed extensively against buffer T.

The dialyzed sample (600 mg) from ammonium sulfate fractionation was loaded to a column (20 × 5.5 cm) of DEAE-cellulose (DE-52) preequilibrated with buffer T. The column was washed with 2 column volumes of buffer T. The enzyme was eluted with a linear gradient (2 L) of NaCl (0–0.3 M) at a flow rate of 60 mL/h. Fractions of 17 mL were collected and assayed. Histidyl-tRNA synthetase activity emerged at 0.14 M NaCl and peaked at 0.15 M. Fractions containing enzyme activity were pooled, diluted 2-fold with buffer T, and loaded directly to a column (35 × 2.5 cm) of phosphocellulose (Sigma) preequilibrated with buffer T. The column was washed with 2 column volumes of buffer T. The enzyme was eluted with a linear gradient (1 L) of NaCl (0–0.5 M) at a flow rate of 30 mL/h. Fractions of 10 mL were collected. Histidyl-tRNA synthetase activity emerged at 0.24 M NaCl and peaked at 0.32 M. The yield of this step was only 33%, but a 20-fold increase in specific activity was achieved. Active fractions were pooled, and ammonium sulfate was added to 80% saturation to precipitate the protein. The precipitate was dissolved in buffer T and dialyzed extensively against buffer T. The protein was concentrated 20-fold by ammonium sulfate precipitation with 80% recovery of activity.

The protein from phosphocellulose column chromatography was loaded to a column of Bio-Rex 70 (Bio-Rad) preequilibrated with buffer T. The column was washed with buffer T containing 50 mM NaCl. The enzyme was eluted with a linear gradient (140 mL) of NaCl (50–500 mM). Fractions of 3 mL were collected and assayed. Histidyl-tRNA synthetase activity emerged at 0.25 M NaCl and peaked at 0.3 M.

The Bio-Rex pool that contained the activity peak of histidyl-tRNA synthetase was about 70% pure, as determined from polyacrylamide gel electrophoresis. The purified histidyl-tRNA synthetase was stored in buffer T with 50% glycerol at −20 °C and was used for characterization in most cases unless specified otherwise.

Further Purification and Identification

Bio-Gel TSK DEAE-5-pw high-pressure liquid column chromatography, affinity chromatography on tRNA–Sepharose, and gel filtration on Sephacryl S-300 were used to further purify and identify histidyl-tRNA synthetase.

HPLC. The HPLC apparatus consisted of a Perkin-Elmer delivery system, a Model 7125 Rheodyne sample injection valve, and a UV detector. The flow rate of the column was 0.7 mL/min. The column (Bio-Gel TSK DEAE-5-pw, 75 × 7.5 mm) was equilibrated with 0.1 M KCl in buffer P. A sample from the Bio-Rex 70 pool (300 μL) was diluted 2-fold and injected into the column. The column was washed with 0.1 M KCl in buffer P, and the enzyme was eluted with a linear gradient (60 mL) of KCl (0.1–0.4 M) in buffer P. Fractions of 1.25 mL were collected and assayed.

tRNA–Sepharose. The tRNA–Sepharose 4B using *E. coli* tRNA was prepared according to the method of Remy et al. (1972). A sample of the Bio-Rex pool was diluted 7-fold and loaded to the tRNA–Sepharose column (20 mL). The column was washed with buffer T. The enzyme was eluted by a linear gradient (100 mL) of NaCl (0–0.5 M NaCl) in buffer T. Fractions of 2.5 mL were collected and assayed.

Gel Filtration. The Bio-Rex fractions were concentrated by using a small column (0.5 mL) of hydroxyapatite preequilibrated with buffer T. The enzyme was eluted by 0.5 M KCl in buffer T. The concentrated sample was loaded to a column of Sephacryl S-300 (45 × 1.5 cm) equilibrated with 200 mM KCl in buffer T. The flow rate of the column was 20 mL/h. Fractions of 1.6 mL were collected and assayed. The same column was used to determine the Stokes radius of histidyl-tRNA synthetase. The void volume and the total volume of the column were determined with Blue dextran and phenol red.

Extraction and Renaturation from SDS–Polyacrylamide Gels. Partially purified histidyl-tRNA synthetase was subjected to electrophoresis in a 10% preparative polyacrylamide slab (10 × 14 × 0.075 cm) in the presence of sodium dodecyl sulfate. The M_r 50000 protein and the M_r 60000 protein were extracted according to the method of Hager and Burgess (1980). Pieces of the sliced gel after electrophoresis were incubated in the elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1 mM EDTA, 0.1 mg/mL BSA) for 12 h at 37 °C and filtered through glass wool with the aid of a small syringe. The extracted protein was then precipitated with 4 volumes of cold acetone at −80 °C. The suspension was centrifuged at 10000*g* for 15 min. The pellet was rinsed with 80% acetone in buffer T and dried in vacuo.

The precipitate was dissolved in 25 μL of 6 M guanidine hydrochloride in the renaturing buffer (50 mM Tris-HCl, pH

Table I: Purification of Histidyl-tRNA Synthetase from Rat Liver[a]

steps	protein (mg)	total units	sp act. (units/mg)	yield (%)	purification (x-fold)
crude extract	72400	3830	0.053	100	1
80000*g* centrifugation	17700	2360	0.133	61.5	2.5
ammonium sulfate fractionation	5540	2200	0.399	57.5	7.5
DEAE-cellulose chromatography	950	1140	1.20	29.7	22.6
phosphocellulose chromatography	14.9	374	25.1	9.7	473
Bio-Rex 70 chromatography	0.424	264	622	6.8	11700

[a] 100 g of liver was used.

7.5, 150 mM KCl, 1 mM DTE, 0.1 mM EDTA, 0.1 mg/mL BSA, 20% glycerol), diluted 50-fold in renaturing buffer, and incubated at 20 °C for periods of 2 and 6 h. Aliquots of renatured proteins were assayed for histidyl-tRNA synthetase activity.

Hydrodynamic Parameters

The sedimentation coefficients were determined by the method of Martin and Ames (1961) using galactosidase (16 S), catalase (11.2 S), alcohol dehydrogenase (7.6 S), and hemoglobin (4.6 S) as standards. The Stokes radius was determined by analytical gel filtration at 4 °C with a column of Sepharcyl S-300 (Pharmacia). Standards included catalase (55 Å), alcohol dehydrogenase (45 Å), hemoglobin (31 Å), and cytochrome *c* (16 Å). The void volume was determined with Blue dextran 2000. The data were analyzed according to the formulation of Ackers (1967).

Inhibition Patterns by Anti-Jo-1 IgG

The initial velocity of aminoacylation was determined for each substrate by varying its concentration while the other two substrates were kept at saturating concentrations as described previously (Wahab & Yang, 1986). This was carried out in the absence or presence of purified anti-Jo-1 IgG.

When tRNA was the variable substrate, the assay mixture contained, in 32 μL, 18 μL of standard assay mixture minus tRNA (50 mM Tris, 6 mM $MgCl_2$, 4 mm DTT, 25 mM ATP, 0.03 mM [^{14}C]histidine, 1 mg/mL BSA), 10 μL of varying concentrations of tRNA, and 2 μL of either buffer or anti-Jo-1 IgG (0.5 mg/mL). The reaction was initiated by the addition of 2 μL of purified histidyl-tRNA synthetase and incubation at 37 °C for 2 min. Aliquots of 25 μL of the incubation mixtures were then spotted on the Whatman 3MM paper pads, and washing was carried out as in standard assay procedure. When ATP or histidine was used as the variable substrate, the assay mixture contained, in 40 μL, standard assay mixture with varying concentrations of ATP or histidine and 2 μL of either buffer or anti-Jo-1 IgG. The reaction was initiated by adding a limiting amount of histidyl-tRNA synthetase. After incubation at 37 °C for 2 min, 30 μL of the incubation mixture was spotted on a paper pad and processed as in the standard assay.

Enzyme-Linked Immunoassay

Nitrocellulose sheets were cut into 1-cm strips and soaked in 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl (TBS) and then left to air-dry on a filter paper. Samples of the antigen to be tested were spotted (2–10 μL) on the nitrocellulose strips and air-dried. The nitrocellulose membrane was then incubated in the blocking solution (3% gelatin in TBS) with shaking for 40 min. The strips were removed from the blocking solution and incubated with the anti-Jo-1 antibodies diluted with 10 volumes of 1% gelatin in TBS for 40 min. The strips were then rinsed with water and with TBS twice for 10 min each time. The strips were then incubated with affinity-purified horseradish peroxidase conjugated goat anti-human immunoglobulin antibodies (Bio-Rad GAH-HRP) at 0.01 mg/mL

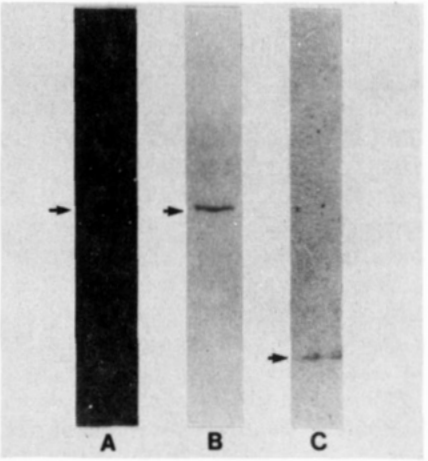

FIGURE 1: SDS–polyacrylamide gel electrophoresis of fractions containing histidyl-tRNA synthetase: (lane A) purified histidyl-tRNA synthetase from column chromatography on Bio-Rex BR70; (lane B) extracted M_r 50000 protein from SDS–polyacrylamide gel of partially purified histidyl-tRNA synthetase; (lane C) active fraction eluted from Sephacryl S-300 column chromatography. The arrows indicate the position for M_r 50000 when standards including galactosidase, phosphorylase *a*, bovine serum albumin, catalase, and carbonic anhydrase are used.

for 1 h and washed with water and with TBS for 10 min each time. It was then incubated in the freshly prepared color development solution (1.5 mg/mL chloronaphthol, 0.018% H_2O_2 in TBS). Color develops in 10–30 min.

The immunoassay was also performed in microtiter plates following the same steps as on nitrocellulose sheets, but soluble substrates including 2,2′-azinobis(3-ethylbenzothiazoline-sulfonate) and hydrogen peroxide were used. When these substrates are mixed in equal volumes and added to the immobilized GAH-HRP in the wells of the titer plates, the positives develop green color. The extent of reaction was quantitated by absorbance at 415 nm.

RESULTS

Purification of Histidyl-tRNA Synthetase. Histidyl-tRNA synthetase was purified from rat liver according to the procedure modified from Kanes et al. (1978) for the reticulocyte histidyl-tRNA synthetase. A typical purification is summarized in Table I.

The histidyl-tRNA synthetase activity was completely separated from the main protein peak during the chromatography on Bio-Rex 70 and was purified more than 10000-fold after this step. The protein concentration under the peak of activity was less than 10 μg/mL. When the purified fractions were subjected to electrophoresis on 7.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (Figure 1), a major protein band (~70%) with a molecular weight of 50000 correlated with the activity of histidyl-tRNA synthetase. The major contaminant has an M_r of 60000, which copurified with the M_r 50000 protein throughout the purification. As an attempt to demonstrate the identity of the Jo-1 antigen, Western blot analysis was carried out. A 7.5%

SDS–polyacrylamide gel of purified histidyl-tRNA synthetase was blotted onto nitrocellulose membrane. The membrane was incubated with anti-Jo-1 antibodies, followed by incubation with ^{125}I-labeled protein A from *Staphylococcus aureus*. No protein band was specifically labeled as detected by autoradiography. It appears that the anti-Jo-1 antibodies did not recognize the denatured form of histidyl-tRNA synthetase.

The enzyme purified after Bio-Rex column chromatography had a high specific activity (622 units/mg), which is comparable to that of most highly purified aminoacyl-tRNA synthetases. Such preparations were used for characterization of histidyl-tRNA synthetase and its interaction with anti-Jo-1 antibodies. Further purification by a variety of procedures described as follows showed that the M_r 50 000 protein band is histidyl-tRNA synthetase. However, such steps invariably yielded unstable, dilute preparations with low yields.

Identification of Histidyl-tRNA Synthetase. The Bio-Rex fractions contained partially purified histidyl-tRNA synthetase, and additional steps were used to purify the enzyme to homogeneity. Various procedures were examined for further purification.

Purified histidyl-tRNA synthetase from the Bio-Rex chromatography was concentrated on a small column of hydroxyapatite and further purified by gel filtration on Sephacryl S-300. The active fractions were analyzed by electrophoresis on 7.5% and 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. The only detectable protein band showed a molecular weight of 50 000 and coeluted with the histidyl-tRNA synthetase activity.

A sample from the Bio-Rex column chromatography was also analyzed by HPLC on Bio-Gel TSK DEAE-5-pw and eluted with a linear gradient of KCl. The synthetase activity was eluted as a sharp peak at 0.15 M KCl. The peak of activity, which eluted mainly in one fraction, was analyzed by SDS–polyacrylamide gel, and a faint protein band with a molecular weight of 50 000 was observed.

Finally both M_r 50 000 and M_r 60 000 protein bands were extracted from a preparative SDS–polyacrylamide gel of purified histidyl-tRNA synthetase. A sample of extracted proteins was electrophoresed to ascertain the band extracted. The recovery of the protein is about 50%. The proteins were renatured, and aliquots were assayed for histidyl-tRNA synthetase activity. Activity was observed for the M_r 50 000 protein (1200 cpm), which corresponded to 10% recovery of the total activity. No activity was observed for the M_r 60 000 protein.

The subunit molecular weight of histidyl-tRNA synthetase is thus 50 000 on the basis of HPLC, gel filtration, and extraction of the protein from SDS gels. Further purification by chromatography on tRNA–Sepharose, Cibacron blue–Sepharose, DEAE-cellulose, and hydroxyapatite was unsuccessful.

Hydrodynamic Properties. The sedimentation constant for histidyl-tRNA synthetase was determined to be 6.0 S relative to the standards. The Stokes radius for histidyl-tRNA synthetase was found to be 38 Å.

The native molecular weight of histidtyl-tRNA synthetase was calculated by using the equation (Siegel & Monty, 1966)

$$M_r = (6\pi\eta Nas)/(1 - \bar{v}\rho)$$

where s, the corrected sedimentation coefficient, is 6.0 S; a, the Stokes radius, is 38 Å; N is Avogadro's number; η, the viscosity, is 0.01 P; ρ, the density of water, is 1.00 g/mL; and $\bar{v}$, the partial specific volume, is assumed to be 0.73 mL/g. The estimated molecular weight of native histidyl-tRNA synthetase is 96 000. This result is consistent with an α_2-type subunit structure of histidyl-tRNA synthetase with a subunit molecular weight of 50 000.

Table II: Molecular Properties of Histidyl-tRNA Synthetase

sedimentation coeff $s_{20,w}$ (S)	6.0
Stokes radius R_S (Å)	38
M_r by gel filtration	115 000 ± 5000
M_r calcd from $s_{20,w}$ and R_S	96 000
subunit M_r (SDS gel electrophoresis)	50 000 ± 2000
frictional coeff ratio f/f_0	1.14
axial ratio a/b	3.5
turnover no. (s^{-1})	1.24

Table III: Inhibition of *E. coli* and Rat Liver Histidyl-tRNA Synthetases by Anti-Jo-1 Antibodies[a]

	liver His RS (% of activity)	*E. coli* His RS (% of activity)
no anti-Jo-1	100	100
with anti-Jo-1	59	95
preincubation with calf liver tRNA	27	ND[b]
preincubation with yeast tRNA	45	ND[b]

[a] Aliquots of histidyl-tRNA synthetase with or without preincubation were assayed for aminoacylation activity in the absence or presence of the anti-Jo-1 serum. Preincubation of histidyl-tRNA synthetase with 5 mg/mL unfractionated tRNA from calf liver or yeast was carried out at 4 °C for 10 min. Liver histidyl-tRNA synthetase does not aminoacylate *E. coli* tRNA, and *E. coli* histidyl-tRNA synthetase does not aminoacylate calf liver tRNA but does aminoacylate up to 24% of yeast tRNA. [b] Not determined.

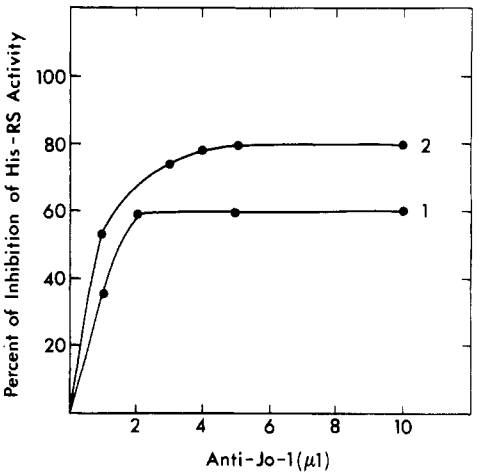

FIGURE 2: Inhibition of histidyl-tRNA synthetase activity by anti-Jo-1 antibody. The percentage of inhibition of histidyl-tRNA synthetase was determined by assaying the enzyme activity in the presence of varying amounts of anti-Jo-1 serum (1) or purified anti-Jo-1 IgG (2; 0.5 mg/mL).

The axial ratio (a/b) of an equivalent prolate is calculated to be 3.5. Histidyl-tRNA synthetase appears to be a highly unsymmetric ellipsoid as compared to other mammalian synthetases. The hydrodynamic properties of histidyl-tRNA synthetase are summarized in Table II.

Inhibition by Anti-Jo-1 Antibodies. The inhibition of histidyl-tRNA synthetase is examined by assaying the enzyme activity in the presence of varying amounts of anti-Jo-1 sera or the purified anti-Jo-1 IgG (Figure 2). An 80% inhibition of the activity was achieved by the purified antibody (1.9 mg/mL) and 60% inhibition by the serum (21.6 A_{280}/mL). A preparation of unfractionated aminoacyl-tRNA synthetases from *E. coli* and purified histidyl-tRNA synthetase from rat liver were compared for the extent of inhibition by the anti-Jo-1 antibody. The results summarized in Table III show that anti-Jo-1 antibodies do not inhibit histidyl-tRNA synthetase

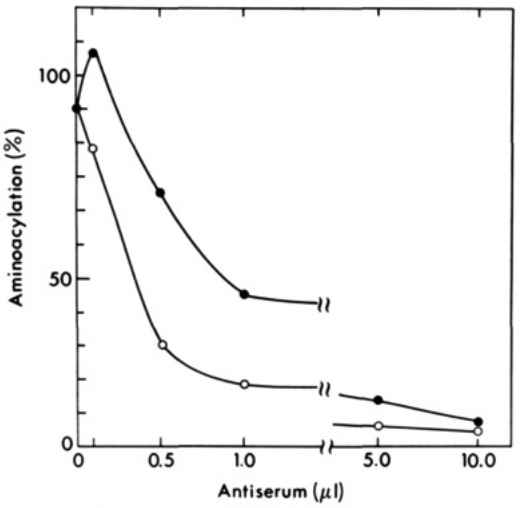

FIGURE 3: Inhibition of rat liver histidyl-tRNA synthetase by anti-Jo-1 antibodies in the presence of tRNA. Rat liver histidyl-tRNA synthetase activity was preincubated with 5 mg/mL calf unfractionated liver tRNA for 10 min and then assayed in the presence of 2 μL of anti-Jo-1 serum (O–O) or in the presence of 2 μL of anti-Jo-1 serum without preincubation (●–●).

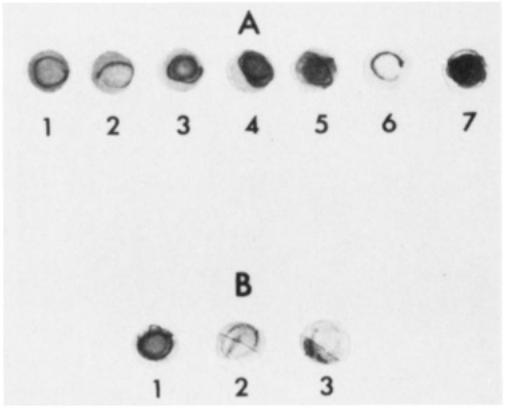

FIGURE 4: Immunodot assay of histidyl-tRNA synthetase. The immunodot assay was performed on different samples of histidyl-tRNA synthetase. (A) Detection limit of the assay for different amounts of the enzyme: (1) 0.001 μg, (2) 0.003 μg, (3) 0.01 μg, (4) 0.025 μg, and (5) 0.05 μg of histidyl-tRNA synthetase from Bio-Rex chromatography, (6) 0.07 μg of denatured enzyme, and (7) 0.07 μg of histidyl-tRNA synthetase from phosphocellulose pool. (B) Immunodot assay performed on (1) native histidyl-tRNA synthetase, (2) M_r 60000 protein, and (3) M_r 50000 protein, extracted from polyacrylamide gel and renatured.

from *E. coli*. Preincubation of liver histidyl-tRNA synthetase with tRNA augmented the inhibitory effect of anti-Jo-1 antibodies. Histidyl-tRNA synthetase was preincubated with a saturating amount (5 mg/mL) of calf liver tRNA and then assayed in the absence or presence of varying amounts of anti-Jo-1 antibodies. As shown in Figure 3, the concentration of anti-Jo-1 IgG required for 50% inhibition of histidyl-tRNA synthetase activity is apparently reduced after preincubation of the synthetase with tRNA. The extent of inhibition of the enzyme activity was also appreciably higher after preincubation with tRNA than that without preincubation.

An immunodot assay using the horseradish peroxidase conjugated goat anti-human antibodies was developed in order to assay low amounts of histidyl-tRNA synthetase or anti-Jo-1 antibodies and to study their interaction. When 4-chloronaphthol was used as the peroxidase substrate, the lower limit of detection of the immunoassay was 0.01 μg of histidyl-tRNA synthetase. The Bio-Rex pool which contained 10 μg/mL protein could also be detected to the same limit (0.01 μg) as shown in Figure 4A. The optimal dilution to achieve a clear detection of the enzyme was a 10-fold dilution for the anti-Jo-1 sera.

Table IV: Michaelis–Menten Constants of Histidyl-tRNA Synthetase[a]

variable substrate	Michaelis–Menten constants		inhibition patterns
	–anti-Jo-1	+anti-Jo-1	
ATP	0.38 mM	2.0 mM	competitive
histidine	0.07 mM	0.41 mM	competitive
tRNA	12.5 mg/mL 0.84 mg/mL	12.5 mg/mL 0.84 mg/mL	noncompetitive

[a] Michaelis–Menten constants were determined from Eadie–Hofstee plots. Unfractionated calf liver tRNA was used throughout the experiment. Aminoacylation was for 2 min at 37 °C after the addition of histidyl-tRNA synthetase to aminoacylation mixture with or without anti-Jo-1 IgG. The histidine acceptor activity of unfractionated calf liver tRNA was found to be 35 pmol/A_{260}.

The sensitivity of the immunoassay can be enhanced 2–3-fold when the incubation of histidyl-tRNA synthetase with anti-Jo-1 antibodies is carried out in the presence of 1 mg/mL yeast tRNA (data not shown). This is likely due to the higher affinity of the anti-Jo-1 antibodies toward the tRNA–synthetase complex than toward the synthetase alone.

Histidyl-tRNA synthetase from the pool of phosphocellulose chromatography was denatured with sodium dodecyl sulfate. The final concentration of SDS was 1%. The denatured enzyme was then spotted on the nitrocellulose membrane (10 μL). A very faint immunoreaction was observed with the denatured enzyme compared to the strong reaction of the native histidyl-tRNA synthetase, which can be detected after 50-fold dilution (Figure 4). No reaction was observed for the denatured form at 0.02% SDS either. These results suggest that the anti-Jo-1 antibodies do not recognize the denatured form of histidyl-tRNA synthetase. This confirms the earlier explanation of why the immunoblotting of histidyl-tRNA synthetase after SDS–polyacrylamide gel electrophoresis was unsuccessful.

In order to determine whether the renatured enzyme, which gives a 10% recovery of activity, can restore the ability of the enzyme to recognize the antibody, the immunodot assay was carried out on the M_r 50000 and M_r 60000 proteins extracted from SDS preparative polyacrylamide gels and subsequently renatured. A more intense color reaction was observed for the M_r 50000 protein than for the M_r 60000 protein, but the result was not conclusive due to the high background observed (Figure 4B). The assay was then followed colorimetrically with the soluble substrate [2,2′-azinobis(3-ethylbenzothiazolinesulfonate)]. The absorbance at 415 nm for the M_r 50000 protein (0.60) was 2-fold higher than that for the M_r 60000 protein (0.31), which was comparable to the absorbance observed for sample without proteins. These results confirm that the M_r 50000 protein is immunoreactive with the anti-Jo-1 antibodies.

Inhibition Patterns of Histidyl-tRNA Synthetase. The effects of anti-Jo-1 antibodies on the kinetic parameters of histidyl-tRNA synthetase were determined at variable concentration of one substrate and saturating concentrations for the other two substrates. The Lineweaver–Burk plots for tRNA, ATP, and histidine as the variable substrates in the presence and absence of anti-Jo-1 IgG were obtained. The inhibition patterns obtained were competitive when ATP and histidine are the variable substrates.

In the case of tRNA as the variable substrate a more complex pattern was obtained (Figure 5). Two Michaelis–Menten constants were obtained for tRNA in the absence and

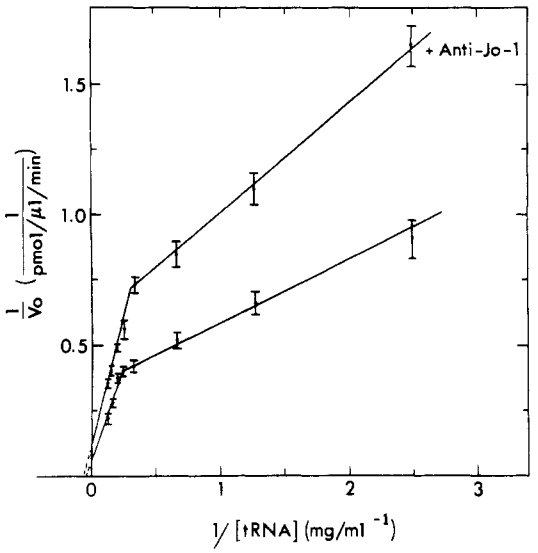

FIGURE 5: Inhibition pattern of histidyl-tRNA synthetase activity by anti-Jo-1 antibodies. The initial velocity of aminoacylation by histidyl-tRNA synthetase was determined in the presence and absence of anti-Jo-1 IgG with varying concentrations of tRNA. The error bar represents the maximal range of the results from three sets of experiments.

presence of anti-Jo-1 IgG. Noncompetitive inhibition by anti-Jo-1 antibodies was obtained. The kinetic parameters of the histidyl-tRNA synthetase in the presence or absence of the anti-Jo-1 antibodies are summarized in Table IV.

Discussion

While Rosa et al. (1983) isolated $tRNA^{His}$ from the immunoprecipitate using anti-Jo-1 antibodies, Mathews and Bernstein (1983) identified the protein moiety of the Jo-1 antigen as histidyl-tRNA synthetase with a subunit molecular weight of 50 000. However, there are some disparities with the molecular weights previously reported for the Jo-1 antigen (M_r 150 000) (Nishikai & Reichlin, 1980) and for reticulocyte histidyl-tRNA synthetase (subunit M_r 64 000) (Kane et al., 1978). Furthermore, since crude HeLa extract was used in correlating the histidyl-tRNA synthetase activity with the Jo-1 antigen, the possibilities of immunoprecipitation of contaminant proteins or ancillary proteins for histidyl-tRNA synthetase activity are yet to be completely eliminated.

As a result of the purification of histidyl-tRNA synthetase from rat liver in the present investigation, the subunit molecular weight of histidyl-tRNA synthetase is shown to be 50 000 by three independent procedures. The major protein contaminant with a subunit molecular weight of 60 000 is apparently inactive in both histidyl-tRNA synthetase activity and anti-Jo-1 antibodies reactivity. The contamination of the M_r 60 000 protein accounts for the erroneous assignment of the molecular weight of histidyl-tRNA synthetase (Yang et al., 1984). The hydrodynamic properties of histidyl-tRNA synthetase suggest a rather high axial ratio of the equivalent ellipsoid. The elongated shape of histidyl-tRNA synthetase gave an overestimated native molecular weight on the basis of gel filtration alone that is similar to the previously reported native molecular weight of histidyl-tRNA synthetase (Kane et al., 1978) and Jo-1 antigen (Nishikai & Reichlin, 1980) for a combined M_r of tRNA and synthetase. Combination of sedimentation coefficient and Stokes radius, which corrects for the shape effect, gives a native molecular weight of 96 000 for liver histidyl-tRNA synthetase. These results suggest that histidyl-tRNA synthetase has an α_2-type subunit structure with a subunit molecular weight of 50 000.

The purification of histidyl-tRNA synthetase, the identification of the M_r 50 000 proteins as histidyl-tRNA synthetase, and the reaction of purified histidyl-tRNA synthetase with anti-Jo-1 antibodies excluded the possibilities that the Jo-1 antigen may be an ancillary protein of histidyl-tRNA synthetase or contaminant protein in immunoprecipitation. Thus, the histidyl-tRNA synthetase is indeed at least one of the Jo-1 antigens, as previously concluded by Mathews and Berstein (1983).

It should be noted that rat liver was used in the present investigation because it is readily accessible. It is possible that conclusions drawn from the present investigation may be different for human enzymes. Nonetheless, mammalian aminoacyl-tRNA synthetases from different sources showed remarkably similar subunit structure for the eight synthetases in the multienzyme complex of aminoacyl-tRNA synthetases [e.g., Mirande et al. (1982)].

The anti-Jo-1 antibodies apparently recognize the native form of histidyl-tRNA synthetase, but do not or very poorly recognize the denatured form. This in turn suggests that the antigenic determinants are primarily conformational epitopes instead of a sequential type. This differs from the antibodies against mammalian synthetases raised in rabbits, which showed normally a reduction of the order of 50% in reactivities with the denatured form in immunoassays (Yang, unpublished results). In addition, the anti-Jo-1 antibodies do not inhibit the bacterial histidyl-tRNA synthetase, in contrast to the immuno-cross-reactivities between bacterial and silkworm alanyl-tRNA synthetases using antibodies raised in rabbits (Regan et al., 1986). Anti-Jo-1 antibodies may be useful as a screening probe in the molecular cloning of mammalian histidyl-tRNA synthetase (Tsui et al., 1985).

The observation that anti-Jo-1 antibodies showed competitive inhibition with histidine and ATP suggests that anti-Jo-1 antibodies bind close to the binding sites of histidine and ATP in the free form of histidyl-tRNA synthetase and that histidine and ATP are unlikely parts of the antigenic determinants. Both of the K_m's for tRNA are not affected in the presence of anti-Jo-1 antibodies, but the V_{max}'s are significantly reduced. Although it is not clear that the two K_m's reflect different populations of $tRNA^{His}$ or unsymmetric reactivity of the two subunits of histidyl-tRNA synthetase, a noncompetitive inhibition is consistent with the fact that anti-Jo-1 antibodies bind to both free and tRNA-bound histidyl-tRNA synthetase. However, the interpretation can only be considered as tentative until more detailed kinetic studies are carried out.

A higher extent of inhibition of histidyl-tRNA synthetase activity was observed when the histidyl-tRNA synthetase was preincubated with tRNA before the addition of anti-Jo-1 antibodies. We believe this is due to the anti-Jo-1 antibodies binding to the tRNA-synthetase complex with a significantly higher affinity than to the free synthetase, as results of its interaction with tRNA and tRNA-induced conformational changes in histidyl-tRNA synthetase. Conformational changes in both synthetase and tRNA have been demonstrated for bacterial enzymes [e.g., Ferguson and Yang (1986a,b)].

Immunoprecipitation of only one of the minor species of $tRNA^{His}$ in HeLa cells by anti-Jo-1 antibodies (Rosa et al., 1983; Mathews & Bernstein, 1983) suggests that some undetermined structural features in this particular species of $tRNA^{His}$ may have contributed additional antigenic determinants. Several unusual features are known (Rosa et al., 1983). The 5′ end has a methylated guanine. This guanine has been shown to be added posttranscriptionally (Cooley et al., 1982) but was not found to be methylated in $tRNA^{His}$ from

other sources. The wobble position in the anticodon sequence is an unmodified guanine and not the hypermodified base queuine that occurs in $tRNA^{His}$ from several species including the two isoacceptor $tRNA^{His}$ present in rabbit reticulocytes. It appears that these structural features in $tRNA^{His}$ (Rosa et al., 1983) present a unique tRNA–enzyme complex, which binds to the anti-Jo-1 antibodies as a ternary complex with a sufficiently high affinity to sustain the rather drastic conditions of immunoprecipitation.

The presence of the methylated base at the 5′ end and the unmodified anticodon in the immunoprecipitable $tRNA^{His}$ is intriguing. The ribonucleoprotein complex, which induces the formation of anti-Jo-1 antibodies, may contain an RNA with a low degree of modifications in contrast to the high degree of enzymatic modifications in tRNA.

The present approach in the studies of anti-Jo-1 antibodies and histidyl-tRNA synthetase may be applicable to other autoantibodies directed toward other synthetases, including threonyl- (Okada et al., 1983) and alanyl-tRNA synthetases (Mathews et al., 1984). The studies of the autoantibodies with their respective antigens by physical–chemical methods are hampered by the heterogeneity of the antibodies. The observation that anti-Jo-1 antibodies behave as a competitive or a noncompetitive inhibitor of the enzyme suggests the reversibility in the early stages of the binding of the antigen and antibody. It appears that the Jo-1 system may be particularly useful for studies of the interaction of autoantibodies with the respective antigens.

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Registry No. ATP, 56-65-5; His, 71-00-1; histidyl-tRNA synthetase, 9068-78-4.

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