Purification and Characterization of the Isoleucyl-tRNA Synthetase Component from the High Molecular Weight Complex of Sheep Liver: A Hydrophobic Metalloprotein†

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ABSTRACT: Native isoleucyl-tRNA synthetase and a structurally modified form of methionyl-tRNA synthetase were purified to homogeneity following trypsinolysis of the high molecular weight complex from sheep liver containing eight aminoacyl-tRNA synthetases. The correspondence between purified isoleucyl-tRNA synthetase and the previously unassigned polypeptide component of M_r 139 000 was established. It is shown that dissociation of this enzyme from the complex has no discernible effect on its kinetic parameters. Both isoleucyl- and methionyl-tRNA synthetases contain one zinc ion per polypeptide chain. In both cases, removal of the metal ion by chelating agents leads to an inactive apoenzyme. As the trypsin-modified methionyl-tRNA synthetase has lost the ability to associate with other components of the complex [Mirande, M., Kellermann, O., & Waller, J. P. (1982) *J. Biol. Chem. 257*, 11049–11055], the zinc ion is unlikely to be involved in complex formation. While native purified isoleucyl-tRNA synthetase displays hydrophobic properties, trypsin-modified methionyl-tRNA synthetase does not. It is suggested that the assembly of the aminoacyl-tRNA synthetase complex is mediated by hydrophobic domains present in these enzymes.

The existence, in extracts from mammalian cells, of high molecular weight complexes containing several aminoacyl-tRNA synthetases has been extensively documented in the past few years (Dang et al., 1982a; Dang & Dang, 1983). In particular, a highly purified complex has been isolated from sheep liver and spleen, as well as from rabbit liver and reticulocytes (Kellermann et al., 1982), from rat liver (Johnson & Yang, 1981), and from cultured Chinese hamster ovary (CHO) cells (Mirande et al., 1985). The eight aminoacyl-tRNA synthetases from sheep liver (Mirande et al., 1985), specific for arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, lysine, and methionine, are associated within the same complex (Mirande et al., 1982a) and are functionally independent (Mirande et al., 1983).

Little is known about the structural features involved in the association of these synthetases into a multienzyme structure. It has been suggested that lipids (Bandyopadhyay & Deutscher, 1973; Saxholm & Pitot, 1979) or carbohydrates (Dang et al., 1982b) may play a role in aminoacyl-tRNA synthetase assembly, but that these components actually were implicated in complex formation was not demonstrated. However that may be, hydrophobic interactions were supposed to contribute to the stability of the synthetase complex (Johnson et al., 1980; Sihag & Deutscher, 1983; Deutscher, 1984).

In order to gain more insight into the structural features responsible for aminoacyl-tRNA synthetase complex formation, we have purified to homogeneity one of the components of the complex. Isoleucyl-tRNA synthetase was isolated in the native state after trypsinolysis of the purified complex from sheep liver. It corresponds to the previously unidentified polypeptide component of M_r 139 000 common to the complexes from rabbit and sheep liver (Mirande et al., 1982b). Characterization of this enzyme was followed in parallel with that of trypsin-modified methionyl-tRNA synthetase from sheep liver, which was purified as a fully active monomer of M_r 68 000 after proteolysis of the high molecular weight complex (Kellermann et al., 1978). One of the distinctive features that characterize homogeneous isoleucyl-tRNA synthetase is its ability to bind to hexylagarose in conditions where trypsin-modified methionyl-tRNA synthetase does not. As the ability of the latter enzyme to associate with other components of the complex is lost on conversion of its associated form (M_r 103 000) to the monomeric form (M_r 68 000) (Mirande et al., 1982c), we propose that these mammalian enzymes are composed of a structural domain encoding catalytic functions accompanied by another domain that participates in complex formation through hydrophobic interactions.

Experimental Procedures

Materials. Isoleucyl- and methionyl-tRNA synthetases from *Escherichia coli* and bakers' yeast phenylalanyl-tRNA synthetase were gifts of Drs. P. Plateau, C. Hountoundji, and J. P. Pailliez (Palaiseau), respectively. The aminoacyl-tRNA synthetase complex from rabbit liver was purified as previously described (Kellermann et al., 1982). Bovine trypsin (TPCK treated), from Merck, was titrated by the method of Chase & Shaw (1970). The preparation used was 40% active.

Unfractionated tRNA from brewers' yeast was purchased from Boehringer-Manheim. Unfractionated tRNA from beef liver was a gift from Dr. J. Labouesse (Bordeaux). Partially purified isoleucine-specific tRNA from beef liver (275 pmol of isoleucine acceptance/A_{260} unit) was obtained by benzoyl-DEAE chromatography according to Fournier et al. (1976). ^{14}C-Labeled amino acids were from the Commissariat à l'Energie Atomique (Saclay, France).

Sepharose-bound tRNA containing 70–75 A_{260} units of unfractionated brewers' yeast tRNA/mL of gel bed was prepared as previously described (Kellermann et al., 1979). Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia, Ultrogel A6 was from IBF (Industrie

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Biologique Française), hydroxylapatite, Chelex 100, and Bio-Gel A-5m were from Bio-Rad, and hexylagarose was from Miles.

Soybean trypsin inhibitor and ATP[1] were from Boehringer, bovine serum albumin, Brij 30, 35, 56, and 72, and Triton X-100 and X-114 were from Sigma, and EDTA was from Prolabo. Other chemicals were from Merck.

Analytical Methods. Protein concentration was determined according to Lowry et al. (1951) with bovine serum albumin as the standard.

Enzymatic activities were measured at 25 °C under standard conditions optimized for methionyl-tRNA synthetase (Kellermann et al., 1982). The reaction mixture contained, in a final volume of 0.1 mL, 20 mM imidazole hydrochloride at pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 60 μM ^{14}C-labeled amino acid (50 Ci/mol), 0.5 mM dithioerythritol, and saturating amounts of tRNA (150 μM unfractionated yeast tRNA or 35 μM partially purified beef liver tRNA). Initial rates were measured with catalytic amounts of enzymes, appropriately diluted in 10 mM Tris-HCl, pH 7.5, containing 10 mM 2-mercaptoethanol and bovine serum albumin at 4 mg/mL. For the determination of the Michaelis constants for tRNA of isoleucyl-tRNA synthetase, the reaction mixture contained 2.5 nM of either the purified complex or purified isoleucyl-tRNA synthetase and variable concentrations of partially purified beef liver tRNA (0.046–10.9 μM, expressed as isoleucine-acceptor tRNA).

Zinc concentrations were measured by flame atomic absorption spectroscopy according to Mayaux & Blanquet (1981). Standard solutions of zinc were prepared by diluting a Titrisol solution (Merck) in buffer Z (20 mM Tris-HCl, pH 7.5, 150 mM KCl, Chelex 100 treated). Dialysis tubings were washed extensively with metal-free water, and prerinsed plastic tubes and pipetting material were used.

SDS–polyacrylamide gel electrophoresis and protein blotting experiments were conducted as previously described (Mirande et al., 1982c).

The apparent native molecular weight of purified isoleucyl-tRNA synthetase was determined by gel filtration through Sephadex G-200. The column (1.6 × 85 cm), equilibrated in 25 mM potassium phosphate, pH 7.5, 2 mM DTE, and 10% glycerol and developed at a flow rate of 6 mL/h, was calibrated with *E. coli* isoleucyl-tRNA synthetase (M_r 110000), *E. coli* methionyl-tRNA synthetase (M_r 152000), and yeast phenylalanyl-tRNA synthetase (M_r 274000).

Large-Scale Purification of Aminoacyl-tRNA Synthetase Complex from Sheep Liver. The high molecular weight complex containing the eight aminoacyl-tRNA synthetases specific for arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, lysine, and methionine was purified essentially as described earlier (Kellermann et al., 1979; Mirande et al., 1985). To perform a large-scale purification, starting from 5 kg of fresh sheep liver, the following modifications were introduced to the standard procedure.

After preparation of the crude extract by Waring Blendor homogenization, the material precipitating between 2% and 5% (w/v) of PEG [poly(ethylene glycol) 6000] was recovered in buffer A (25 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 10% glycerol) at a protein concentration of 10 mg/mL (5100 mL) and subjected to a second fractional precipitation with PEG. The aminoacyl-tRNA synthetase complex, which precipitated between 9% and 22% of PEG in buffer A, was recovered in 500 mL of buffer A lacking glycerol and applied on a column (25 × 45 cm) of Ultrogel A6 equilibrated in buffer A. Ascending chromatography was performed at a flow rate of 400 mL/h. Fractions containing the complex were combined (3590 mL) and directly applied on 1000 mL of Sepharose-bound yeast tRNA, as previously described (Kellermann et al., 1979). The complex emerged as two partially overlapping peaks (A and B), which were separately submitted to an additional fractionation step on hydroxylapatite. The combined fractions corresponding to peaks A (2040 mL) and B (1640 mL) were diluted to 100 mM potassium phosphate by slow addition of buffer B (10 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 10% glycerol) and applied on columns (2.5 × 12 cm) of hydroxylapatite equilibrated in buffer C (100 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 10% glycerol). The columns were washed with 30 volumes of buffer C, at a flow rate of 3 column volumes/h. Elutions were performed by linear gradients (850 mL) of 100–400 mM potassium phosphate, pH 7.5, containing 10 mM 2-mercaptoethanol and 10% glycerol. The aminoacyl-tRNA synthetase complexes emerged as single, symmetrical peaks at phosphate concentrations of about 250 and 350 mM, respectively, for peaks A and B. Fractions were pooled, concentrated by vacuum dialysis, dialyzed against buffer D (50 mM potassium phosphate, pH 7.5, 2 mM DTE, 50% glycerol), and stored at −20 °C.

Purification of Trypsin-Modified Methionyl-tRNA Synthetase from Sheep Liver. Homogeneous trypsin-modified methionyl-tRNA synthetase was purified starting from the purified aminoacyl-tRNA synthetase complex from sheep liver, fraction A. All steps were carried out at 0–4 °C, unless otherwise stated.

The complex from sheep liver (fraction A, 204 mg at 4 mg/mL in 30 mM potassium phosphate, pH 7.5, 2 mM DTE, and 25% glycerol) was incubated with trypsin (0.4 mg) for 30 min at 25 °C. After inactivation of trypsin by addition of 4-fold excess of soybean trypsin inhibitor, the digest was applied to a column (5 × 89 cm) of Bio-Gel A-5m equilibrated in buffer E (200 mM potassium phosphate, pH 7.5, 2 mM DTE, 10% glycerol) and developed at a flow rate of 50 mL/h. Methionyl-tRNA synthetase was eluted as a single symmetrical peak at an elution volume corresponding to an apparent molecular weight of about 70000. Fractions containing activity were pooled (172 mL), 5-fold diluted by slow addition of buffer B lacking glycerol, and subjected to chromatography on hydroxylapatite. The column (2.4 × 35 cm) was equilibrated in 50 mM potassium phosphate, pH 7.5, and 10 mM 2-mercaptoethanol and developed at a flow rate of 300 mL/h. The flow-through fraction, which contained methionyl-tRNA synthetase activity, was directly applied on a column (2.4 × 35 cm) of DEAE-Sephadex A-50 equilibrated in the same buffer. After the column was washed with 4 column volumes of this buffer, at a flow rate of 80 mL/h, elution was achieved by a linear gradient (2000 mL) of KCl (0–500 mM) in the same buffer. Methionyl-tRNA synthetase emerged as a single, symmetrical peak at a KCl concentration of about 120 mM. Fractions containing activity were pooled, concentrated by vacuum dialysis, dialyzed against buffer D, and stored at −20 °C. From the absorbance at 280 nm and the protein concentration measured according to Lowry et al. (1951), a specific optical extinction coefficient of 1.38 mg^{-1} cm^2 was determined for the purified enzyme.

[1] Abbreviations: tRNA, transfer ribonucleic acid; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; DTE, dithioerythritol; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

Purification of Native Isoleucyl-tRNA Synthetase from Sheep Liver. Native isoleucyl-tRNA synthetase was purified following controlled proteolysis of the purified aminoacyl-tRNA synthetase complex from sheep liver, fraction B. The complex [22 mg at 2.4 mg/mL in buffer F (25 mM potassium phosphate, pH 7.5, 2 mM DTE, 10% glycerol)] was incubated with trypsin (44 μg) for 60 min at 25 °C. Proteolysis was arrested by addition of a 4-fold excess of soybean trypsin inhibitor, and the digest was cooled in an ice–water bath. All subsequent steps were performed at 0–4 °C. The trypsin-modified complex was subjected to molecular sieve chromatography on Bio-Gel A-5m (1.6 × 83 cm) equilibrated in buffer G (100 mM potassium phosphate, pH 7.5, 2 mM DTE, 10% glycerol) and developed at 6 mL/h. Isoleucyl-tRNA synthetase was eluted as a single peak, with an apparent molecular weight of about 200 000. The eluate (30 mL) was diluted with 120 mL of buffer H (5 mM potassium phosphate, pH 7.5, 2 mM DTE, 10% glycerol) and applied on a column (2.6 × 15 cm) of Sepharose-bound yeast tRNA equilibrated in buffer I (30 mM potassium phosphate, pH 7.5, 2 mM DTE, 10% glycerol). After the column was washed with 4 column volumes of buffer I, isoleucyl-tRNA synthetase was eluted with a linear gradient (800 mL) of potassium phosphate, pH 7.5 (30–350 mM), containing 2 mM DTE and 10% glycerol, as a single peak at a phosphate concentration of about 100 mM. Fractions were combined (98 mL), dialyzed for 3 h against 2 × 2 L of buffer H, applied on a column (1.1 × 8 cm) of hydroxylapatite equilibrated in buffer F, and developed at a flow rate of 18 mL/h. After the column was washed with 8 column volumes of buffer F, the enzyme was eluted with a linear gradient (160 mL) of potassium phosphate, pH 7.5 (25–350 mM), containing 2 mM DTE and 10% glycerol. Isoleucyl-tRNA synthetase, emerging as a single symmetrical peak, was concentrated by vacuum dialysis and stored at −20 °C after dialysis against buffer D. The specific optical extinction coefficient of the purified enzyme, determined as in the case of the trypsin-modified methionyl-tRNA synthetase, was 1.22 mg^{-1} cm^2.

Results

Purification of Aminoacyl-tRNA Synthetases from Sheep Liver. The high molecular weight complex from sheep liver, containing the eight aminoacyl-tRNA synthetases specific for arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, lysine, and methionine (Mirande et al., 1985), was purified essentially as described by Kellermann et al. (1979). The purification procedure was adapted to the treatment of 5 kg of sheep liver, leading to the obtention of 63 mg of purified complex, fraction B. In addition to the four steps previously described (Kellermann et al., 1979), a second poly(ethylene glycol) precipitation and chromatography on hydroxylapatite were introduced, as described under Experimental Procedures and summarized in Table I. Methionyl-tRNA synthetase displayed the same specific activity (60 units/mg of protein) (Table I) as that previously reported (Kellermann et al., 1979), as did the other aminoacyl-tRNA synthetases (results not shown). Furthermore, the purified complex, fraction B, analyzed by SDS–polyacrylamide gel electrophoresis (Figure 1A), displayed a polypeptide pattern identical with that previously obtained. In addition to this highly purified fraction, the same eight aminoacyl-tRNA synthetases were also eluted from Sepharose-tRNA with 2-fold lower specific activities (5-fold for lysyl-tRNA synthetase). The origin of this material, corresponding to fraction A, is unclear. It may be the result of uncontrolled proteolysis encountered in the course of purification. It is noteworthy that only minor amounts of material corresponding to fraction A were encountered during the purification of the complexes from rabbit liver (Kellermann et al., 1982) or CHO cells (Mirande et al., 1985). Fraction A was used to purify the fully active monomeric form of methionyl-tRNA synthetase obtained after mild trypsinolysis of the complex. This modified enzyme was previously purified after subjecting the crude extract of sheep liver to proteolysis (Kellermann et al., 1978). The procedure described in this paper, summarized in Table II, allows the rapid recovery of homogeneous monomeric methionyl-tRNA synthetase (Figure 1C). The release of monomeric methionyl-tRNA synthetase

Table I: Large-Scale Purification of the Aminoacyl-tRNA Synthetase Complex from Sheep Liver[a]

purification step	tot protein (mg)	sp act. (units/mg)[b]	yield (%)	purification factor
10000*g* supernatant	380 000	0.11	100	1
2–5% poly(ethylene glycol) precipitate	51 000	0.55	70	5
9–22% poly(ethylene glycol) precipitate	16 000	1.61	59	15
Ultrogel A6 eluate	3 690	4.60	39	42
Sepharose–tRNA eluate				
peak A			10	
peak B			10	
total			20	
hydroxylapatite eluate				
peak A	113	35	9	318
peak B	63	60	9	545
total			18	

[a] The purification was monitored by following methionyl-tRNA synthetase activity. [b] A unit of activity corresponds to the formation of 1 nmol of aminoacyl-tRNA/min at 25 °C.

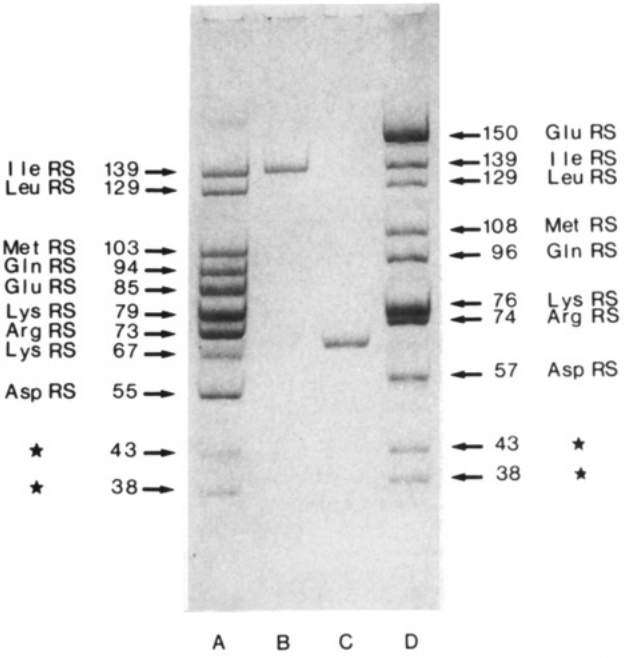

FIGURE 1: Polypeptide compositions of the purified aminoacyl-tRNA synthetases. The purified aminoacyl-tRNA synthetases were analyzed by electrophoresis on a gel of polyacrylamide, in the presence of SDS: (A) aminoacyl-tRNA synthetase complex from sheep liver (15 μg); (B) isoleucyl-tRNA synthetase from sheep liver (1.5 μg); (C) trypsin-modified methionyl-tRNA synthetase from sheep liver (1.5 μg); (D) aminoacyl-tRNA synthetase complex from rabbit liver (15 μg). The molecular weights of the polypeptide components of the complexes and their aminoacyl-tRNA synthetase assignments (amino acid RS) were determined previously (Mirande et al., 1982a), except for isoleucyl- and aspartyl-tRNA synthetase assignments [this study and A. Delcayre and J.-P. Waller (unpublished results), respectively].

Table II: Purification of Trypsin-Modified Methionyl-tRNA Synthetase from Sheep Liver

purification step	tot protein (mg)	sp act. (units/mg)[a]	yield (%)	purification factor
purified complex from sheep liver (fraction A)	204	26	100	1
trypsinolysis and Bio-Gel A-5m chromatography	26	142	79	5
hydroxylapatite chromatography			75	
DEAE-Sephadex A-50 chromatography	6.6	565	69	22

[a] Units of activity are as defined in Table I.

Table III: Purification of Native Isoleucyl-tRNA Synthetase from Sheep Liver

purification step	tot protein (mg)	sp act. (units/mg)[a]	yield (%)	purification factor
purified complex from sheep liver (fraction B)	22	33	100	1
trypsinolysis and Bio-Gel A-5m chromatography	7.4	41	40	1.2
Sepharose–tRNA chromatography			24	
hydroxylapatite chromatography	0.435	260	15	8

[a] Units of activity are as defined in Table I.

(M_r 68 000) from the high molecular weight complex was shown to proceed through the deletion of a third of its native polypeptide chain (M_r 103 000) (Mirande et al., 1982c).

Isoleucyl-tRNA synthetase was purified to homogeneity, in the native state, after mild trypsinolysis of the purified complex, fraction B. Purification was conducted as described under Experimental Procedures, by the three chromatographic steps summarized in Table III. Conservation of isoleucyl-tRNA synthetase activity required the use of 2 mM DTE in all buffers. This enzyme was estimated to be at least 90% homogeneous by SDS–polyacrylamide gel electrophoresis (Figure 1B). The purified product displayed a major polypeptide of M_r 139 000, which comigrated with the component of M_r 139 000 of the complexes from sheep and rabbit liver (parts A and D of Figure 1, respectively). The homology between these three polypeptides was further demonstrated by the protein blotting procedure (Figure 2), using antibodies directed against the component of M_r 139 000 originating from sheep liver (Mirande et al., 1982b). The minor contaminant of $M_r \simeq 70\,000$ present in the purified preparation of isoleucyl-tRNA synthetase was also recognized by these antibodies, indicating that it corresponds to a fragment generated during trypsinolysis of the complex. The apparent molecular weight of purified native isoleucyl-tRNA synthetase was estimated to be $\simeq 200\,000$ by gel filtration through a precalibrated column of Sephadex G-200.

This enzyme could also be isolated starting from the purified complex, fraction A, as a side product of the purification of trypsin-modified methionyl-tRNA synthetase (Table II). Upon chromatography on Bio-Gel A-5m, isoleucyl-tRNA synthetase was eluted ahead of monomeric methionyl-tRNA synthetase and could be further purified by chromatography on Sepharose–tRNA and hydroxylapatite. However, due to the presence of several contaminants in the purified complex fraction A, this procedure led to an enzyme estimated to be only 70% homogeneous.

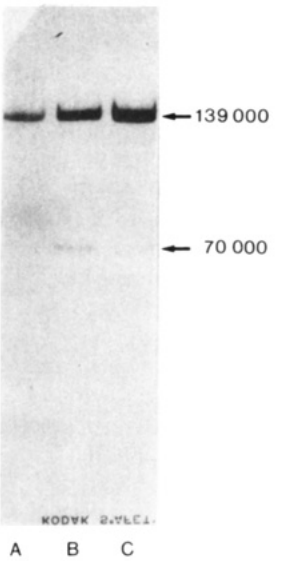

FIGURE 2: Homology between the purified isoleucyl-tRNA synthetase and the polypeptide components of M_r 139 000 of the purified complexes from sheep or rabbit liver. The aminoacyl-tRNA synthetase complex from rabbit liver (10 μg), isoleucyl-tRNA synthetase from sheep liver (1 μg), and the aminoacyl-tRNA synthetase complex from sheep liver (10 μg) (lanes A–C, respectively) were analyzed by the protein blotting procedure, using antibodies directed against the polypeptide of M_r 139 000 originating from sheep liver.

Determination of Kinetic Parameters of Isoleucyl-tRNA Synthetase. The apparent Michaelis constants for tRNA of the purified high molecular weight complex from sheep liver containing isoleucyl-tRNA synthetase and of homogeneous isoleucyl-tRNA synthetase were determined with a tRNA preparation enriched in $tRNA^{Ile}$ (275 pmol of isoleucine acceptance/A_{260} unit). The free and associated forms displayed nearly identical K_m values for $tRNA^{Ile}$ (2.0 and 2.5 μM, respectively) and similar turnover numbers (1.10 and 1.45 s^{-1}, respectively). These values were calculated with the assumption that 1 mol of complex (M_r 1.2×10^6) contains 1 mol of isoleucyl-tRNA synthetase (M_r 139 000) (Mirande et al., 1982c). It was shown previously that the aminoacyl-tRNA synthetase components remained associated under conditions prevailing in the tRNA aminoacylation assay (Mirande et al., 1983).

Zinc Content of Isoleucyl- and Methionyl-tRNA Synthetases from Sheep Liver. The zinc content of homogeneous isoleucyl-tRNA synthetase and trypsin-modified methionyl-tRNA synthetase were estimated from the data presented in Figure 3. Both isoleucyl- and methionyl-tRNA synthetases from sheep liver were found to contain tightly bound zinc at a molar ratio close to unity (1.1 and 1.2 zinc ions/monomeric enzyme, respectively). This metal ion could not be removed by overnight dialysis against buffer Z containing 1 mM EDTA. In addition, when these two enzymes were incubated in the presence of 10 mM 1,10-phenanthroline, the tRNA aminoacylation activities were rapidly decreased (Figure 4). Similar results were obtained in presence of 10 mM 2,2′-bipyridine. On the contrary, 1,7-phenanthroline, a nonchelating isomer of 1,10-phenanthroline, did not affect isoleucyl- and methionyl-tRNA synthetase activities. Identical irreversible inactivations in the presence of either 1,10-phenanthroline or 2,2′-bipyridine were observed for these two enzymes, when associated within the complex from sheep liver.

In another set of experiments, isoleucyl-tRNA synthetase and trypsin-modified methionyl-tRNA synthetase were dialyzed 10 h at 4 °C against buffer Z, with or without 10 mM 1,10-phenanthroline. After dialysis and centrifugation for 15

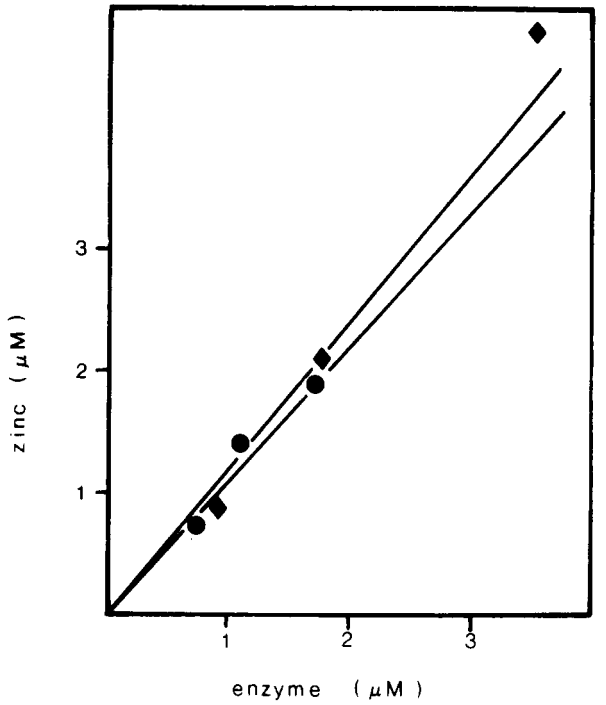

FIGURE 3: Zinc content of isoleucyl- and methionyl-tRNA synthetases from sheep liver. Enzyme samples were dialyzed 16 h against 2 × 1 L of buffer Z and then centrifuged 15 min at 10000*g* to remove insoluble material. The enzyme and zinc concentrations were measured by absorbancy at 280 nm and atomic absorption at 213.9 nm, respectively. Data for trypsin-modified methionyl-tRNA synthetase (♦) and purified isoleucyl-tRNA synthetase (●) are plotted on the figure.

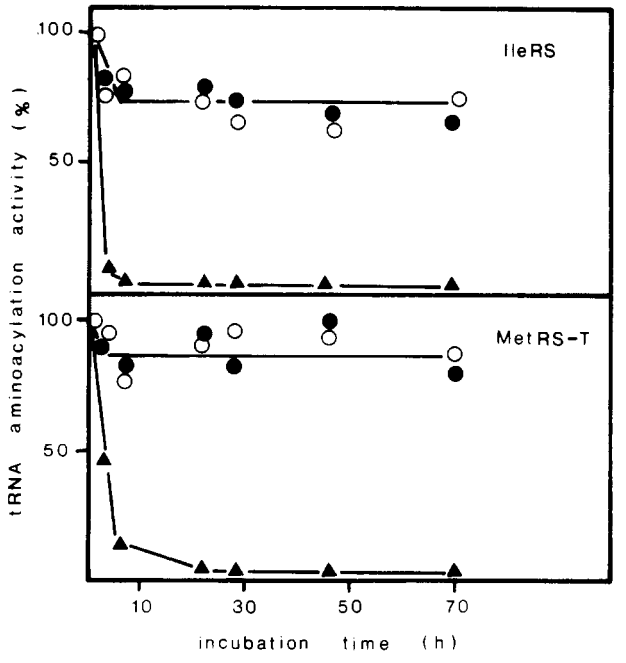

FIGURE 4: Effect of chelating agents on isoleucyl- and methionyl-tRNA synthetase activities. Purified isoleucyl-tRNA synthetase (Ile RS, 1 μM) and trypsin-modified methionyl-tRNA synthetase (Met RS-T, 1.5 μM) were incubated at 4 °C in buffer Z containing 1 mM DTE, in the absence (○) or in the presence of 10 mM 1,10-phenanthroline (▲) or 10 mM 1,7-phenanthroline (●). Initial rates of tRNA aminoacylation, expressed as percentage of the rates at $t = 0$, were measured as a function of time, after appropriate dilution.

min at 10000*g*, fractions were assayed for aminoacyl-tRNA synthetase activities, protein concentration, and zinc content. Control samples, dialyzed in the absence of phenanthroline, contained approximately 1 zinc ion/monomeric enzyme and

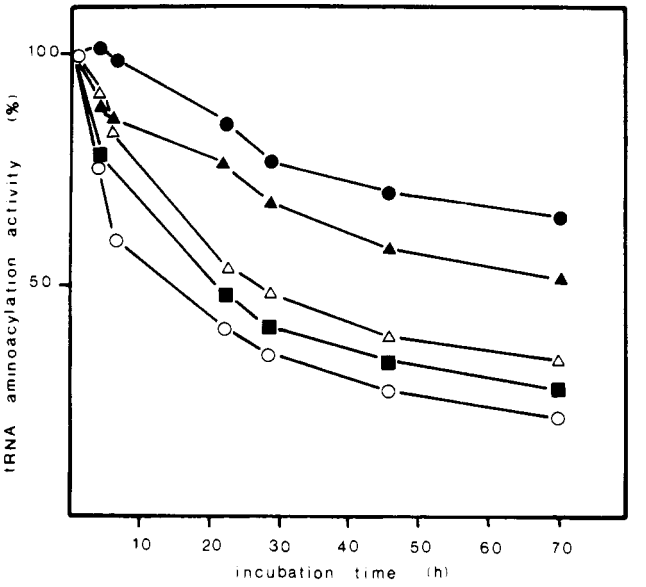

FIGURE 5: Effect of nonionic detergents on the stability of isoleucyl-tRNA synthetase activity. The purified low molecular weight form of isoleucyl-tRNA synthetase (0.1 μM) was incubated at 4 °C in 25 mM potassium phosphate, pH 7.5, 2 mM DTE, and 10% glycerol in the absence (○) or in the presence of nonionic detergents: 1.5 mM Brij 56 (●), 0.5 mM Brij 30 (▲), 0.03 mM Brij 72 (△), and 1.7 mM Brij 35 (■). Initial rates of tRNA aminoacylation, expressed as percentage of the rates at $t = 0$, were measured as a function of time, after appropriate dilution.

retained full activity (90% of their initial specific activities). While the input protein was fully recovered after dialysis in the above conditions, approximately 50% of the protein from samples dialyzed in presence of phenanthroline was lost as insoluble material after centrifugation at 10000*g*. In the soluble fraction of those samples, the zinc contents of methionyl- and isoleucyl-tRNA synthetases were only 0.5 and 0.4 zinc ion/molecule, respectively. Specific activities of both enzymes were largely decreased (30% of their initial specific activities), corresponding to 15% recovery of the input activity. Thus, the loss of activity observed in Figure 4 in the presence of 1,10-phenanthroline (10% residual activity after 10-h incubation) appears to be related to the removal of zinc, leading to a rapid denaturation of the apoenzyme.

Hydrophobic Properties of the Low Molecular Weight Isoleucyl-tRNA Synthetase from Sheep Liver. The stability of the low molecular weight form of isoleucyl-tRNA synthetase was investigated in the conditions described in the legend of Figure 5. The enzyme (0.1 μM) was incubated in the absence or presence of various nonionic detergents, at concentrations that depended upon their solubilities. In all cases, they were above their critical micelle concentrations. Using a Brij series, we found that isoleucyl-tRNA synthetase activity can be stabilized in the presence of detergent, depending upon the hydrophile–lipophile balance (HLB) (Becher, 1966) of the detergent used. Brij 72 (HLB = 4.9) and Brij 30 (HLB = 9.7) were less effective than Brij 56 (HLB = 12.9), which was more effective than Brij 35 (HLB = 16.9) (Figure 5). Tween 80 (1.5 mM) and digitonin (0.4 mM) (HLB = 11.0 and 12.7, respectively) were as effective as Brij 56. However, Triton X-114 and X-100 (3 mM each) (HLB = 12.4 and 13.5, respectively) did not prevent isoleucyl-tRNA synthetase inactivation, possibly due to the chemical structure of their nonpolar *p-tert*-octylphenol moiety. While isoleucyl-tRNA synthetase at 0.1 μM was unstable in the absence of nonionic detergents, trypsin-modified methionyl-tRNA synthetase (0.1 μM) remained stable when incubated in the absence of de-

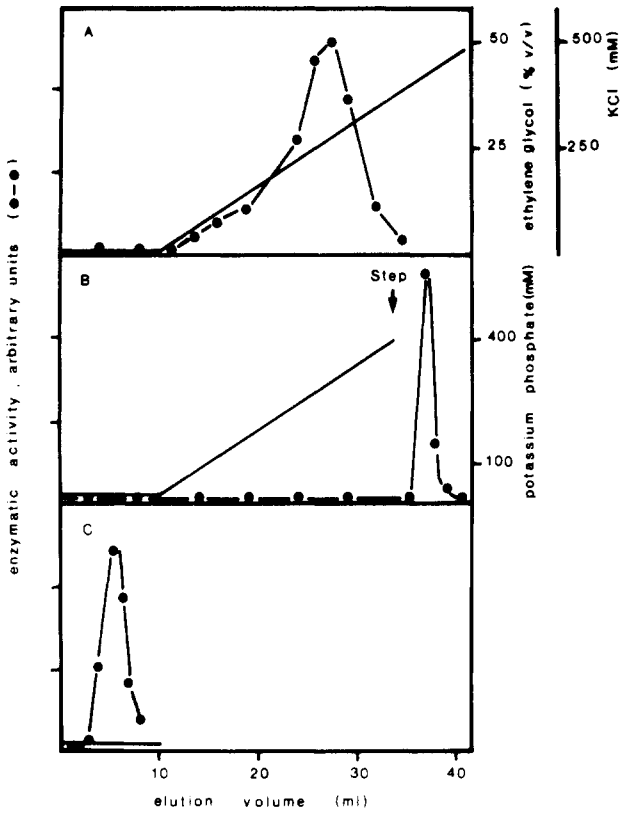

FIGURE 6: Hexylagarose chromatography of isoleucyl- and methionyl-tRNA synthetases from sheep liver. The purified low molecular weight form of isoleucyl-tRNA synthetase (A and B) or the trypsin-modified methionyl-tRNA synthetase (C) was applied on a column (1.1 × 1.0 cm) of hexylagarose equilibrated in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 2 mM DTE, and 10% glycerol (A and C) or in 25 mM potassium phosphate, pH 7.5, 2 mM DTE, and 10% glycerol (B) and developed at a flow rate of 2 mL/h. A total of 30 μg of protein was loaded. Elution was achieved by a linear gradient (40 mL) of KCl (25–500 mM) and ethylene glycol (0–50% v/v) in 50 mM Tris-HCl, pH 7.5, and 2 mM DTE (A) or by a linear gradient (40 mL) of potassium phosphate, pH 7.5 (25–450 mM), containing 2 mM DTE and 10% glycerol, followed by a step of 500 mM KCl and 50% ethylene glycol in 50 mM Tris-HCl, pH 7.5, and 2 mM DTE (B). Recovery of synthetase activities was (A) 54%, (B) 45%, and (C) 100%.

tergent (10–15% loss of activity after 70 h of incubation).

Increasing the concentration of purified isoleucyl-tRNA synthetase in the incubation mixture to 1.0 μM, in the absence of detergent, led to stabilization of its enzymatic activity (only 20–25% loss of activity after 70 h of incubation). In addition, when the aminoacyl-tRNA synthetase complex from sheep liver (0.1 μM) was incubated for 70 h in absence of detergents, in the conditions described in Figure 5, only 30% of isoleucyl-tRNA synthetase activity was lost. These results suggest that hydrophobic interactions (synthetase–synthetase or synthetase–detergent) may stabilize isoleucyl-tRNA synthetase activity.

The hydrophobic character of purified isoleucyl-tRNA synthetase was further demonstrated by hydrophobic interaction chromatography. When the low molecular weight form of isoleucyl-tRNA synthetase was applied to hexylagarose as described in the legend of Figure 6, its elution required the use of ethylene glycol and KCl (Figure 6A). Raising the phosphate concentration up to 450 mM was not effective for its recovery (Figure 6B). On the other hand, the trypsin-modified monomeric form of methionyl-tRNA synthetase did not bind to hexylagarose (Figure 6C). Thus, the free form of isoleucyl-tRNA synthetase, isolated from the high molecular weight aminoacyl-tRNA synthetase complex without proteolytic modification, displayed hydrophobic properties, whereas monomeric methionyl-tRNA synthetase, isolated from the complex after proteolytic deletion of a third of its polypeptide chain (Mirande et al., 1982c), did not.

Resistance to Proteolysis of Isoleucyl-tRNA Synthetase from Sheep Liver. To search for an active protease-modified form of isoleucyl-tRNA synthetase that might have lost its hydrophobic character, the purified low molecular weight form was subjected to controlled proteolysis. After incubation at a protein concentration of 1.1 μM in 25 mM potassium phosphate, pH 7.5, 2 mM DTE, and 10% glycerol for 30 min at 25 °C in presence of trypsin (1/10 w/w), elastase (1/2 w/w), subtilisin (1/20 w/w), thermolysin (1/2 w/w), chymotrypsin (1/30 w/w), or protease K (1/20 w/w), approximately 50% of the original polypeptide of M_r 139 000 was proteolyzed, as visualized by Coomassie blue staining after SDS–polyacrylamide gel electrophoresis of the digests. Proteolysis of the polypeptide was accompanied by a parallel loss of isoleucyl-tRNA synthetase activity (results not shown). It is noteworthy that much higher levels of proteases were necessary to achieve partial proteolysis of the purified enzyme than were required for its liberation from the high molecular weight complex. Thus the stability of isoleucyl-tRNA synthetase during trypsinolysis of the purified complex from sheep liver is not due to a protection by its neighboring components but is an intrinsic property of this enzyme.

Discussion

The extensive purification from sheep liver of a high molecular weight complex containing eight aminoacyl-tRNA synthetases specific for arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, lysine, and methionine was previously described (Kellermann et al., 1979; Mirande et al., 1985). The efficiency of this procedure was further demonstrated by its application to the purification of similar complexes from sheep spleen, rabbit liver, or rabbit reticulocytes (Kellermann et al., 1982), cultured CHO cells (Mirande et al., 1985), and rat liver (Johnson & Yang, 1981; Cirakoglu & Waller, 1985a). The large-scale purification of the aminoacyl-tRNA synthetase complex was a prerequisite for biochemical characterization of individual components. The scaled-up procedure described in this paper leads to the obtention of sufficient amounts of the purified complex to undertake such studies. In addition, fraction A obtained as a side product of purification can serve as starting material for the rapid purification of large amounts of trypsin-modified monomeric methionyl-tRNA synthetase.

An essential step in the characterization of this multienzyme complex is the assignment of the eight aminoacyl-tRNA synthetase activities to the polypeptides revealed by SDS–polyacrylamide gel electrophoresis. Since the complex had so far resisted dissociation under nondenaturing conditions to allow isolation and characterization of individual components in their native state, indirect procedures were used to correlate the polypeptide components with the aminoacyl-tRNA synthetase activities (Mirande et al., 1982b). Six of the relevant enzymes, specific for arginine, glutamic acid, glutamine, leucine, lysine, and methionine, were unambiguously identified. The isoleucyl-tRNA synthetase component could not be identified by the procedures used in that study but was presumed to correspond to the unidentified polypeptide of M_r 139 000 (Mirande et al., 1982b). The approach used in the present study to establish the correspondance between isoleucyl-tRNA synthetase activity and the polypeptide of M_r 139 000 was founded on the observation that this polypeptide was very resistant to tryptic digestion of the purified complex,

under conditions that led to extensive alterations of the original SDS–polyacrylamide gel electrophoretic pattern (Mirande et al., 1982c). This suggested the possibility that proteolytic degradation of the purified complex may have released isoleucyl-tRNA synthetase as a fully active, structurally intact component, amenable to purification by conventional procedures. Following this approach, the present study describes the successful purification of the free form of isoleucyl-tRNA synthetase. It is shown that this enzyme does indeed correspond to the polypeptide of M_r 139 000.

We previously reported that the eight aminoacyl-tRNA synthetases that were copurified from sheep liver as a heterotypic complex (Mirande et al., 1982a) were functionally independent (Mirande et al., 1983). In addition, we showed that proteolytically modified free forms of methionyl- and lysyl-tRNA synthetases, derived from the complex, displayed kinetic parameters indistinguishable from those of their native, associated counterparts. However, that study was based on the comparison of native associated and proteolyzed free forms of aminoacyl-tRNA synthetases. Thus, the possibility that catalytic functions of native enzymes might be impaired when the portion of the structure excisable by proteolysis is not engaged in the macromolecular structure was left open. The demonstration that the free and associated forms of native isoleucyl-tRNA synthetase display identical kinetic parameters clearly establishes that association of these enzymes within the high molecular weight complex is not a prerequisite for full expression of their catalytic functions.

It was noted elsewhere (Mirande et al., 1983) that the proteolytically modified free forms of lysyl-tRNA synthetase (M_r 2 × 64 000) and methionyl-tRNA synthetase (M_r 68 000) released from the complex of sheep liver have structural properties similar to those of the corresponding enzymes from *E. coli*: M_r 2 × 67 000 for native lysyl-tRNA synthetase (Hirschfield et al., 1976); M_r 64 000 for the trypsin-modified monomeric methionyl-tRNA synthetase (Cassio & Waller, 1971). The homology between prokaryotic and eukaryotic aminoacyl-tRNA synthetases is further emphasized by showing that native isoleucyl-tRNA synthetase and trypsin-modified methionyl-tRNA synthetase from sheep liver contain one zinc ion per polypeptide chain, as in the case of their prokaryotic counterparts (Posorke et al., 1979; Mayaux & Blanquet, 1981). It was shown that the loss of zinc from *E. coli* trypsin-modified methionyl-tRNA synthetase was directly proportional to its inactivation (Mayaux et al., 1982). The irreversible loss of activity observed after incubation of the low molecular weight form of isoleucyl-tRNA synthetase from sheep liver with chelating agents such as 2,2′-bipyridine or 1,10-phenanthroline, but not with the nonchelating isomer 1,7-phenanthroline, is also related to the removal of zinc ion from the enzyme. Similarly, zinc is a requirement for the expression of the enzymatic activity of the trypsin-modified monomeric methionyl-tRNA synthetase from sheep liver. Since this enzyme has lost its ability to associate with other components of the complex, these results strongly suggest that the enzyme-bound zinc ions are not involved in the association of these aminoacyl-tRNA synthetases within the multienzyme structure.

Despite these similarities, prokaryotic and eukaryotic aminoacyl-tRNA synthetases differ widely in their subunit molecular weights (Dang & Dang, 1983; Mirande et al., 1982b), which are significantly higher for eukaryotic enzymes. It was previously proposed that this chain elongation may be implicated in the assembly of the multienzyme complex (Mirande et al., 1983; Deutscher, 1984). Moreover, involvement of hydrophobic interactions in the formation of this complex was suggested by several authors (Johnson et al., 1980; Sihag & Deutscher, 1983; Deutscher, 1984). In agreement with these predictions, the results presented in this study show that isoleucyl-tRNA synthetase isolated from the complex in its native state displays hydrophobic properties, while the proteolytically modified methionyl-tRNA synthetase does not.

These observations support a structural model for the multienzyme complex, according to which the constituent aminoacyl-tRNA synthetases are each composed of a catalytic domain freely accessible to substrates and a hydrophobic domain responsible for complex formation (Cirakoglu et al., 1985). Associations between aminoacyl-tRNA synthetases may occur through direct interactions between their hydrophobic domains or else through participation of other components, such as one or more of the three proteins of unknown function that are present in these complexes (Mirande et al., 1982b).

Studies are presently in progress in our laboratory to further demonstrate the involvement of hydrophobic interactions in the aminoacyl-tRNA synthetases assembly. In particular, lysyl- and leucyl-tRNA synthetases were purified to homogeneity, in their native state, after dissociation of the purified complex from sheep liver by hydrophobic interaction chromatography (Cirakoglu & Waller, 1985b). As in the case of isoleucyl-tRNA synthetase, the two enzymes displayed hydrophobic properties.

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Registry No. Isoleucyl-tRNA synthetase, 9030-96-0; methionyl-tRNA synthetase, 9033-22-1; aminoacyl-tRNA synthetase, 9028-02-8.

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Probing Histidine–Substrate Interactions in Tyrosyl-tRNA Synthetase Using Asparagine and Glutamine Replacements†

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ABSTRACT: We have analyzed the interactions of a histidine residue with a substrate using site-directed mutagenesis. Previous studies on tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* have shown that a histidine residue (His-48) makes an interaction with ATP, which is improved on mutating Thr-51 → Pro-51. We find on replacing His-48 in wild-type enzyme with either asparagine or glutamine that Asn-48 is equally as good as His-48 but His-48 → Gln-48 leads to a far lower activity. The side chain of an asparagine residue may be superimposed on that of a histidine so that the amide $-NH_2$ group of asparagine occupies the same position as the π-N of histidine, whereas the equivalent $-NH_2$ group of glutamine may be superimposed upon the τ-N. This suggests that it is the π-N of histidine that hydrogen bonds with ATP and that there is no significant electrostatic interaction between the histidine and ATP. Incorporating the Pro-51 mutation into each of the Asn-48 and Gln-48 mutants gives an improvement in the affinity of the enzyme for ATP, but this improvement is less than that seen with the wild-type enzyme.

Residues in the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* that are known from protein crystallographic studies to interact with the substrates are being systematically altered by site-directed mutagenesis of the gene (Winter et al., 1982; Fersht et al., 1984). The effects of these mutations on substrate binding and the enzyme kinetics allow a detailed analysis of the roles of such contacts in catalysis. This enzyme catalyzes the aminoacylation of $tRNA^{Tyr}$ in a two-step reaction (Fersht & Jakes, 1975) in which the tyrosine is activated to give enzyme-bound tyrosyl adenylate (eq 1) before being transferred to $tRNA^{Tyr}$ (eq 2).

$$E + Tyr + ATP = E\cdot Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\cdot Tyr\text{-}AMP + tRNA^{Tyr} = Tyr\text{-}tRNA^{Tyr} + AMP + E \quad (2)$$

It is known from the crystal structure of the enzyme-bound tyrosyl adenylate (Rubin & Blow 1981) that a histidine at position 48 is in close proximity to the ribose ring oxygen of ATP (Figure 1). Mutagenesis experiments are consistent with there being a hydrogen bond from the histidine (Carter et al., 1984). It was not possible, however, to identify which of the two nitrogens of the imidazole ring is likely to be making a hydrogen-bond contact with the ribose ring oxygen or whether the imidazole ring is positively charged and contributes an electrostatic interaction to the binding of the negatively charged phosphate groups of ATP. In an attempt to answer these questions, we have made mutations at position 48 to asparagine and glutamine. These amino acids have a nitrogen atom placed, respectively, three and four atoms distant from the α-carbon atom, in equivalent positions to the nitrogens in a histidine imidazole (see Figure 2). By studying the energetics of the mutated enzymes, we have deduced the most likely orientation of the histidine ring and have ruled out any large electrostatic contributions in its binding to ATP in the wild-type enzyme.

In a previous study it was shown that mutation of residue 51 from a threonine to a proline greatly improves the strength of the interaction of His-48 with ATP in the transition state

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