A tRNA Identity Switch Mediated by the Binding Interaction between a tRNA Anticodon and the Accessory Domain of a Class II Aminoacyl-tRNA Synthetase[†]

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ABSTRACT: Identity elements in tRNAs and the intracellular balance of tRNAs allow accurate selection of tRNAs by aminoacyl-tRNA synthetases. The histidyl-tRNA from *Escherichia coli* is distinguished by a unique G-1·C73 base pair that upon exchange with other nucleotides leads to a marked decrease in the rate of aminoacylation in vitro. G-1·C73 is also a major identity element for histidine acceptance, such that the substitution of C73 brings about mischarging by glycyl-, glutaminyl-, and leucyl-tRNA synthetases. These identity conversions mediated by the G-1·C73 base pair were exploited to isolate secondary site revertants in the histidyl-tRNA synthetase from *E. coli* which restore histidine identity to a histidyl-tRNA suppressor carrying U73. The revertant substitutions confer a 3−4-fold reduction in the Michaelis constant for tRNAs carrying the amber-suppressing anticodon and map to the C-terminal domain of HisRS and its interface with the catalytic core. These findings demonstrate that the histidine tRNA anticodon plays a significant role in tRNA selection in vivo and that the C-terminal domain of HisRS is in large part responsible for recognizing this trinucleotide. The kinetic parameters determined also show a small degree of anticooperativity ($\Delta\Delta G = -1.24$ kcal/mol) between recognition of the discriminator base and the anticodon, suggesting that the two helical domains of the tRNA are not recognized independently. We propose that these effects substantially account for the ability of small changes in tRNA binding far removed from the site of a major determinant to bring about a complete conversion of tRNA identity.

Although many proteins interact with RNA during the expression of genetic information, the molecular basis of these interactions is poorly understood. The joining of amino acids to their cognate transfer RNAs by the aminoacyl-tRNA synthetases (aaRS)[1] is a model for this problem, involving a two-step reaction of high fidelity in which specificity is exerted in the selection of both amino acid and tRNA (Schimmel & Soll, 1979). Through use of amber suppressors in the study of tRNA identity and in vitro aminoacylation studies using tRNA transcripts, defined sets of nucleotides have been identified in numerous tRNA isoacceptor groups that permit recognition by the cognate aaRS and discourage interactions with noncognate aaRS in vivo (Giege et al., 1993; McClain, 1993; Saks et al., 1994). These sequence elements are typically localized to the single-stranded anticodon trinucleotide and/or the acceptor helix, with additional contributions from the tertiary core, the variable arm, or modified bases. For a number of systems, substitutions of recognition determinants preferentially lead to decreases in k_{cat}, but the precise role of individual tRNA nucleotides in the rate-determining steps of aminoacylation remains to be elucidated.

The interaction between histidyl-tRNA synthetase and its cognate tRNA constitutes one system in which a single base pair apparently dominates recognition in vitro and in vivo. A unique G-1·C73 base pair caps the acceptor helix in all sequenced prokaryotic histidyl-tRNAs (Sprinzl et al., 1989) and serves as the principal recognition element. The presence of nucleotides other than cytosine at position 73 decreases k_{cat} by 2−3 orders of magnitude (Himeno et al., 1989) and confers mischarging by glycyl-, leucyl-, and glutaminyl-tRNA synthetases in vivo (Yan & Francklyn, 1994). Moreover, HisRS efficiently aminoacylates small helical RNAs resembling the tRNA acceptor stem which contain G-1·C73 (Francklyn & Schimmel, 1990). The efficient aminoacylation of minihelices by HisRS is shared by other aaRS (Martinis & Schimmel, 1995) and is consistent with the observation that both the amber and opal suppressor of $tRNA^{His}$ are quantitatively charged with histidine in vivo (Normanly et al., 1990; Yan and Francklyn, unpublished). Accordingly, a major role for the anticodon in recognition of $tRNA^{His}$ has not been proposed.

Details of the molecular basis of recognition of tRNA by aaRS have emerged in recent years from the crystallographic analysis of enzyme−RNA complexes (Carter, 1993; Arnez & Moras, 1994). In the complexes between GlnRS, AspRS, and their cognate tRNAs, nucleotides that serve as principal determinants provide functional groups which make hydrogen bonds to individual side chains and backbone functional groups or else enable conformational changes in the tRNA that promote greater complementarity in the enzyme−tRNA complex (Rould et al., 1991; Ruff et al., 1991). In both the glutaminyl and aspartyl systems, the anticodon and the discriminator base are crucial for recognition but play different structural roles in the two complexes. Moreover,

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetases; HisRS, histidyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; DHFR, dihydrofolate reductase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; Ni-NTA, nickel nitrilotriacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction.

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the precise manner in which recognition of these two elements is coupled to the kinetics of the aminoacylation reaction is unknown.

The strong dependence of histidine identity on the acceptor stem base pair G-1·C73 prompted us to examine recognition of this element by HisRS in vivo. The selection of secondary site suppressors in other tRNA systems has been shown to be a powerful approach for identifying residues in aminoacyl-tRNA synthetases that are important for tRNA discrimination (Rogers et al., 1990, 1994; Meinnel et al., 1991). A genetic approach was therefore designed for the histidine system in which growth on minimal media is dependent on the insertion of histidine by an amber suppressor tRNA. Three independent secondary site revertants were obtained by this selection that convert the identity of a U73 amber suppressor tRNA from glycine to histidine identity. The aminoacylation kinetics of the resulting mutant proteins were characterized with wild-type and mutant $tRNA^{His}$ transcripts and confirmed that the observed phenotype is the direct result of an altered synthetase−tRNA interaction. When analyzed in light of the crystal structure of HisRS, these mutant proteins provide direct evidence that binding interactions with the anticodon are important for tRNA selection in vivo by HisRS.

MATERIALS AND METHODS

General. The immobilized methotrexate affinity column matrix was purchased from Pierce and the Ni-NTA affinity resin was purchased from Qiagen. Restriction enzymes and other molecular biology reagents were obtained from Boehringer or New England Biolabs and were used according to the manufacturer's instructions. Hydroxylamine was a product of EM Science.

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Strain DF502 carries a chromosomal deletion of the *rha-pfk-tpi* locus and can be complemented by the *tpi* gene from *Saccharomyces cerevisiae* (Straus & Gilbert, 1985). Strain CJ236 is defective for dUTPase and uracil *N*-glycosylase and was used to propagate uracil containing DNA arising from hydroxylamine mutagenesis (Kunkel et al., 1987). Strain JM105 was used as the standard host for M13 derivatives and was also used as the background for expression of mutant HisRS proteins.

The plasmids pWY10 through pWY13 were constructed by inserting the *Pvu*II fragment containing the tRNA gene from its corresponding pGFIB:$tRNA^{His}_{CUA}$ construct (Yan & Francklyn, 1994) and inserting it into the unique *Pvu*II site of pACYC184. Plasmid pWY20, the starting plasmid for mutagenesis, was constructed by cloning a 1.2 kb fragment corresponding to the *Escherichia coli hisS* gene (Freedman et al., 1985) into the *Pst*I−*Hin*dIII restriction sites of pTIM:UAG95 (Yan & Francklyn, 1994). In the resulting construct, both $tpi95_{am}$ and *hisS* are under control of the strong *trp-lac* promoter. The expression plasmids for the wild-type and mutant HisRS (pWY30−32) were derived from the commercial plasmid pQE-30 (Qiagen), which contains a multiple cloning site downstream of a leader sequence encoding six tandem histidine residues. Expression of foreign coding sequences is controlled by a synthetic T5/lac promoter which can be regulated by IPTG.

The plasmid templates for in vitro synthesis of wild-type $tRNA^{His}$ and its variants (pWY40 to pWY43) were constructed by insertional oligonucleotide-directed mutagenesis using the Kunkel method (Kunkel et al., 1987). The starting plasmid was a variant of pGFIB (Normanly et al., 1986a) constructed by cloning a wild-type *E. coli* $tRNA^{His}$ gene with a GUG anticodon into the *Eco*RI-*Pst*I sites of phagemid pGFIB. A 48-mer oligonucleotide of the sequence 5′-CTGAGCTATAGCCACCTATAGTGAGTCGTATTAGAATTCAGCGTTACA-3′ was used to introduce the T7 promoter (underlined) adjacent to the 5′-end of the $tRNA^{His}$ gene. At the 3′-end of the tRNA gene, a mixed site oligonucleotide of the sequence 5′-GCTAAGGATCTGCAGATGCATGGG(A,U,C)GTGGCTAATGG-3′ was used to insert an *Eco*T22I restriction site to allow generation of a 3′-CCA end. The mixed bases in this second oligonucleotide were designed to introduce mutations at position 73 of the $tRNA^{His}$ gene. An oligonucleotide of the sequence 5′-ACTGGAATTAGAATCCAGC-3′ was used to change the tRNA anticodon sequence from GUG to CUA for the synthesis of wild-type and U73 $tRNA^{His}$ amber suppressor.

Table 1: List of Bacterial Strains and Plasmids

strain or plasmid	genotype	reference or source
XAC-1	*argE F′lac1373 lacZ*$_{u118am}$ *proB+/ D(lac pro)*$_{x11}$ *gyrA rpoB ara*	*a*
DF502	*galK35 purD34 pfkB1 edd-1his-68 rpsL125 l-D(rha-pfkA-tpi)269*	*b*
CJ236	*dut ung thi relA; pCJ105 (Cm*^{r}*) thi rpsL (Str*^{r}*)endA sbcB15 hsdR4 supE D(lac-proAB) F′:traD36 proAB lacl*^{q}*ZDM15*	*c*
JM105	*thi rpsL (Str*^{r}*)endA sbcB15 hsdR4 supE D(lac-proAB) F′:traD36 proAB lacl*^{q}*ZDM15*	*d*
pTrc99	*bla trp-lac expression vector*	*e*
pGBIB	bla; derivative of pEMBL+	*f*
pTIM:UAG95	*pTrc99 (Nco*I-*Pst*I*::tpi UAG95)*	*g*
pQE-30	*bla, derivative of pDS56/RBS11*	Qiagen
pWY10	*pACYC184 (Pvu*II*::*$tRNA^{His}_{CUA}$*)*	this work
pWY11	*pACYC184 (Pvu*II*::U73* $tRNA^{His}_{CUA}$*)*	this work
pWY12	*pACYC184 (Pvu*II*::A73* $tRNA^{His}_{CUA}$*)*	this work
pWY13	*pACYC184 (Pvu*II*::G73* $tRNA^{His}_{CUA}$*)*	this work
pWY20	*pTIM*$_{95am}$ *(Pst*I-*Hin*dIII*::hisS)*	this work
pWY21	*pTIM*$_{95am}$ *(Pst*I-*Hin*dIII*::hisS M2)*	this work
pWY22	*pTIM*$_{95am}$ *(Pst*I-*Hin*dIII*::hisS M4)*	this work
pWY23	*pTIM*$_{95am}$ *(Pst*I-*Hin*dIII*::hisS M5)*	this work
pWY24	*fol*$_{10am}$ *(Pst*I-*Hin*dIII*::hisS M2)*	this work
pWY25	*fol*$_{10am}$ *(Pst*I-*Hin*dIII*::hisS M5)*	this work
pWY30	*pQE-30 (hisS)*	this work
pWY31	*pQE-30 (hisS M2)*	this work
pWY32	*pQE-30 (hisS M5)*	this work
pWY40	*pGFIB (*$tRNA^{His}_{GUG,}$ *T7 promoter)*	this work
pWY41	*pGFIB (U73* $tRNA^{His}_{GUG,}$ *T7 promoter)*	this work
pWY42	*pGFIB (*$tRNA^{His}_{CUA,}$ *T7 promoter)*	this work
pWY43	*pGFIB (U73* $tRNA^{His}_{CUA,}$ *T7 promoter)*	this work

[a] Kleina et al. (1990). [b] Straus and Gilbert (1985). [c] Kunkel et al. (1987). [d] Yanisch-Perron et al. (1985). [e] Brosius and Holy (1984). [f] Normanly et al. (1986). [g] Yan and Francklyn (1994).

Mutagenesis and Selection of hisS Revertants. The histidine-specific suppression system using pTIM:UAG95/DF502 has been described previously (Yan & Francklyn, 1994). In this construct, an amber version of the yeast *tpi* gene ($tpi95_{am}$) is unable to complement strain DF502 unless a functional histidine-inserting suppressor tRNA is provided. Complementation is monitored by growth on M63 glycerol-selective medium, which consists of M63 salts (Miller, 1972), 2 mM $MgSO_4$, 0.2% glycerol, 1 mg/mL vitamin B_1, 80 μg/mL histidine, and antibiotics for maintenance of plasmids (100 μg/mL ampicillin and 12.5 μg/mL tetracycline). Random mutagenesis of pWY20 was performed by modification of the method of Davis et al. (1980). Plasmid DNA was resuspended in 0.5 M sodium phosphate, pH 6.0, at a

concentration of 200 μg/mL and treated with 50 mM hydroxylamine at 65 °C for 20 min. The hydroxylamine was then removed from the DNA by use of a Centri-Sep column (Princeton Separations) and then passaged through strain CJ236 to prevent the accumulation of short deletions at the site of the lesion. For genetic selection of *hisS* revertants, 1.5 μg of plasmid DNA prepared from strain CJ236 was used to transform pWY11/DF502 by electroporation. The transformants were spread directly on M63 glycerol plates and scored for growth at 37 °C after 2−3 days. Plasmid DNAs isolated from fast-growing colonies were subsequently used to retransform fresh pWY11/DF502 cells to confirm linkage to the plasmid. In order to rule out potential *tpi* amber gene revertants that would also confer growth on the selective medium, the *hisS* gene fragments from putative revertants were excised from pWY20 and recloned into an unmutagenized $pTIM_{95am}$ vector. The reconstructed pWY20 (with *hisS**) plasmid DNAs were then retransformed into pWY11/DF502. Growth of the transformants on glycerol plates in this second round verified linkage of the selected phenotype to *hisS*.

Sequence Analysis of hisS Revertants. Clones for which the phenotype could be shown to be directly linked to *hisS* were analyzed by dideoxy chain terminated DNA sequencing. The *Pst*I−*Hin*dIII fragments from the pWY20 derivatives were subcloned into M13mp18 to allow full-length sequencing of both strands of the complete *hisS* genes. A set of eight sequencing primers spanning *hisS* was synthesized using the sequence of wild-type *E. coli hisS* (Freedman et al., 1985). The cycle sequencing reactions were performed using the Applied Biosystems Taq DyeDeoxy sequencing kit and a Perkin Elmer/Cetus thermocycler. The reactions were analyzed using an ABI 370 DNA sequencer.

Determination of tRNA Identity. The in vivo amino acid identity of the U73 $tRNA^{His}_{CUA}$ amber suppressor in the presence of the isolated *hisS* revertants was determined by the standard DHFR assay (Normanly et al., 1986b). The fol_{10am} gene fragment was PCR amplified and used to replace the $tpi95_{am}$ gene in pWY21 and pWY23. The resulting constructs, pWY24 and pWY25, were then transformed into pWY11/XAC-1. Transformed cells were grown in large culture in M9 medium supplemented with ampicillin and tetracycline, and the suppressed DHFR protein was purified to homogeneity as described previously (Yan & Francklyn, 1994). The N-terminal 11 amino acid sequence of DHFR protein was determined, and the identities and relative amounts of amino acid inserted at position 10 were quantitated.

Synthesis of $tRNA^{His}$ in Vitro Transcripts and Aminoacylation Assay. Plasmid DNAs for transcription of $tRNA^{His}$ in vitro (plasmids pWY40 to pWY43) were purified by a $CsCl_2$ gradient prior to in vitro tRNA synthesis. The tRNA transcripts were prepared according to standard published protocols (Francklyn & Schimmel, 1990) with some modifications. Plasmids were linearized by digestion with *Eco*T22I, and the resulting 3′ single-stranded extensions were end-filled using the 3′−5′ exonuclease activity of the Klenow fragment of DNA polymerase I. To obtain tRNA transcripts initiating with a 5′ guanosine monophosphate, a 5-fold molar excess of GMP (20 mM) over GTP was added, as described by Milligan and Uhlenbeck (1989). The reaction mixtures were incubated for 4 h at 37 °C and terminated by addition of Na_2EDTA to a final concentration of 50 mM, followed by extraction with phenol/chloroform and precipitation with ethanol. The transcripts were purified on 12% denaturing polyacrylamide gels and then isolated by diffusion from the crushed gel fragments. The concentration of each tRNA transcript preparation was determined by its specific histidine acceptance. For typical preparations, values in the range of 1200 pmol/A_{260} were obtained.

Protein Expression and Purification. T7 RNA polymerase was purified from strain pAR1219/BL-21 by the method of Grodberg and Dunn (1988). The wild-type and mutant HisRS proteins were overexpressed and purified by use of the QIAExpress System (Qiagen), which fuses a His6 affinity tag to the amino terminus, allowing purification by Ni-NTA agarose chromatography. Fragments corresponding to *hisS M2* and *hisS M5* were PCR amplified from pWY21 and pWY23 and inserted into the *Bam*HI−*Kpn*I sites of pQE-30. The presence of the appropriate *hisS* mutations in pWY31 and pWY32 was confirmed by sequencing.

E. coli JM105 cells that carried the appropriate HisRS overproducer plasmids (pWY30-32) were grown in rich media to log phase at 37 °C before induction by 1 mM IPTG for 5 h. All subsequent purification steps were carried out at 4 °C. Crude cell extracts were prepared in the same manner as for the wild-type protein (Francklyn et al., 1994). The extracts were mixed with 5 mL of Ni-NTA resin that had been preequilibrated in sonication buffer. After a 1 h incubation of the protein on ice to allow binding to the resin, this mixture was poured into a 10 mL column. Weakly bound proteins were removed with wash buffer (50 mM potassium phosphate, pH 6.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol) until the A_{280} of the eluate dropped below 0.1. The protein was eluted with a gradient of 0−0.5 M imidazole in wash buffer. Peak fractions identified by SDS−PAGE were pooled together and dialyzed against buffer containing 50 mM potassium phosphate, pH 7.0, 100 mM KCl, and 10 mM β-mercaptoethanol at 4 °C overnight. Protein was then concentrated to a final concentration of 10 mg/mL in 40% glycerol and then stored at −20 °C. The wild-type HisRS protein lacking an affinity tag was purified according to previously published methods (Francklyn et al., 1994). Protein concentrations were determined by absorbance using a molar extinction coefficient of 127 097 obtained by composition analysis. When measured by active site titration, the enzymes were typically 95% active at a stoichiometry of two active sites per dimer.

In Vitro Aminoacylation Assays. In vitro aminoacylations of selected $tRNA^{His}$ transcripts by the pure wild-type or mutant HisRS proteins were carried out as described previously (Francklyn & Schimmel, 1990). The standard aminoacylation assays were performed in a buffer containing 50 mM HEPES, pH 7.5, 8.3 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2.5 mM ATP, 22.4 μM [^{3}H]histidine, and various amounts of enzyme and tRNA. The reactions were incubated at 37 °C and terminated by trichloroacetic acid quench using standard procedures. The specific activity of the tritiated amino acid was corrected for the variation in scintillation counting efficiency for the free amino acid versus amino acid esterified to the tRNA.

Western Blotting. Sonicated cell lysates in 10 mM potassium phosphate (pH 7.2) containing 10 mM β-mercaptoethanol were prepared, and the protein concentration of each extract was measured using purified HisRS as standard (Bradford, 1976). Proteins were separated on a 12.5% SDS−PAGE gel and transferred to Scheichler & Schuell Nytran

membranes with a Mini-V Blot module (Gibco-BRL) according to the manufacturer's instructions. Mouse-derived MRGS:His antibody used for detecting recombinant proteins containing the epitope MRGS$(His)_6$ was purchased from Qiagen. Goat anti-mouse IgG conjugated with horseradish peroxidase secondary antibody (Pierce) was incubated with the immobilized MRGS:His antibody. Subsequent addition of Renaissance Western Blot Chemiluminescence Reagent (DuPont) allowed for visualization of bound protein by autoradiography.

RESULTS

Mutagenesis and Selection. The identity of histidine tRNA is dependent on C73, and the introduction of other nucleotides at the discriminator position leads to mischarging by other aaRS (Yan & Francklyn, 1994). This loss of identity or conversion of identity allows the formal possibility of isolating compensatory mutations in the corresponding aminoacyl-tRNA synthetase. Accordingly, a genetic selection was devised in which growth on selective media was dependent on the histidine identity of a mutant $tRNA^{His}$ amber suppressor. This requirement for histidine insertion arises from the use of an amber version of the yeast *tpi* gene with a stop codon at position 95, corresponding to an essential catalytic histidine in the gene product triosephosphate isomerase (Komives et al., 1991). The activity of triosephosphate isomerase is required to allow glycerol to enter the glycolytic pathway, such that cells lacking this activity are unable to grow on glycerol. The experimental system consisted of two separate plasmids, the first of which (series pWY10−pWY13) carries the gene for the $tRNA^{His}$ amber suppressor and the second of which (pWY20) carries the tpi_{95am} and *hisS* genes. Both plasmids are harbored in strain DF502, whose chromosomal copy of *tpi* is deleted but whose *hisS* gene is intact. Under these conditions, growth on glycerol is strictly dependent on histidine insertion by the mutant amber suppressor (Figure 1).

Random mutagenesis of the *E. coli hisS* gene carried on pWY20 was carried out using several different approaches, including error-prone PCR, passage through mutator strains, and classical chemical mutagenesis. Unfortunately, no revertants were obtained with the first two methods, which have relatively broad mutagenic specificities. Despite its limitation to transition mutations, hydroxylamine mutagenesis was chosen to obtain a higher efficiency of mutagenesis. Plasmid pWY20 was treated with hydroxylamine as described in Materials and Methods, stabilized by passage through CJ236, and then used to transform a tester strain (pWY11/DF502) containing plasmid pWY11, which encodes the gene for U73 $tRNA^{His}_{CUA}$. Five colonies appeared after 2−3 days of incubation that grew at rates comparable to pWY10/DF502. Cells transformed with unmutagenized pWY20 did not yield colonies under identical conditions.

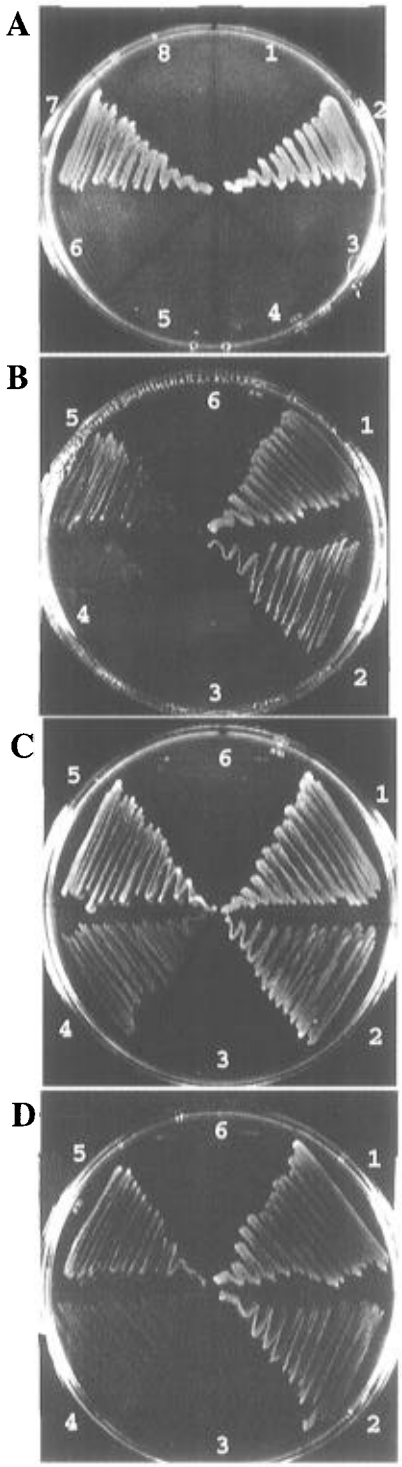
Plasmid DNA corresponding to pWY20 was isolated from each of the five putative revertants, purified by preparative agarose gel electrophoresis, and then used to retransform pWY11/DF502. In each case, the revertant phenotype could be shown to be linked to plasmid. However, subcloning of the putative mutant *hisS* genes into unmutagenized $pTIM_{95am}$ showed that only three of the mutants were authentic *hisS* revertants. These putative revertant plasmids were designated pWY21 to pWY23, and the mutant alleles were designated *hisS M2*, *hisS M4*, and *hisS M5*. Plate assays revealed that *hisS M2* was a weak suppressor requiring 3 days at 37 °C for colonies to appear, while the other two mutant alleles (*hisS* and *hisS M5*) conferred growth comparable to pWY10/DF502 (Figure 1). The remaining clones were not characterized further.

The tRNA allele specificity of the mutants was determined by examining the ability of each of the three *hisS* mutants to confer glycerol prototrophy on DF502 in the presence of the different discriminator variants of $tRNA^{His}_{CUA}$. The *hisS M2* mutant was allele specific and did not confer growth on glycerol with either A73 or G73 $tRNA^{His}_{CUA}$ (Figure 1B). By contrast, the *hisS M4* and *hisS M5* mutants were less selective and conferred growth with both U73 and A73 but not with G73 $tRNA^{His}_{CUA}$ (Figure 1C,D). The unmutagenized wild-type allele of *hisS* did not confer suppression with tRNAs containing U, A, or G73 (Figure 1A). Moreover, some inhibition of growth was observed when both the wild-type protein and C73 $tRNA^{His}_{CUA}$ were overexpressed, reflecting the high efficiency of the wild-type amber suppressor.

Sequence Analysis of the Revertants. The 1.2 kb fragment corresponding to the entire *hisS* gene of each revertant was subcloned into M13 and then subjected to dye-terminated dideoxy DNA sequencing using a set of primers spanning the length of the gene. For both of the strong suppressors (*hisS M4* and *hisS M5*), the single transition mutation of G1214 to A, encoding the amino acid substitution Gly405 to Asp, was found to be the only mutation in the sequence of *hisS* (Table 2). In the weaker *hisS M2* gene, two transition mutations were observed, G287 to A and G311 to A. These latter mutations encode Gly96 to Asp and Arg104 to His. All of the mutations obtained were transition mutations, consistent with the mutagenic specificity of hydroxylamine.

Identity Analysis of the hisS Revertants. Although the genetic linkage of the suppression phenotype to *hisS* strongly suggested the involvement of HisRS, these plate assays provide indirect evidence at best of a switch in the identity of $tRNA^{His}$. If the mutant proteins were directly aminoacylating the mutant tRNAs, then the switch from glycine to histidine identity demanded by the test system should be independent of the tpi_{am} reporter system. To experimentally verify this prediction, the tpi_{95am} genes of pWY21 and pWY23 were excised and replaced with the DHFR fol_{10am} gene. The resulting plasmids (pWY24 and pWY25) were introduced into strain pWY11/XAC-1; samples of the suppressed DHFR protein were prepared and then sequenced up to the first 11 residues. When the *hisS M5* allele was present, U73 $tRNA^{His}_{CUA}$ was quantitatively aminoacylated with histidine, as opposed to an identity of 83% glycine and 17% glutamine obtained in the absence of an exogenous *hisS* allele (Table 2). In the presence of the *hisS M2* allele, the identity of U73 $tRNA^{His}_{CUA}$ was mixed, comprising 75% histidine and 25% glycine. These results confirmed that the suppression observed was a direct result of the switch of the identity of the amber suppressor and that the growth rate correlated with the extent of conversion to histidine identity. Thus, the genetic system is sensitive to changes in the identity of suppressor tRNAs and can be used to detect a range of responses.

In Vitro Aminoacylation Studies. The in vivo identity studies provided strong evidence that the phenotype observed was the direct result of an altered interaction between the mutant synthetases and the U73 amber suppressor. This mutant tRNA differs from the canonical histidine tRNA at



Relevent Genetic Markers and tRNAs

A			
1.	*tpi95am*		
2.	*tpi+*		
3.	*tpi95am*	*hisS+*	G73 $tRNA^{His}_{CUA}$
4.	*tpi95am*	*hisS+*	U73 $tRNA^{His}_{CUA}$
5.	*tpi95am*	*hisS+*	A73 $tRNA^{His}_{CUA}$
6.	*tpi95am*	*hisS+*	C73 $tRNA^{His}_{CUA}$
7.	*tpi95am*	--	C73 $tRNA^{His}_{CUA}$
8.	*tpi95am*	*hisS+*	----------
B			
1.	*tpi95am*	--	C73 $tRNA^{His}_{CUA}$
2.	*tpi95am*	*hisSM2*	C73 $tRNA^{His}_{CUA}$
3.	*tpi95am*	*hisSM2*	G73 $tRNA^{His}_{CUA}$
4.	*tpi95am*	*hisSM2*	A73 $tRNA^{His}_{CUA}$
5.	*tpi95am*	*hisSM2*	U73 $tRNA^{His}_{CUA}$
6.	*tpi95am*	*hisS+*	U73 $tRNA^{His}_{CUA}$
C			
1.	*tpi95am*	--	C73 $tRNA^{His}_{CUA}$
2.	*tpi95am*	*hisSM4*	C73 $tRNA^{His}_{CUA}$
3.	*tpi95am*	*hisSM4*	G73 $tRNA^{His}_{CUA}$
4.	*tpi95am*	*hisSM4*	A73 $tRNA^{His}_{CUA}$
5.	*tpi95am*	*hisSM4*	U73 $tRNA^{His}_{CUA}$
6.	*tpi95am*	*hisS+*	U73 $tRNA^{His}_{CUA}$
D			
1.	*tpi95am*	--	C73 $tRNA^{His}_{CUA}$
2.	*tpi95am*	*hisSM5*	C73 $tRNA^{His}_{CUA}$
3.	*tpi95am*	*hisSM5*	G73 $tRNA^{His}_{CUA}$
4.	*tpi95am*	*hisSM5*	A73 $tRNA^{His}_{CUA}$
5.	*tpi95am*	*hisSM5*	U73 $tRNA^{His}_{CUA}$
6.	*tpi95am*	*hisS+*	U73 $tRNA^{His}_{CUA}$

FIGURE 1: Genetic selection for secondary site suppressors in *hisS* that compensate for the discriminator base mutation in $tRNA^{His}_{CUA}$. The relevant genetic markers carried by the plasmids of each strain are indicated in the legends to the right of the panels. (A) Controls demonstrating the histidine-specific suppression system. (B−D) Phenotypes of the *hisS M2*, *hisS M4*, and *hisS M5* revertants. This figure was created by photographing the plates and then scanning the resulting slides into a digital format using Adobe Photoshop.

both the discriminator nucleotide and at two of the three nucleotides of the anticodon. From the in vivo studies alone, it could not be determined whether the revertant substitutions in HisRS confer altered specificity for the mutant discriminator base, the amber anticodon, or both elements. To address this question, in vitro aminoacylation studies were carried out.

The mutant proteins HisRS G96D/R104H and HisRS G405D were prepared from pWY31/JM105 and pWY32/JM105 cells following growth at 37 °C and induction with IPTG. Purification to near homogeneity was accomplished by chromatography on Ni-NTA agarose. To provide strict comparisons between the wild-type and mutant proteins, versions of the wild-type protein that both contained and lacked the N-terminal histidine affinity tag were purified. The first three residues at the amino terminus are disordered in the X-ray structure of HisRS, suggesting that the amino terminus is not directly required for maintaining the folded structure of the enzyme (Arnez et al., 1995). Four different transcripts of $tRNA^{His}$ were prepared by standard methods

Table 2: Sequence and tRNA Identity Analysis of the *hisS Revertants*

plasmid *hisS* allele	deduced amino acid substitution	in vivo identity of U73 $tRNA^{His}_{CUA}$ [a]
none		83% glycine, 17% glutamine[b]
hisS M2	Gly96 → Asp Arg104 → His	77% histidine, 23% glycine
hisS M4	Gly405 → Asp	ND
hisS M5	Gly405 → Asp	100% histidine

[a] Determined by sequencing DHFR as described in Materials and Methods. Only values greater than or equal to 5% are shown. [b] Data reported previously in Yan and Francklyn (1994). ND, not determined

to allow the contribution of U73 and the amber anticodon to be evaluated by determination of apparent kinetic parameters. These transcripts included the wild-type $tRNA^{His}$, a $tRNA^{His}$ amber suppressor, and two variants with the C73 to U mutations in these two contexts. This analysis by definition excluded the role of modified bases in recognition by HisRS, which remains to be determined. A previous study on the role of the G-1•C73 base pair compared native and synthetic transcripts and did not observe a significant change in kinetic parameters (Himeno et al., 1989).

A comparison of kinetic parameters obtained with the unmodified wild-type enzyme and those determined with the affinity-tagged version of the wild-type enzyme showed that the His6 affinity tag introduces a 1.5−2.5-fold increase in the K_m parameter which varied slightly depending on the transcript tested (Table 3). The activities of the tagged wild-type protein with the various transcripts with respect to the k_{cat} parameter were not significantly different from those of the unmodified wild-type enzyme. Substitution of the C73 discriminator base by uracil brought about a decrease in k_{cat} of 3 orders of magnitude but had a negligible effect on the K_m parameter (Table 3, compare no. 1, 5 to 2, 6). This value is in good agreement with previously published values (Himeno et al., 1989). The introduction of the amber suppressor anticodon, by contrast, conferred an increase of 8−20-fold in K_m but had little effect on k_{cat}. For single nucleotide substitutions of the *E. coli* $tRNA^{His}$ anticodon, 5−7-fold increases have been observed (Nameki et al., 1995). When U73 and the amber anticodon were both present, the k_{cat} and K_m parameters were both affected, but the k_{cat}/K_m for this mutant was 7.5-fold higher than what would have been observed had the two mutations been acting independently. Thus, recognition of the acceptor stem and the anticodon is slightly anticooperative, with a $\Delta\Delta G = -1.24$ kcal/mol [calculated as described in Putz et al. (1993)]. The acceptor stem and the anticodon therefore both serve as important recognition elements for $tRNA^{His}$, with the former playing a greater role in the catalytic steps and the latter a greater role in binding steps.

Both mutant proteins were selected against the mutant tRNA containing the discriminator base substitution U73 in the context of the amber suppressor. Given the significant effect on catalysis imparted by the U73 substitution, the aminoacyl-tRNA synthetase revertants might be expected to aminoacylate U73 transcripts with higher rates of aminoacylation than the wild-type protein. Analysis of the apparent kinetic parameters for aminoacylation catalyzed by the mutant proteins showed that this was not the case, and other effects were observed. Both G405D HisRS and G96D/R104H HisRS showed decreases in the apparent K_m for aminoacylation of tRNAs bearing the amber-suppressing (CUA) anticodon, relative to the parameter determined on the wild-type tRNA. For the U73 CUA transcripts (no. 12 and 16) in particular, the K_m parameters were only 2−3-fold higher than those for the wild-type transcript, while 7−10-fold increases were observed in the same comparison for the wild-type protein. Variations in k_{cat} were also observed but were less systematic. G405D HisRS showed 1.5−2-fold decreases in k_{cat} with each of the transcripts tested, and this did not appear to be the result of the affinity tag. The G96D/R104H HisRS mutant gave minor decreases in k_{cat} for the two transcripts with the GUG anticodon (no. 9, 10) but 12−40-fold decreases with substrates containing the CUA anticodon (no. 11, 12). Calculation of free energy changes for the mutants showed that G405D HisRS retains the anticooperativity characteristic of the wild-type protein ($\Delta\Delta G = -1.59$ kcal/mol), while anticooperativity has apparently been abolished in the case of G96D/104H HisRS ($\Delta\Delta G = -0.103$ kcal/mol). Both mutant proteins appear to suppress the mutant tRNAs by virtue of reduced discrimination between the GUG and CUA anticodon. The reduced discrimination is largely manifested through a reduced K_m parameter for the aminoacylation of the suppressor relative to the wild type, but there are also effects on K_{cat} that may arise from differences in the anticooperativity between recognition of the two domains of the tRNA.

The aminoacylation assays described above were conducted under standard reference conditions in the absence of additional NaCl or KCl. While not strictly physiological conditions, they provide a basis for comparing the efficiency of aminoacylation under a single fixed condition. Both mutant proteins possess amino acid substitutions that introduce additional charged side chains, raising the question of whether or not changes in the electrostatic component of binding are altered in these mutants. A preliminary study probing the importance of electrostatics in the aminoacylation of tRNA was therefore carried out by measuring the aminoacylation velocities of the wild-type and G405D HisRS proteins on wild-type tRNA and the U73 CUA tRNA substrates over a range of KCl concentrations from 0 to 400 mM. These measurements showed that the aminoacylation of the wild-type transcript by the wild-type (affinity-tagged) enzyme at a concentration of 1 μM was 3-fold higher at 100 mM KCl than at 0 or 250 mM KCl (1.38 s^{-1} versus 0.35 and 0.30 s^{-1}, respectively), while aminoacylation of the U73 $tRNA^{His}_{CUA}$ transcript at a concentration of 3.5 μM was 20-fold higher at 0 mM KCl than at 100 mM (2.0 versus 0.1 × 10^{-3} s^{-1}). Thus, aminoacylation of the mutant transcript showed a greater sensitivity to salt concentration than the wild-type transcript. By contrast, aminoacylation velocities by G405D HisRS were higher at 0 mM KCl than at 100 mM for both substrates (0.404 versus 0.328 s^{-1} for the wild-type transcript and 7.7 versus 0.78 × 10^{-3} s^{-1} for the mutant transcript). A comparison of the activity on the U73 CUA transcript versus that observed with the wild-type transcript at the two salt concentrations indicates that the ratio for G405D HisRS decreases by a factor of 8 from 0 to 100 mM KCl and decreases by a factor of 28 for the wild-type protein. These observations indicate that the conclusions drawn from the kinetic determinations in the absence of the additional KCl are likely to be valid at higher salt concentrations that may be characteristic of physiological conditions.

Intracellular Levels of the Mutant Enzymes. The change in the Michaelis constant for the aminoacylation of the

Table 3: Apparent Kinetic Parameters for the Aminoacylation of $tRNA^{His}$ Transcripts[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
wild-type HisRS (lacking affinity tag)			
(1) $tRNA^{His}_{GUG}$ C73	0.49 (±0.15)	1.70 (±0.14)	3.5×10^6
(2) $tRNA^{His}_{GUG}$ U73	0.86 (±0.1)	0.00176 (±0.00013)	2.0×10^3
(3) $tRNA^{His}_{CUA}$ C73	10.74 (±0.65)	2.05 (±0.01)	1.9×10^5
(4) $tRNA^{His}_{CUA}$ U73	4.98 (±1.6)	0.007 (±0.00079)	1.4×10^3
wild-type HisRS (with His6 affinity tag)			
(5) $tRNA^{His}_{GUG}$ C73	1.3 (±0.7)	2.1 (±0.4)	1.6×10^6
(6) $tRNA^{His}_{GUG}$ U73	1.6 (±0.4)	0.0050 (±0.00056)	1.9×10^3
(7) $tRNA^{His}_{CUA}$ C73	13.0 (±0.11)	1.49 (±0.3)	1.1×10^5
(8) $tRNA^{His}_{CUA}$ U73	9.0 (±3.7)	0.0029 (±0.0005)	3.2×10^2
G96D/R104H HisRS			
(9) $tRNA^{His}_{GUG}$ C73	0.67 (±0.007)	1.09 (±0.003)	1.6×10^6
(10) $tRNA^{His}_{GUG}$ U73	0.90 (±0.09)	0.00095 (±0.00025)	1.1×10^3
(11) $tRNA^{His}_{CUA}$ C73	0.66 (±0.27)	0.29 (±0.038)	4.3×10^5
(12) $tRNA^{His}_{CUA}$ U73	1.66 (±0.87)	0.00058 (±0.00010)	3.5×10^2
G405D HisRS			
(13) $tRNA^{His}_{GUG}$ C73	0.49 (±0.185)	1.11 (±0.17)	2.57×10^6
(14) $tRNA^{His}_{GUG}$ U73	0.70 (±0.28)	0.00075 (±0.000071)	1.0×10^3
(15) $tRNA^{His}_{CUA}$ C73	5.33 (±1.3)	1.26 (±0.11)	2.3×10^5
(16) $tRNA^{His}_{CUA}$ U73	1.19 (±0.3)	0.00147 (±0.00010)	1.2×10^3

[a] Aminoacylation reactions were carried out as described in Materials and Methods. The concentration ranges of tRNA substrates were as follows: $tRNA^{His}$, 0.2−5.0 μM; $tRNA^{His}_{CUA}$, 0.5−30.0 μM; U73 $tRNA^{His}$, 0.2−10.0 μM; U73 $tRNA^{His}_{CUA}$, 0.5−20.0 μM. Enzyme concentrations used in the reactions were 0.5−5 nM for C73 tRNA substrates and 100−250 nM for U73 tRNA substrates. ATP concentrations of 2.5 mM, and histidine concentrations of 22.4 μM were used in all reactions. The K_m for histidine in the aminoacylation reaction has been reported to be 6 μM (Kalousek & Konigsberg, 1974) but was not determined here explicitly. The kinetic parameters determined should therefore be considered apparent. Kinetic parameters of HisRS proteins for the selected $tRNA^{His}$ substrates were determined by measuring the initial rates of histidylation of tRNA substrates over the first 2.5 or 5 min in the presence of catalytic amounts of synthetases. Initial rates for each tRNA concentration determined from plots of the quantity of aminoacylated tRNA versus time typically varied by no more than 10%. Kinetic parameters were derived from fits to the Michaelis−Menten equation by nonlinear regression using the program Enzfitter (Elsevier). Standard experimental errors calculated by the program are shown in parentheses.

mutant tRNAs observed with both G405D and G96D/R104H HisRS provided one explanation for the observed suppression. In previous work, it has been shown that the identity of suppressor tRNAs can also be altered by manipulating the levels of cognate and noncognate aaRS (Swanson, 1988; Hou & Schimmel, 1989). Although control experiments in the original selection ruled out the possibility that overproduction of the wild-type enzyme is responsible for the suppression (Figure 1A), the presence of substantially higher levels of the mutant proteins relative to wild type remained an alternative explanation that needed to be examined. To address this question, levels of wild-type and mutant HisRS enzymes were analyzed by immunoblotting analysis of uninduced crude cellular extracts from strains harboring the appropriate plasmids. All of the strains used in this study express a chromosomal copy of *hisS* that would potentially complicate any immunological analysis using antibodies directed against epitopes of unmodified HisRS, so the antibodies used were directed against the His6 affinity tag. The crude extracts were prepared from uninduced JM105 strains harboring the pWY30-32 overproducer plasmids. Efforts to transform DF502 with pWY31 and pWY32 were unsuccessful, perhaps because this strain lacks the *lacI*^{q} allele required to maintain the synthetic T5/lac promoter present in pWY30-32 plasmids in a fully repressed state. Despite these differences from the original selection background, this test system allowed us to determine directly whether the mutations isolated in *hisS* confer any increased stability to HisRS. Under the conditions tested, the intracellular levels of wild-type, G96D/R104H, and G405D HisRS were not significantly different (Figure 2). In the absence of exogenous HisRS expressed from the plasmid, no signal was observed, demonstrating that the antibody is specific for the

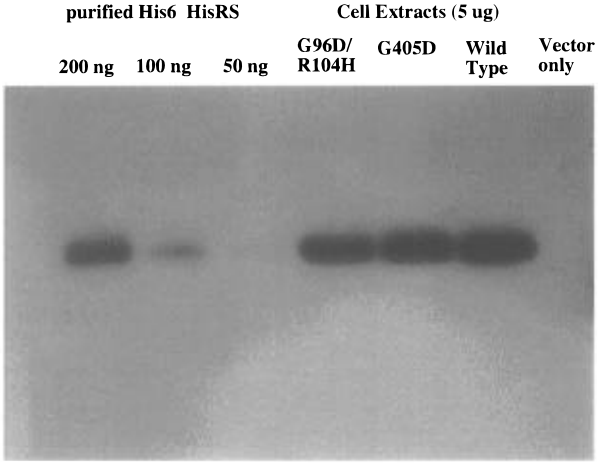


FIGURE 2: Analysis of intracellular levels of wild-type and mutant HisRS. Affinity-purified (His6) HisRS was loaded in lanes 1−3 in the amounts indicated. Five microgram aliquots of crude extracts prepared from JM105 bacterial strains carrying the appropriate overproducer plasmids were loaded in lanes 4−7. The proteins were visualized by the Western blotting procedure using antibodies directed against the (His6) affinity tag as described in Materials and Methods. Duplicate polyacrylamide gels stained with Coomassie Blue (not shown) further verified that equivalent amounts of total protein derived from the crude extracts were loaded in each lane.

His6 affinity tag and does not cross-react with the unmodified wild-type enzyme.

DISCUSSION

Histidyl-tRNA synthetase from *E. coli* is a class II homodimeric enzyme in which two principal domains make up each monomer: a canonical class II antiparallel β-sheet that comprises the catalytic domain and a C-terminal α/β

domain topologically related to the nucleotide binding fold (Arnez et al., 1995). The presence of histidyl adenylate in the crystal complex allows the active site to be unequivocally identified, including putative tRNA contact regions. A docking model of the HisRS·tRNA^{His} complex prepared by using the coordinates of HisRS and those of the AspRS·tRNA^{Asp} complex (Ruff et al., 1991) suggests that the C-terminal domain of HisRS is responsible for binding the anticodon arm of the tRNA (Figure 3). Accordingly, the substitutions identified by our genetic selection provide an independent means of testing the validity of this model. It should be emphasized that this docking model is not based on X-ray measurements, and significant structural differences between this approximation and the actual complex are likely. Proposals about the direct role of the revertant substitutions in direct tRNA contact should be considered with this in mind.

The docking model suggests that the HisRS dimer can bind two tRNA molecules, with each tRNA making contacts to portions of both monomers. The three areas of principal contact proposed consist of the tRNA acceptor helix and the active site cleft, contacts between the back face of the catalytic domain and the tRNA tertiary core, and interactions between the anticodon arm and the C-terminal domain. None of the amino acids substituted in the mutants is within 20 Å of the active site, accounting for the absence of increased specificity for the discriminator nucleotide. The G405D substitution introduces an aspartate residue into the surface loop between β strands CS4 and CS5 in the C-terminal domain. In the docking model, this loop is predicted to be in close proximity of G36. On the basis of phosphorothioate footprinting experiments (Yan and Francklyn, in preparation), G36 is protected by HisRS in solution. Thus, the G405D substitution is appropriately situated to exert a direct effect on anticodon binding.

The substitutions encoded by the *hisS M2* allele (Gly96 → Asp, Arg104 → His) are not in the C-terminal domain and are thus unlikely to be in direct contact with the anticodon loop. Glycine 96 is located at the C-terminal end of helix AH3; residues situated at the amino-terminal end of this helix are directly involved in contacts with the histidyl adenylate (Arnez et al., 1995). In the docking model, Gly96 is predicted to be in close proximity to the 5′-phosphate of C11, which is also protected in solution by HisRS (Yan and Francklyn, unpublished). Arginine 104 is located at the amino-terminal end of β strand AS5, a structural element of motif 2, but also plays an important structural role in the interface between the C-terminal domain and the catalytic domain (Arnez et al., 1995).

The separate contributions of G96D and R104H to the altered tRNA binding observed in the M2 mutant have not been fully assessed. Introduction of the R104H substitution into *hisS* independently by oligonucleotide mutagenesis reveals that this substitution alone is sufficient to confer glycerol prototrophy with U73 tRNA$^{His}_{CUA}$ (Yan and Francklyn, unpublished observation), raising the possibility that indirect effects on structure are able to affect tRNA selection. The location of Arg104 suggests two possible models. One possibility is that the Arg104 → His substitution propagates a structural change through β strand AS5 to the active site, altering tRNA selection. A more likely explanation given the increased affinity for the CUA anticodon is that R104H reorients the packing of the C-terminal domain against the catalytic core. HisRS and GlyRS share related C-terminal domains which are oriented differently with respect to the

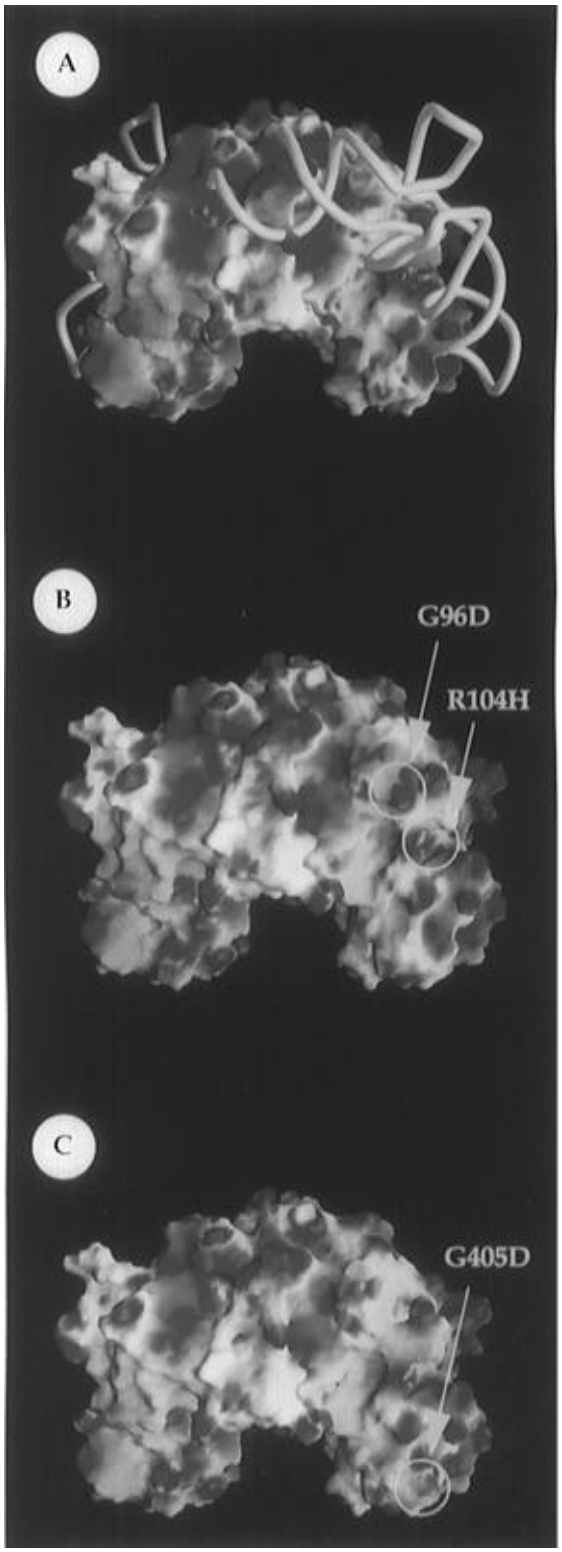


FIGURE 3: Comparison of the electrostatic surface potentials of the wild-type and mutant HisRS. (A) Model of the wild-type complex. (B) G96D/R104H HisRS. Regions corresponding to the mutant substitutions are indicated. (C) G405D HisRS. The model of the complex was prepared using the program O (Jones et al., 1993), the coordinates of *E. coli* HisRS (Arnez et al., 1995) and the yeast AspRS·tRNA^{Asp} complex (Ruff et al., 1991), and a model of tRNA^{His} constructed on the basis of the yeast tRNA^{Phe} structure (Kim et al., 1974). The molecular surfaces were calculated and displayed using GRASP (Nicholls et al., 1991). The surface is colored on the basis of the electrostatic potential, with red representing negative charge and blue representing positive charge.

catalytic domain (Arnez et al., 1995; Logan et al., 1995), and these structural variations may contribute to differences in the tRNA selection by these enzymes. This model may account for the reduced anticooperativity observed with the G96D/R104H mutant. The significance of interdomain communication for integrating tRNA binding and catalysis has been raised before in the case of GlnRS (Rould et al., 1991; Rogers et al., 1994).

The perturbation of individual enzyme−tRNA contacts may comprise an important component of altered tRNA binding observed in the mutants but may not fully account for these properties. Calculation of the surface potential of wild type, HisRS G405D, and HisRS G96D/R104H suggests that both mutant proteins possess regions of additional negative charge in the vicinity of the tRNA backbone, and this change in surface charge may also contribute to the altered specificity for the anticodon (Figure 3). Evidence for this model is provided by a preliminary analysis of the salt dependence of the synthetase−tRNA interaction for four different cases, which showed that G405D HisRS had a lower optimum salt concentration for aminoacylation than the wild-type protein. Although this behavior is consistent with an additional ionic interaction with the phosphodiester backbone, recall that neither mutant protein exhibited a nonspecific decrease in the K_m for aminoacylation for all of the transcripts tested (Table 3). Clearly, further studies of dependence of rate constants upon salt concentration as well as the effects of substituting other amino acids at the positions identified by the mutations will have to be performed to establish the molecular basis of these effects. Moreover, the significance of these differences in the ionic component of binding with respect to the cellular environment is difficult to estimate, because the free concentrations of electrolytes are unknown and tRNAs apparently do not freely diffuse throughout the cell, making their effective concentrations difficult to estimate.

While tRNA recognition is principally concerned with contacts between a single synthetase and tRNA, identity is a system property that reflects the outcome of some 1200 possible interactions in vivo between synthetases and tRNAs (Yarus, 1972; Normanly & Abelson, 1989). Of these, only a small fraction are between cognates. In order to rationalize changes in the identities of suppressor tRNAs, one must consider the effects of both endogenous and exogenous HisRS and histidyl-tRNAs. For the endogenous enzyme, the 10-fold difference in K_m between the wild-type tRNA and the amber suppressor will tend to saturate the wild-type protein with its cognate substrate, making the mutant amber suppressor available for binding and aminoacylation by noncognate enzymes. This holds true even when the wild-type enzyme is overproduced, so that the reversion of the U73 suppressor cannot be due only to the availability of excess HisRS (Figure 1A). Both G405D and G96D/R104H HisRS bind the wild-type and mutant tRNAs with nearly equal affinities and may therefore outcompete noncognate enzymes for binding to the amber suppressor. The more effective competition by G405D HisRS may reflect its higher catalytic efficiency relative to HisRS G96D/R104H (Table 3). These slight binding effects may also be enhanced by the overexpression of the mutant proteins relative to the chromosomally produced endogenous HisRS and GlyRS in the DF502 test strain.

Our results provide direct evidence that misacylation can result from relatively small changes in the binding equilibria between aaRS and tRNA which originate from mutations in the C-terminal anticodon binding domain. Misacylation has previously been associated with altered levels of the macromolecules, with mutations in the tRNA, or from a combination of these effects (Rogers & Soll, 1990). As pointed out by Yarus (1972), misacylation in normal cells may be suppressed by parallel aminoacylation systems that tend to limit the amounts of free macromolecules available for noncognate interactions.

Major determinants for tRNA recognition which promote interaction with the cognate synthetase typically affect catalytic rather than binding steps (Ebel et al., 1973). For histidyl-tRNAs, contacts to the acceptor stem are responsible for the major fraction of transition state free energy (Francklyn et al., 1992). Nonetheless, sequences outside of this region, including the anticodon, contribute a small (∼3.2 kcal/mol) but significant fraction of transition state stabilization energy. On the basis of the kinetic analysis presented here, recognition of the acceptor stem and the anticodon is slightly anticooperative, and the U73 suppressor is aminoacylated more efficiently than what would be observed if these domains were being recognized independently. By contrast, the converse is observed in the complex between the class II *S. cerevisiae* AspRS and its cognate tRNA, in which anticodon recognition contributes a major fraction of transition state stabilization and multiple acceptor stem and anticodon mutations interact cooperatively (Putz et al., 1993). The presence of similar contextual effects in the HisRS complex may explain why C73 is necessary but not sufficient to confer histidylation in vivo (Hou & Schimmel, 1988; Sherman et al., 1992) and why the contribution of the anticodon arm of $tRNA^{His}$ may be important. Thus, while the $tRNA^{His}$ acceptor stem may recapitulate a large fraction of the specificity interactions in vitro, the anticodon is also clearly important and plays an essential role in dictating selection of $tRNA^{His}$ in vivo.

The apparent absence of compensatory mutations at the site of HisRS responsible for direct contact of C73 remains unaccounted for. We have not ruled out the trivial possibility that the relatively limited scope of the mutagenic specificity of hydroxylamine precluded isolation of a rare substitution in close proximity to the discriminator base. Alternatively, substitution of the putative direct contact side chain may confer a lethal or inactive phenotype, or a main chain functional group may directly contact the discriminator base. Validation of these proposals awaits the structure of the HisRS·$tRNA^{His}$ complex.

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