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Isolation and Analysis of Mutated Histidyl-tRNA Synthetases from *Escherichia coli*

Andreas Rühlmann,*[,1] Friedrich Cramer,† and Uwe Englisch‡

†*Max-Planck-Institut für Experimentelle Medizin, Department of Chemistry, Hermann-Rein-Strasse 3, 37075 Göttingen, Germany;* **Medizinische Hochschule Hannover, Institut für Molekularbiologie, OE5250, 30623 Hannover, Germany; and* ‡*Fachhochschule Lübeck, FB Angew. Naturwissenschaften, Stephensonstrasse 3, 23562 Lübeck, Germany*

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Amino terminally deleted and point-mutated histidyl-tRNA synthetases were purified from *E. coli* via βGal fusion proteins. A hinge region proximal and distal to the factor Xa cleavage region was necessary to cut the βGal-fusion proteins efficiently under very mild nondenaturing conditions. N-terminal addition of either methionine or valine to this enzyme (its starting N-formyl-methionine is *in vivo* post-translationally removed) or the deletion of 6 amino terminal amino acids decreased the specific aminoacylation activity 2- to 7-fold. Further N-terminal deletions of 10 or 17 amino acids caused significantly reduced aminoacylation (100-fold) and ATP/PPi exchange (10-fold) activities, and a reduced binding affinity for histidine. Removal of 18 or more amino acids from the N-terminus thereby removing residues from MOTIF 1 resulted in inactive histidyl-tRNA synthetase mutants. Two point mutations within the histidyl-adenylate binding pocket, R259Q and R259K, also blocked histidyl-tRNA synthetase activity without affecting histidine or ATP binding. The experiments shown identify a highly conserved N-terminal R/KG-patch in front of MOTIF 1 as well as R259 as vital for full enzymatic activity.

Aminoacyl-tRNA synthetases catalyze the aminoacylation of a tRNA with the appropriate amino acid in a two-step mechanism [1]: condensation of an amino acid with ATP to an enzyme-bound aminoacyladenylate [2] and esterification of this adenylate with tRNA resulting in a released aminoacyl-tRNA. Despite a common mechanism of action this class of enzymes shows diversity of both size and quarternary structure [3]. Sequence comparisms and structure-function studies reveal that some domain elements are common to most synthetases and suggest that two classes gave rise to the diverse set of aminoacyl-tRNA synthetases during evolution [4-8]. Each class consists of 10 members easily characterized by their signature sequences. Class I bears according to the one letter amino acid abbreviation code the HIGH and KMSKS regions which are integral parts of the Rossmann fold. Class II enzymes are characterized by the sequence motifs 1, 2 and 3.

Histidyl-tRNA synthetase (HisRS) from *E. coli* is one of the smallest bacterial synthetases. It belongs to the class IIa of these enzymes and its crystal structure complexed with histidyl-adenylate was recently elucidated [9]. HisRS is a homodimeric protein of 92 kDa with 424 amino acids in each subunit and bears two sequence motifs Histidine A and Histidine B conserved among all known HisRS. Most interestingly within Histidine A, arginine 259 makes a direct substrate interaction to histidyl adenylate as suggested by the X-ray structure [9]. Similarly the most recently determined crystal structure of histidyl-tRNA synthetase from *Thermus thermophilus* [10] complexed with histidine or histidine-adenylate shows a direct interaction of R259, which compares to R259 in the *E. coli* enzyme, with the α-phosphate of the adenylate. Therefore arginine 259 is most likely of paramount importance for the function of HisRS its exchange should change enzyme function and an experimental verification is eminent. A sequence comparison among the known histidyl-tRNA syntethases reveals a striking sequence preservation within the N-terminal domain. N-terminal deletions might therefore lead to clear effects on enzymatic activity. The N-terminal formyl-methionine is post-transcriptionally removed [11]. To investigate the functional effect of this removal, the role of the N-terminus, and the conserved arginine 259 for enzymatic activity

[1] Corresponding author. Fax: (+49 511) 532 4283. E-mail: RHL@IFM.MH-Hannover.de.

Abbreviations: aa, amino acid; APTG, *p*-aminophenyl-β-D-thiogalactoside; βME, β-mercaptoethanol; βGal, β-galactosidase; bp, base pair(s); factor Xa, bovine blood coagulation factor Xa; HisRS, histidyl-tRNA synthetase; hisS, gene encoding for HisRS; IPTG, isopropyl-β-D-thiogalactoside; PVDF, polyvinylidene fluoride; ss-DNA, single stranded DNA; XaaRS, Xaa(yl)-tRNA synthetase.

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we investigated a number of point and deletion mutants of HisRS. Here we present evidence that the first 17 N-terminal amino acids and arginine 259 are vital for the function of HisRS and are first experimental verifications of the X-ray structure of this enzyme and additionally give an experimental support for the interpretation of the crystal structure of the very similar HisRS from *Thermus thermophilus* [10].

MATERIALS AND METHODS

Plasmid construction. Oligodeoxynucleotides were synthesized by the solid phase phosphoamidite method [12] using either an Applied Biosystems model 380A (Weiterstadt, Germany) or Biosearch model Sam One DNA synthesizer (San Rafael, CA, USA).

DNA was manipulated according to standard techniques [13, 14] using the conditions recommended by the suppliers. DNA fragments were isolated from agarose gels with gene clean (Bio 101, La Jolla, CA, USA). Ligated DNAs were transformed in the appropriate *E. coli* strains (TG1 or JM109) made competent as described by Hanahan [15]. Enzymes were purchased from Boehringer-Mannheim, Gibco BRL (Eggenstein, Germany) and New England Biolabs (Schwalbach, Germany), chemicals from Baker (Deveter, Netherlands), Biomol, Fluka (Ulm, Germany), Merck (Darmstadt, Germany), Riedel-de Haen (Seelze, Germany) or Serva (Heidelberg, Germany). T4 DNA Ligase, the Klenow Fragment of DNA polymerase I and Sp-dCTPαS was a gift of Dr. F. Eckstein (Max-Planck-Institut für experimentelle Medizin, Göttingen).

The production of single-stranded plasmid DNA was performed according to Vieira and Messing [16] using M13K07 as a helper phage. Site-directed mutagenesis procedures were performed according to Sayers and Eckstein [17].

To facilitate the purification of foreign proteins without vector-encoded amino acids, the vectors pAX5+/− (Amersham, Braunschweig, Germany; MobiTec, Göttingen, Germany) were constructed. A *Nru* I site (TCG′CGA) was mutated within the coding sequence of the factor Xa recognition sequence [18] of the vector pAX4c+ [19] via phosphorothioate-based oligonucleotide directed mutagenesis using the mutation primer 5′-TCCATGGTCGTCGCGACCCTCGATT. The resulting vector was called pAX5+ (6201 bp). The pAX5- was constructed by ligation of the 915 bp-*Xmn* I fragment from pAX4c-, which includes the intergenic region of the bacteriophage f1 (f1 IG) and the 5286 bp-*Xmn* I fragment from pAX5+. The +/− indicates the orientation of the f1 IG. The + orientation leads to a rescue of the coding strand for βGal as ss-DNA.

Construction of the vector pAR15+N, pARQ259 and pARK259: The 1.79 kbp *Pvu* II-*Sal* I fragment from pSE411 [20] was ligated into the *Sal* I and *Sma* I cleaved vector M13mp8 [21], generating pTU801. Using single-stranded pTU801-DNA, the recognition sequence for the restriction endonuclease *Nru* I was mutated within the first codon (GTG) of the *hisS* gene [11] with the mutation primer 5′-TGAATGTTTTTCGCGACGTTTATTCT, resulting in the vector pTU801/NruI. The vector pTU801/NruI was cleaved with *Nru* I and *Pst* I and the 1.44 kbp *Nru* I-*Pst* I-*hisS* gene fragment was isolated and ligated into the *Nru* I and *Pst* I linearized vector pAX5+, resulting in the phagemid pAR15+. In order to maintain the reading frame with the *lacZ* gene, two additional 5′-nucleotides (5′-CG-*hisS*-3′) were inserted via phosphorothioate-based insertional mutagenesis using the mutation primer 5′-GGCTTGAATGTTTTTCGCGCGACCCTCGATTGC, generating the vector pAR15+N. The codon R259 CGT was mutated into a CAG codon in case of glutamine or in AAG in the case of lysine (removing the Eco NI site) using site-directed mutagenesis in pTU801/NruI with the mutation primer RQ 5′-GTAATCCAGACCCTGCA CCAGGCGCTGGTTTAC or QK 5′-GTAATCCAGACCCTTCACCAGGCGCTGGTTTAC, respectively. The mutations were verified by sequencing and inserted as *Nru* I-*Pst* I fragments into pAX1 [19], resulting in the plasmid pARQ259 and pARK259.

Generation of 5′deleted *hisS* gene fusion expression vectors and pAR11+: Eight 5′-deletion mutants of the *hisS* gene were constructed using restriction sites within the *hisS* gene (Table 2). The 1.44 kbp *Nru* I-*Pst* I-*hisS* gene fragment from pTU801/NruI was ligated into the *Kpn* I (flushed) and *Pst* I cleaved vector pMEX7 [22] (Amersham, Braunschweig, Germany; MobiTec, Göttingen, Germany). This vector pAR301- was digested as follows: *Nco* I (addition of methionine), *BspM* I (deletion of 17 codons of the *hisS* gene), *Eae* I (deletion of 19 codons), *Nae* I (deletion of 90 codons) or partially digested with *Ple* I (deletion of 76 codons). Klenow-fragment of DNA polymerase I and dNTP were used to generate blunt ended DNA. After a second cut with *Pst* I the resulted 1.3-1.4 kbp *hisS* fragments were isolated. The 1.44 *Nco* I(PolK)-*hisS* fragment was ligated into the *Nru* I and *Pst* I cleaved vector pAX5+ resulting in pAR11+. The plasmid pAR10 was described elsewere [19]. The 1396 bp *BspM* I(PolK)- and the 1390 bp *Eae* I-*Pst* I-*hisS* fragment was ligated into the *Nru* I and *Pst* I cleaved vector pAX5-. The vectors were named pAR5ΔBspMI- and pAR5ΔEaeI-, respectively. The *Ple* I-*Pst* I and the *Nae* I -*Pst* I-*hisS* -fragments were ligated into the Klenow-fragment treated *Nco* I and *Pst* I cleaved pAX4a+ vector. The plasmids were named pAR4ΔNaeI+ and pAR4ΔPleI+, respectively. The plasmid pAR5Δ23- was generated by deleting the *Eae* I cleaved pAR301- vector with S1 nuclease, recleaving with *Pst* I and inserting this *hisS* fragment into the *Nru* I and *Pst* I cleaved pAX5-. The plasmid pARΔEaeI was obtained in the ligation of the *Eae*I-*Hin*dIII fragment from pAR301- with the *Pst*I (T4 blunted) and *Hin*dIII treated pAX1 vector [19]. The vector pAR15+ was the target to introduce the coding sequences of the restriction enzymes *Nar* I within the sixth codon or *Sph* I within the tenth codon via site-directed mutagenesis using the mutation primer ONarI 5′-CATGCCGCGAATGGCGCCAATGTTTTTCGC or OSphI 5′-GTAATCGTTCATGCATGCAATGGCTTGAATGTTTTTC, respectively. The 1428 bp *Nar* I(PolK treated)-*Pst* I-*hisS* and the 1416 bp *Sph* I(T4Pol treated)-*Pst* I-*hisS* gene fragments could then be isolated and ligated into the *Nru* I and *Pst* I linearized vector pAX5-. The plasmid were named pAR5ΔNarI- and pAR5ΔSphI- respectively. All plasmids were confirmed by sequencing [23, 24].

Protein expression and isolation. Overnight cultures of *E. coli* containing pAX plasmids or derivatives thereof were1:300 diluted in 750 ml NZCYMT-ampicilin medium (10 g NZ-amine (Otto Aldag, Hamburg, Germany), 1 g casamino acids (Difco), 5 g yeast extract, 2 g $MgSO_4 \cdot 7H_2O$, 5 g NaCl, 60 μg/ml ampicillin-sodium salt, 1 ml 1% (w/v) thiamine-hydrochloride) and were grown with shaking at 25°C until the stationary phase was reached. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and the culture was grown for an additional 5h at 25°C. All subsequent steps were carried out at 4°C. After harvesting the cells at 4000xg, 10 min, the pellet was resuspended in 1/5 volume lysis buffer (50 mM Tris-HCl pH 7.4, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 200 μg/ml lysozyme, 1 mM $PhMeSO_2F$). After adding 10 μg/ml DNAseI the cells were lysed in a french press (SLM aminco, Urbana, IL, USA) (110 MPa). Phenylmethylsulfonyl fluoride ($PhMeSO_2F$) was added to 2 mM final concentration. The cellular debris were removed by centrifugation for 15 min (17000xg). Equal volumes of lysate and DE52 (Whatman, Maidstone, England), equilibrated in lysis buffer, were mixed and transferred to a column. The column was reloaded with the flow through, washed with 2 column volumes lysis buffer/150 mM NaCl and eluted with lysis buffer/400 mM NaCl. Fractions containing the fusion protein were pooled and loaded on a 2 ml p-aminophenyl-β-D-thiogalactoside(APTG)-sepharose column previously equilibrated with lysis buffer/400 mM NaCl. The flow through was loaded a second time on the column, which was then washed with the flow through from the DEAE step and with 100 column volumes of lysis buffer. The βGal-fusion proteins were eluted with elution buffer (100 mM sodium borate pH 10, 10 mM βME in 0.5 volumes of 1 M Tris-HCl pH 7.0) into 1.5 ml tubes containing 200

μl 1 M Tris-HCl pH 6.8. The appropriate fractions were concentrated by ultra filtration (amicon ultra filtration cell with diaflo YM 100 filter, Danvers, Ireland) to 2-5 μg/μl and stored at −20°C after adding glycerol to 40%(v/v) final concentration.

The column material was prepared by coupling APTG (Sigma, Deisenhofen, Germany) to CH-Sepharose 4B (Pharmacia, Freiburg, Germany) according to a preparation of Bastia et al. [25] and used as described by Ullmann [26].

Factor X was purified and activated as described [18, 27, 28]. For site-specific cleavage of fusion proteins 1/100th molar ratio of factor Xa [18] was added to the purified fusion protein and dialysed 24h at 4°C with buffer changes after 1h, 4h, 9h and 14h against Xa buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$). The addition of factor Xa prior to the dialysis was vital in order to prevent insoluble precipitates of inactive proteins. The proteolysis of the fusion proteins with collagenase was performed with 3 U/mg (fusion protein) in 250 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$, 10 mM βME and 0.05 mM $ZnCl_2$ for 30 min at 37°C [29]. The collagenase was a gift from Dr. F. Grosse (Max-Planck-Institut für experimentelle Medizin).

After cleavage of the fusion protein, 0.1 volumes of P300 buffer (300 mM K-phosphate pH 7.4, 10 mM β-mercaptoethanol, 10% (v/v) glycerol) was added and applied to a hydroxylapatite (Pharmacia, Freiburg) column (1 ml/2 mg fusion protein) equilibrated with P30 buffer (30 mM K-phosphate pH 7.4, 10 mM β-mercaptoethanol, 10% (v/v) glycerol). The flow through was reapplied eight times to the same column and afterwards eluted in fractions with buffer P400 (400 mM K-phosphate pH 7.4, 10 mM β-mercaptoethanol, 10% (v/v) glycerol). Fractions were concentrated by ultra filtration (centricon 10, Amicon, Danvers) and stored at −20°C after addition of glycerol to a total of 50% (v/v). Proteins obtained had greater than 95% purity according to densitometric estimations.

Immunostaining and analysis of proteins. Proteins were run on SDS-PAGE and Coomassie-stained using standard protocols [13, 14]. Silver staining was performed after Blum et al. [30], semi-dry Western blotting according to Kyhse-Andersen [31]. Anti-HisRS (*E. coli*) antibodies were produced by *s. c.* injection of a 4 kg white New Zeeland rabbit with 600 μg purified HisRS from *E. coli* [32] in 1 ml complete Freund's adjuvant. A second booster injection was performed after 28 days with 600 μg HisRS in incomplete Freund's adjuvant. After 8 additional days 20 ml blood was taken. This serum could detect 0.5 ng HisRS in a 1:5000 dilution and was used in western blots on nitrocellulose. The staining was done with anti-rabbit-alkaline phosphatase coupled goat antibodies (Serva) and BCIP/NBT staining (Boehringer Mannheim).

Protein sequencing. The proteins were transferred to a PVDF membrane and sequenced with a modified solid-phase degradation [33] on an Applied Biosystems protein sequencer model 477.

Determination of the enzyme activity. The aminoacylation activity and the ATP/PPi exchange activity was measured according to Igloi et al. [34] using bulk *E. coli* tRNA and [U-^{14}C]-(S)-histidine (Amersham) or (S)-histidine and [^{32}P]-$Na_2P_2O_7$ (Amersham), respectively. To determine the kinetic parameters, the substrate concentration was varied with five different substrate conditions in the range of 4-24 μM for (S)-histidine and 0.1-1 mM for ATP. K_m and k_{cat} values were calculated from plots of radioactive incorporation against reaction time and Lineweaver-Burk plots using linear regression. All values were verified at least twice. One unit HisRS ATP/PPi exchange activity is the catalyzed incorporation of 1 nmol ^{32}PPi on coal paper (Binzer, Hatzfeld/Eder) in one minute at 37°C in test mix (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 50 mM βME) with 2.6 mM [^{32}P]-$Na_2P_2O_7$ (9.25 kBq/nmol), 0.1 mM (S)-histidine and 4.6 mM ATP. One unit HisRS aminoacylation activity is the esterification of 1 nmol (S)-histidine to *E. coli* $tRNA^{His}$ in one minute at 37°C in test mix with 50 μM [U-^{14}C]-(S)-histidine, 20 mg/ml *E. coli* $tRNA^{bulk}$ and 5.3 mM ATP. One unit HisRS activity is the esterification of 1 nmol (S)-histidine to *E. coli* $tRNA^{His}$ in one minute at 37°C in test mix with 100 μM (S)-histidine, 20 mg/ml E. coli $tRNA^{bulk}$ and 5 mM ATP [35].

RESULTS

Large-Scale Expression and Isolation of the Histidyl-tRNA Synthetase Mutants

To ease handling and to prevent undesired mutations within *hisS* mutants, homologous expression in the *recA*$^-$ *E. coli* strain JM109 was chosen. The isolation of two synthetases, either the authentic HisRS (pAR15+N) or HisRS with one additional amino terminal valine (pAR10) are shown in Figure 1a and presented in Table 1. In the final purification step the HisRS and their mutants were released out of their βGal-fusion proteins by site specific proteolysis and purified on a hydroxylapatite column. Western blotting was used to differentiate the fusion proteins from possible contaminating wild type HisRS and demonstrate the specific release of the synthetase from the fusion protein (Figure 1b). In addition, the specific cleavage by factor Xa was verified by amino terminal sequencing of the first twenty residues of two isolated HisRS, the authentic synthetase (using pAR15+N) and the Val-HisRS (using pAR10). Fusion proteins were cleaved with factor Xa under very mild conditions at 4°C for 24 h or with collagenase at 37°C (Table 2). Interestingly removal of seventeen or more N-terminal amino acids from HisRS resulted in fusion proteins that could not be cleaved with factor Xa under the used conditions, even after a previous collagenase treatment.

Sequence Comparison of Known Histidyl-tRNA Synthetases

Figure 2 shows an alignment of the seventeen currently known complete sequences of histidyl-tRNA synthetases extracted from available databases or publications [11, 10, 36-49]. These are from sixteen different species, bacteria to mammals. Human HO3 is a recently identified homologue of the human HisRS [48]. Beside the MOTIF 1-3 homology regions typical for class II synthetases, all HisRS show a highly conserved Histidine A and Histidine B sequence motif and a characteristic N-terminal R/KG- and a C-terminal VAILGE-patch. The eukaryotic synthetases can most easily be differentiated from the bacterial and archaebacterial (*Methanococcus jannaschii* [40]) by a N-terminal extension of 45 to 58 amino acids representing most likely mitochondrial import sequences. In addition there is an obvious kingdom specific sequence conservation. The eukaryotic sequences and the bacterial sequences of *E. coli* [11], *Haemophilus influenzae* [36] and *Streptococcus equis* [37] show a high degree of similarity among each other. The mycobacterial sequences are most prominently characterized by an arginine to valine substitution in Histidine B and a weak VAILGE-

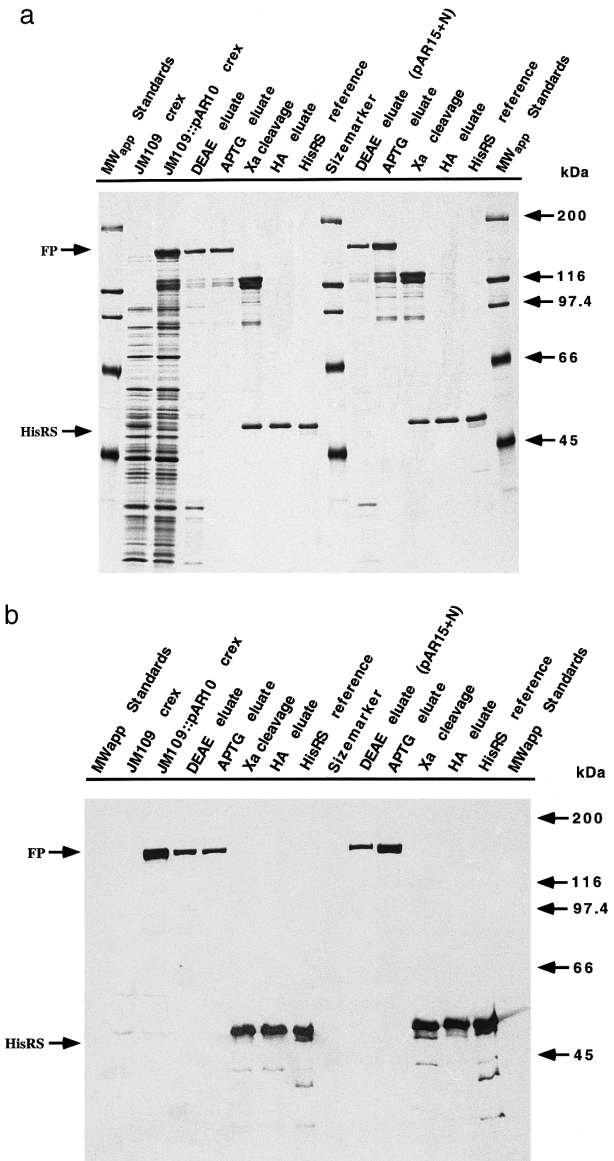

FIG. 1. Isolation of HisRS and Val-HisRS proteins. (**a**) 8% SDS polyacrylamide gel showing the purification steps of the N-terminal valine added HisRS (using the plasmid pAR10) and the authentical HisRS (using the plasmid pAR15+N) via expression and factor Xa cleavage of the appropriate β-galactosidase fusion protein. (**b**) Western blot of an 8% SDS polyacrylamide gel run with same loading and identical conditions as in a), using polyclonal antibodies raised against purified bacterial HisRS.

patch. *Mycobacterium leprae* (Smith and Robinson, unpublished) is more distantly related to *Mycobacterium genitalium* [38] and *Mycobacterium pneumoniae* [39]. Among the eukaryotic sequences the residues and length between Histidine A and Histidine B can easily used as an differentiation marker, separating clearly with a gradual shortening the yeast (*Saccharomyces cerevisiae* [41]) sequence from the sequences of fish (*Fugu rubripes* [44]), worm (*Caenorhabditis elegans* [42, 43]), mammals [45-48] (hamster, mouse, human) and rice (*Oryza sativa;* Akashi and Small, unpublished). The published cyanobacterial *Synethocystis sp.* HisRS [49] has a very unusual composition having a very weak VAILGE-patch and lacking the basic arginine or lysine in the R/KG-patch and several otherwise conserved residues in Histidine A and Histidine B. An enzymatic activity of such a protein remains to be shown. Sequencing errors might be another explanation, similarly to some sequence irregularities to the hamster *Mesocricetus auratus* [45]. Due to the many sequencing errors in the published *Caenorhabditis elegans* sequence from Amaar and Baillie [43], the data from Wilson et al. [42] was used instead.

Functional Analysis of the Histidyl-tRNA Synthetase Mutants

The specific activities of the mutated synthetases for aminoacylation and the ATP-PPi exchange reaction are given in Table 3. The highest specific activity was observed for the authentic synthetase generated from the plasmid pAR15+N, which lacks the N-terminal methionine, like the *in vivo* enzyme [11]. Addition of either methionine or valine to the amino terminus of HisRS lowered the specific aminoacylation activity approximately 4-fold. Deletion of six amino terminal amino acids (including methionine 1) led to a 7-fold depletion in specific aminoacylation activity without affecting the ATP-PPi exchange reaction. Further deletion of ten or seventeen amino acids caused the specific aminoacylation activity to decrease 100-fold, while the ATP-PPi exchange reaction decreased to 18% of wild type activ-

TABLE 1

Purification of HisRS (pAR15+N) and Val-HisRS (pAR10) Protein

Purification step	Total protein (mg)	Volume (ml)	Total HisRS activity (U)	Specific HisRS activity (U/mg)
	pAR15+N (HisRS)			
Crude lysate	280	50	8100	29
DEAE flow through	60	100		
DEAE 150 mM wash	160	200		
DEAE 400 mM wash	22	70		
APTG eluate	5.4	8	4100	750
HA eluate	0.5	6	2700	5400
	pAR10 (V-HisRS)			
Crude lysate	1100	50	5550	5
DEAE flow through	120	200		
DEAE 150 mM wash	700	200		
DEAE 400 mM wash	80	100		
APTG eluate	10	12	2400	250
HA eluate	1.1	7.5	1350	1100

TABLE 2

Plasmids and Corresponding Fusion Proteins with Cleavage Properties

Plasmid	Protein encoded HisRS	Factor Xa cleavage	Collagenase cleavage
pAR15+N	HisRS	+	+
pAR11+	M-HisRS	+	+
pAR10	V-HisRS	+	+
pAR5ΔNarI-	des-(A2-Q6)-HisRS	+	+
pAR5ΔSphI-	des-(A2-G10)-HisRS	+	+
pAR5ΔBspMI-	des-(A2-G17)-HisRS	−	+
pARΔEaeI	VAMS-des-(A2-E18)-HisRS	−	+
pAR5ΔEaeI-	des-(A2-T19)-HisRS	−	+
pAR5Δ23-	des-(A2-R24)-HisRS	−	+
pAR4ΔPleI+	VATM-des(A2-D76)-HisRS	−	+
pAR4ΔNaeI+	VATM-des-(A2-A91)-HisRS	−	+
pARQ259	V-HisRS-R259Q	+	+
pARK259	V-HisRS-R259K	+	+

The βGal-fusion proteins are of the type βGal-crs-IEGR-X: crs - 70 aa collagenase recognition site; IEGR - recognition site for factor Xa, which cleaves after arginine; X - protein encoded HisRS; amino acid(s) are shown in single letter code.

ity. If eighteen or more amino acids are removed from the amino terminus of the synthetase, the enzyme was inactivated.

The kinetic parameters for the aminoacylation reaction of various mutants are shown in Table 4. The k_{cat} values for histidine were similar between the authentic synthetase and the mutants with one amino acid added or six amino acids deleted. In contrast the ten amino acid deleted mutant showed an approximate 6-fold drop in the k_{cat} values of the aminoacylation activity. These attributes were reflected in the k_{cat} values for ATP albeit not entirely. Authentic synthetase showed the highest k_{cat} ATP value, which was clearly affected by the addition of a single amino acid or removal of six amino acids. Deletion of nine amino acids caused a drastic reduction from 34 s^{-1} for the wild type enzyme to 0.2 s^{-1}. The K_m His value for the wild type HisRS is with 8 μM in very good agreement with Kalousek and Konigsberg [50]. Upon the removal of N-terminal amino acids the K_m value for histidine increased. In contrast the K_m for ATP (ATP/PPi exchange reaction) declined for the slightly N-terminally modified mutants from 560 μM to 350 μM, but again increased for the ten amino acid deletion mutant. The authentic synthetase showed the highest k_{cat}/K_m values with 815 $s^{-1}mM^{-1}$ (His) and 61 $s^{-1}mM^{-1}$ (ATP/PPi), respectively. The addition of one amino acid or the deletion of six amino acids caused a 33-64% reduction in both the k_{cat}/K_m values, whereas a deletion of ten or more N-terminal amino acids resulted in drastically less active or inactive synthetases for both activities.

TABLE 3

Specific Activities of the Aminoacylation and ATP/PPi Exchange Reaction for Various HisRS Mutants

Protein	Aminoacylation [U/mg]	ATP/PPi exchange [U/mg]
FP V-HisRS	380 ± 40	n. d.
FP HisRS	800 ± 40	4200 ± 120
HisRS	6000 ± 500	14000 ± 1700
M-HisRS	1400 ± 160	n. d.
V-HisRS	1100 ± 160	n. d.
des-(A2-Q6)-HisRS	700 ± 50	11500 ± 1000
des-(A2-G10)-HisRS	60 ± 10	2500 ± 650
FP des-(A2-G17)-HisRS	42 ± 5	370 ± 100
FP VAMS-des-(A2-E18)-HisRS	4 ± 0.2	20 ± 1
FP des-(A2-T19)-HisRS	2 ± 0.2	13 ± 1
FP des-(A2-R24)-HisRS	2 ± 0.2	58 ± 1
FP VATM-des(A2-D76)-HisRS	3 ± 0.2	46 ± 1
FP VATM-des-(A2-A91)-HisRS	4 ± 0.2	88 ± 1
V-HisRS-R259Q	3 ± 0.2	32 ± 1
V-HisRS-R259K	4 ± 0.2	111 ± 20

FP-βGal-fusion protein (see table 2); amino acid(s) are shown in single letter code.

Within Histidine A in the HisRS from *E. coli* arginine 259 was mutated to glutamine or lysine. These two HisRS point mutants were enzymatically inactive, although the R259K mutant showed ATP/PPi exchange activity slightly above background levels. The K_m values for histidine and ATP (Table 2) were identical to their closest derivative, V-HisRS, suggesting that these mutants had no gross conformational distortion.

DISCUSSION

Choice of and Cleavage Prerequisites for the Fusion Proteins

We used and improved our already established βGal-fusion vector system pAX4 so that an isolation of the HisRS proteins without additional amino acids was possible using mild purification procedures to preserve full enzymatic activity. Renaturation of a denatured

HisRS from *E. coli* does not fully restore its enzymatic competence (data not shown). This isolation via βGal-fusion proteins produces proteins of approximal 700 kDa due to the tetrameric structure of β-galactosidase. We thought to utilize this size difference in comparison to the native 96 kDa HisRS as an additional purification advantage not given in fusion protein vectors like e. g. GST- [51], Thioredoxin- [52], MBP- [53], histidine- [54] tagged fusion proteins.

The properties of the HisRS mutants are summarized in Table 2. The use of the pAX5+/− vector and its derivatives allowed the mild cleavage of the βGal-fusion proteins with factor Xa without a denaturing step. This preserved the enzymatic activity of βGal (data not shown), as well as the attached HisRS (Table 2), despite the fact that the former is a tetrameric and the latter a dimeric protein. Not all βGal-HisRS fusion constructs could be cleaved with factor Xa under the mild conditions used. While the des-(A2-G10)-HisRS mutant was still released from the fusion protein, all further deletion mutants were not, even with increased factor Xa concentrations, incubation times or temperatures. In these cases even a previous collagenase treatment did not facilitate cleavage with factor Xa.

Catalytic Properties of the Isolated Histidyl-tRNA Synthetases

While the deletion of seventeen N-terminal amino acids still resulted in a weakly active HisRS mutant, at least one additional amino acid step inactivated both the aminoacylation and the ATP-PPi exchange reaction (Table 3). The specific activities for the HisRS enzymes clearly discriminated between the authentic HisRS, which started with alanine 2, with 6000 U/mg and the N-terminal methionine added M-HisRS mutant with 1400 U/mg. This implied that it might be advantageous for the enzyme activity to have the starting methionine removed. *In vivo* the formyl-methionine is post-transcriptionally hydrolyzed from the enzyme [11]. This is likely to slightly increase enzyme activity or have some other regulatory function, possibly by freeing a degradation signal. Change of methionine to valine did not alter the catalytic properties of HisRS.

The specificity constants k_{cat}/K_m shown in Table 4 indicate that an N-terminal addition of valine or methionine or a deletion of only six amino acids caused less than 2.8-fold reduction in the catalytic activity of HisRS for both k_{cat}/K_m values. The deletion of six amino acids still leaves a highly conserved R/KG-patch (Figure 2) common to all currently known HisRS [11, 10, 36-49]. The remarkable kingdom specific sequence conservation among the HisRS could be utilized as a differentiation marker to identify a class of an organism. The region between Histidine A and Histidine B seems to be particularly interesting, since both regions are largely totally conserved among all species. The N-terminal extended regions of the eukaryotic sequences probably represent mitochondrial import sequences, as shown for *S. cerevisiae* [55]. The deletion of 60 amino acids from the N-terminus of human HisRS, thereby deleting the K60 in the R/KG-patch, inactivates the enzyme [56]. This observation is somewhat surprising in view of the des-(A2-G10)-HisRS mutant, which clearly showed residual activity. The N-terminal 50 amino acids of *Bombyx mori* GlyRS, a class IIa snthetase, shows similarity with the N-terminal amino acids from human HisRS. Deletion of up to 55 amino acids from *Bombyx mori* GlyRS still results in 15% aminoacyl-tRNA synthetase active mutants [57]. Similarly we would predict a residual catalytic activity for the K60 deletion. One explanation may be the differing reaction conditions, namely 4°C instead of 37°C and the use of yeast tRNA as a substrate instead of the homologous human tRNA.

Figure 3 visualizes the gradual loss of enzymatic activity accompanied with the subsequent removal of N-terminal amino acids. Deletion of seventeen or more amino acids inactivates the synthetase. Two deletion mutants show a drastically loss of enzymatic activity

TABLE 4

Kinetic Parameters of Purified HisRS Proteins

Protein	K_m His [μM]	k_{cat} His [s^{-1}]	k_{cat}/K_m His [$s^{-1}mM^{-1}$]	K_m ATP [μM]	k_{cat}ATP [s^{-1}]	k_{cat}/K_m ATP [$s^{-1}mM^{-1}$]
HisRS	8 ± 2	7 ± 2	815	560 ± 20	34 ± 3	61
M-HisRS	11 ± 4	6 ± 2	550	390 ± 80	9 ± 2	24
V-HisRS	17 ± 9	6 ± 2	370	350 ± 80	8 ± 2	22
des(A2-Q6)-HisRS	20 ± 4	9 ± 2	450	220 ± 40	8 ± 2	30
des(A2-G10)-HisRS	60 ± 30	1 ± 0.3	15	655 ± 100	0.2 ± 0.1	3
V-HisRS-R259Q	n. d.	n. d.		330 ± 40	0.4 ± 0.1	1.2
V-HisRS-R259K	17 ± 6	0.1 ± 0.02	6	300 ± 20	0.5 ± 0.1	1.7
HisRS[1]	6	n. d.		n. d.		

n.d. - not determined; [1] Data from Kalousek and Konigsberg[50]. His describes the observed values of the aminoacylation reaction, ATP describes the observed values of the ATP/PPi exchange reaction; single letter code for amino acids is shown.

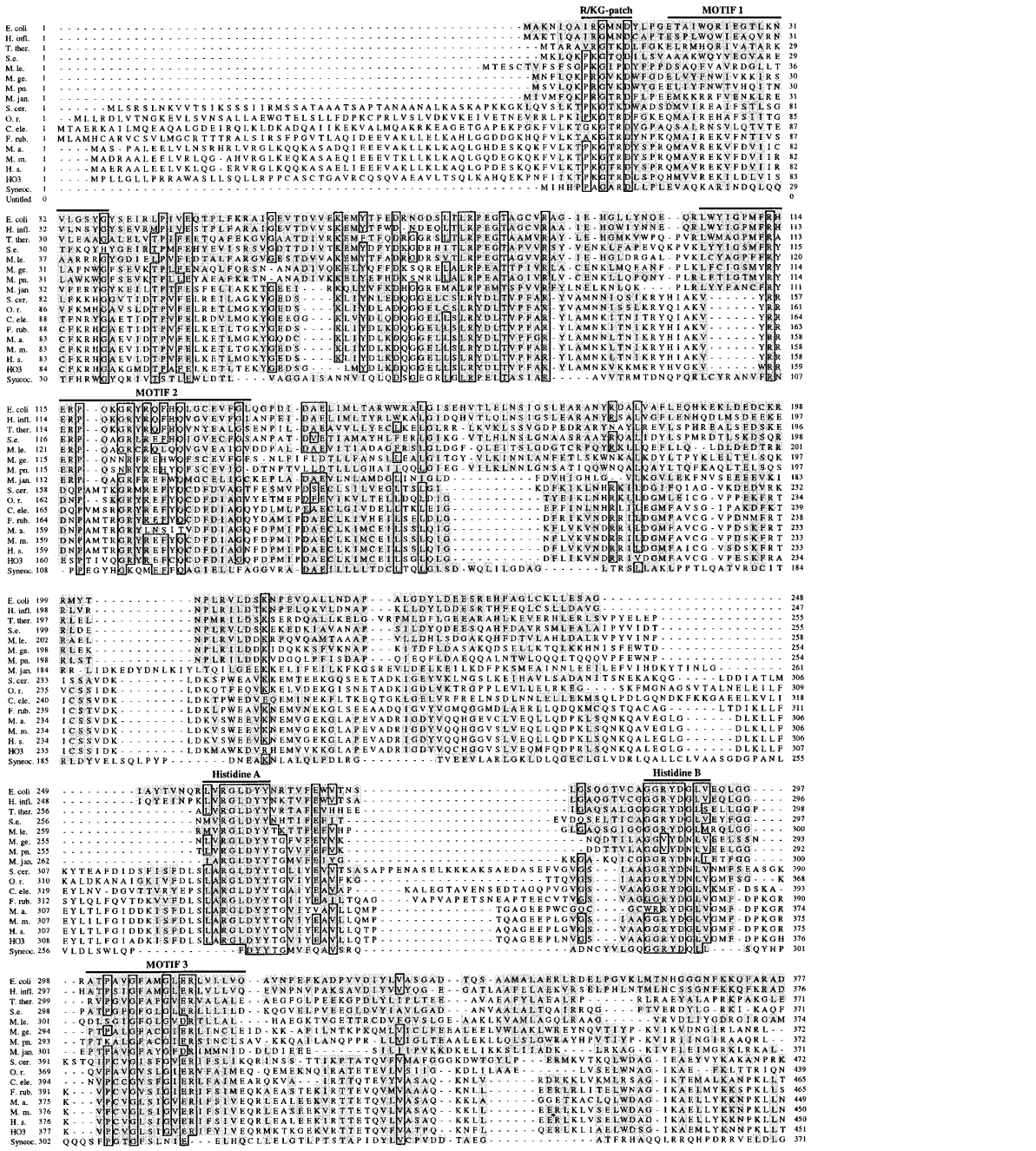

FIG. 2. Sequence alignment of known HisRS. Multiple alignment of HisRS sequences using the program PILEUP (Genetics Computer Group, University of Wisconsin, USA) and manual adjustments: E.coli, *Escherichia coli*, P04804 [11]; H. infl., *Haemophilus influenzae*, P43823 [36]; T. ther., *Thermus thermophilus* [10]; S. e., *Streptococcus equis*, P30053 [37]; M. le., *Mycobacterium leprae*, P46696 (Smith and Robinson, unpublished); M. ge., *Mycobacterium genitalium*, P47281 [38]; M. pn., *Mycobacterium pneumoniae*, U00089 [39]; M. jan., *Methanococcus jannaschii*, U67542 [40]; S. c., *Saccharomyces cerevisiae*, P07263 [41]; O. s., *Oryza sativa*, Z85984 (Akashi, K. and Small, I. D., unpublished); C. e., *Caenorhabditis elegans*, Z69384 [42, 43]; F. rub., Fugu rubripes, Z54243 [44]; M. a., *Mesocricetus auratus*, P07178 [45]; M. m., Mus musculus, U39473 [46]; H. s., *Homo sapiens*, P12081 [45, 47]; HO3, human HisRS homolog, U18937 [48]. Syneoc., Synechocystis sp., D90916 [49]. Regions of identity are boxed, regions of homology are shaded.

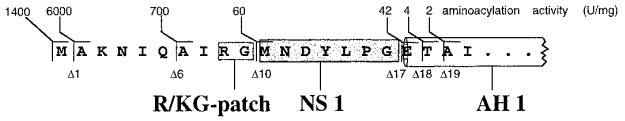

FIG. 3. Aminoacylation activities of selected N-terminal deletion mutants. NS-1, N-terminal sequence 1; and AH 1, alpha helix 1 are abbreviations used in Arnez et al. [9].

the des(A2-G10) HisRS and the des(A2-E18) HisRS mutant. Deletion of G10 in the des-(A2-G10)-HisRS mutant removes a central element within the R/KG-patch resulting in drastically reduced catalytic activity of this enzyme, both in the aminoacylation and the ATP/PPi-exchange reaction. According to the recently published X-ray structure of HisRS [9], G10 stabilizes a bend between the N-terminus and D13, which forms a salt bridge with R90. This may be explained by the ensuing destabilization of the D13-R90 salt bridge, which seems to play an essential role for the catalytic activity of HisRS. The increase of the K_m value from the authentic methionine-deleted HisRS to altered or successively deleted N-terminal ends of HisRS indicate an involvement of this very N-terminal part in the binding of (S)-histidine. Deletion into the MOTIF 1 sequence starting with helix AH1 represented by the des(A2-E18) HisRS mutant inactivates the synthetase. This is an important finding since the deletion of A2-G10 could already interfere with the proposed dimer interface motif 1 starting with the interface helix AH1 (formally H7) at glutamic acid 18 [58]. The GlyRS from *Thermus thermophilus* is another dimeric class IIa synthetase which lacks a proline 66 (compares to proline 44 in *E. coli* HisRS) previously thought to be the only invariant amino acid in MOTIF 1 bearing synthetases. Yet the crystal structure of *T. thermophilus* GlyRS [59] defines a MOTIF 1 structure as an essential element of the catalytic framework. Although several aminoacyl-tRNA synthetases complexed with adenylate have been reported [60-63] the basis for the specific binding of the cognate amino acid is still not resolved [9]. Alternatively the first 10 amino acids might participate in intersubunit interactions and its loss may weaken or even destroy the dimeric structure of the enzyme. Although we can not formally exclude this it has not been shown that any class II tRNA synthetase is active as a monomer. It might be very likely that the dimeric conformation is essential for tRNA binding and therefore aminoacylation activity. In addition a loss of a dimeric structure would imply a sudden total loss of enzymatic activity unless a monomeric HisRS would still maintain a substantial enzymatic activity. This would include binding of histidine, ATP and tRNA. The formation of heterodimers can under our isolation conditions be ruled out. The C-terminal deletion of 140 amino acids from *E. coli* HisRS is a monomeric protein but without any remaining aminoacylation activity (T. Ueda and U. Englisch, unpublished observations). This deletion completely removes the C-terminal domain of HisRS as the only domain which interacts exclusively with the other monomer [9] or as in the case of the dimeric *Thermus thermophilus* HisRS where the C-terminal domain of one subunit has an extensive interface with the catalytic domain of the other subunit [10]. In fact a deletion of 104 C-terminal amino acids results in a functional active monomeric 320-residue Ncat HisRS fragment [64]. This suggests a role for the C-terminus for dimerisation beside its postulated importance for anticodon recognition of $tRNA^{His}$ [8-10]. A function of the C-terminus, as the least conserved domain of HisRS, for dimerization might be less stringent for sequence conservation during evolution than domains involved in binding of identical substrates as histidine and ATP. On the other hand there is the conserved VAILGE-patch common to almost all known HisRS (Fig. 2) which might be responsible for GUG anticodon recognition or for dimerisation.

The binding pocket for histidyl-adenylate [9] is represented by the two most highly conserved regions in all known HisRS (Fig. 2), the two octapeptides L257VRGLDYY (Histidine A) and A283GGRYDGLV (Histidine B). In this active site R259 makes a direct substrate contact to the phosphate oxygen of the histidyl-adenylate. Mutation of arginine 259 into glutamine resulted in an inactive HisRS (Table 3 and 4), indicating that R259 is critical for the catalytic activity of HisRS. The exchange of R259 to a lysine in V-HisRS-R259K resulted in a loss of catalytic activity, although there was a slight increase in the specific ATP/PPi exchange activity from 32 U/mg for the R259Q mutant to 111 U/mg for the R259K mutant (Table 3). The exchange of arginine to a lysine or glutamine within a protein is a mild change according to the amino acid similarity coefficients [65]. Using these stability constants it can be inferred that the exchange of an arginine to a lysine resulted in the loss of one hydrogen bond (−10 kJ/mol) in an hypothetical activation complex [66]. This would agree with the electron density of the active site of HisRS [9]. In conclusion the data we present here are in full aggreement with the HisRS structure predicted by Arnez et al. [9] confirm arginine 259 as a crucial residue in the binding pocket and suggest a vital role of the first 17 N-terminal amino acids for enzymatic activity. In addition our experiments support the interpretation of the crystal structure of HisRS from *Thermus thermophilus* [10] and suggest that the posttranslational removal of the N-terminal formyl-methionine might cause an enhancement of enzyme activity. Future structure-function studies on HisRS especially in view of its dimeric structure and tRNA recognition as well as additional point mutations in its active site might resolve the precise action of this enzyme.

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