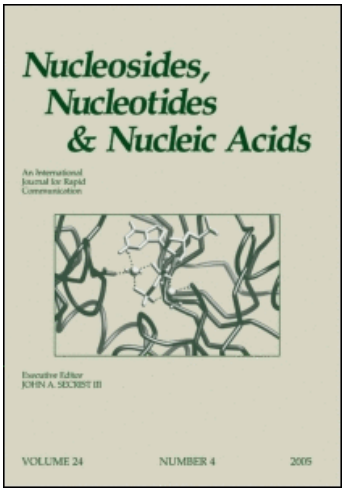

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Domains of *E. Coli* Glutaminyl-tRNA Synthetase Disordered in the Crystal Structure Are Essential for Function or Stability

Jill Conley[a]; Joyce Sherman[a]; Hans-Ulrich Thomann[a]; Dieter Söill[a]
[a] Department of Molecular Biophysics and Biochemistry, Yale University New Haven, Connecticut, USA

To cite this Article Conley, Jill , Sherman, Joyce , Thomann, Hans-Ulrich and Söill, Dieter(1994) 'Domains of *E. Coli* Glutaminyl-tRNA Synthetase Disordered in the Crystal Structure Are Essential for Function or Stability', Nucleosides, Nucleotides and Nucleic Acids, 13: 6, 1581 — 1595
To link to this Article: DOI: 10.1080/15257779408012173
URL: http://dx.doi.org/10.1080/15257779408012173

NUCLEOSIDES & NUCLEOTIDES, 13(6&7), 1581-1595 (1994)

DOMAINS OF *E. COLI* GLUTAMINYL-tRNA SYNTHETASE DISORDERED IN THE CRYSTAL STRUCTURE ARE ESSENTIAL FOR FUNCTION OR STABILITY[1]

Jill Conley, Joyce Sherman, Hans-Ulrich Thomann and Dieter Söll*

Department of Molecular Biophysics and Biochemistry, Yale University
New Haven, Connecticut 06520-8114, USA

Abstract Deletion analysis of *E. coli* glutaminyl-tRNA synthetase indicates that the N- and C-termini of the protein, which appear disordered in the crystal structure, are essential for function and stability *in vivo*. *In vitro* aminoacylation kinetics of a C-terminal deletion mutant exhibit a sharp reduction in the specificity constant. However, an N-terminal extension is catalytically, if not structurally equivalent to wild-type.

The accurate recognition of tRNA by aminoacyl-tRNA synthetases (AARSs) is critical to the fidelity of protein biosynthesis. The crystal structure of the *E. coli* glutaminyl-tRNA synthetase, GlnRS:$tRNA^{Gln}$ complex has been solved and refined to 2.5Å resolution (1, 2). While the vast majority of the amino acid residues in this structure are well-resolved, a number of residues, including those at both the N- and C-termini, are disordered and thus probably highly mobile. The unresolved C-terminal amino acids (#544-553) do not appear from the structure to be essential for tRNA recognition; they are thought to pack on top of the guanine at position 36 in the tRNA which is already specifically bound by the G36 binding pocket (amino acids #398-402) (2). However, the C-terminus is not located on the surface of the protein and could play a role in structural stabilization. On the other hand, the disordered N-terminal amino acids (#1-7) are located on the surface of the protein (2) and are not thought to play a critical role in either structural stabilization or tRNA recognition.

In order to understand what role the N- and C-termini may play in GlnRS, a deletion analysis of these residues was undertaken, initially in the absence of the crystal structure.

[1]Dedicated to Professor Morio Ikehara on the occasion of his 70th birthday.

Deletion analysis of a number of other AARSs indicated that N- and C-terminal deletions were usually functional, as were many internal deletions (3-10; for review, see 11). Thus, it was thought that many parts of AARSs were dispensable for function. However, all the deletion experiments have been performed on large and/or multimeric AARSs (e.g. MetRS, AlaRS, IleRS, GlyRS) which can catalyze amino acid activation in the absence of tRNA (for reviews, see 12, 13). GlnRS, on the other hand, is a 63.5 kD monomer and one of the smallest AARSs in *E. coli* (14). In addition, GlnRS requires tRNA for the formation of the aminoacyl-adenylate (15, 16) and recognizes both the anticodon and the acceptor stem/ discriminator regions of the tRNA (1, 2, 17-20). Thus, can much of GlnRS possibly be dispensable for function?

Many eukaryotic AARSs contain N-terminal extensions which are proposed to play a role in other physiological functions in yeast and in the formation of the multisynthetase complex found in higher eukaryotes (for review, see 21). The gene for the *S. cerevisiae* cytoplasmic GlnRS has been cloned and the enzyme purified (22, 23). The gene encodes a 224 amino acid N-terminal extension and is 40% identical over the remainder of the gene to *E. coli glnS*. Proteolysis and deletion analysis of the yeast GlnRS have demonstrated that this N-terminal extension is not required for AARS function and that the yeast enzyme with the N-terminal extension has catalytic properties that are very similar to the *E. coli* GlnRS (23, 24). Sequence comparisons indicate that the gene encoding the human GlnRS also contains an N-terminal extension (25).

In this paper, we report the results of a deletion analysis of the N- and C-termini, as well as of several internal regions of *E. coli* GlnRS. Only one of the C-terminal truncations was able to complement either a strain harboring a temperature-sensitive GlnRS (26) or a *glnS* deletion strain (27). Interestingly, the region deleted in this active C-terminal mutant corresponds almost exactly to the disordered region in the refined crystal structure (2). This active mutant was purified and its kinetic parameters determined *in vitro*. Neither of the N-terminal deletions were able to complement either the temperature-sensitive or the deletion strains. Thus, even a four amino acid deletion in the N-terminal region which is disordered in the crystal structure was sufficient to destabilize and inactivate GlnRS. As the C-terminal truncations were constructed by inserting an amber codon, a variety of amber suppressors were used to test the tolerance of these positions to amino acid substitutions. All four of the positions tested (residues tryptophan 386 (W386), alanine 484 (A484), alanine 512 (A512), and leucine 544 (L544)) yield active GlnRS mutants when any of six amino acids are inserted.

In the process of attempting to construct a strain for purification of inactive or thermolabile mutants of GlnRS, an N-terminal extension was added to GlnRS in the form of

a tag derived from a hemagglutinin epitope (HAI) (28). The N-terminus was chosen because the yeast enzyme with its N-terminal extension is fully active (23) and the crystal structure indicated that the N-terminus of *E. coli* GlnRS is exposed to solvent (1, 2) and thus accessible to the antibodies needed to separate away the HAI-tagged GlnRS (HAI-GlnRS). While the specific activity of the HAI-GlnRS is equal to that of wild-type GlnRS, the monoclonal anti-HAI antibody cannot immunoprecipitate the HAI-GlnRS, suggesting that the HAI tag is not exposed to solvent. In addition, a polyclonal antibody against GlnRS only weakly recognizes the native HAI-GlnRS, suggesting that the conformation of the HAI-GlnRS may be altered. The results of these experiments clearly demonstrate that the N- and C-termini of the small monomeric GlnRS are essential for stability and activity.

MATERIALS AND METHODS

General. E. coli strains UT172 and X3R2 have been described previously (26, 27) as has the plasmid pGFIB, which was obtained from J. Normanly (29). [^{14}C] and [^{3}H] glutamine and dimethylbenzidine solution (DMB, the peroxidase substrate) were purchased from NEN, Amersham and Kirkegaard & Perry (Gaithersburg, MD) respectively. Mouse ascites fluid containing anti-HAI IgG_{2b} and rabbit serum from an animal immunized with 5 mg purified GlnRS were provided by BabCo (Berkeley, CA). Antibody-horseradish peroxidase conjugates were purchased from Boehringer Mannheim and "Immobilon-P" membranes are a product of Millipore.

Construction of the deletion mutants. The C-terminal truncations (Term1, Term2, Term3 and Term4) were constructed by performing Kunkel mutagenesis (30) on single-stranded DNA from an M13*glnS* clone. The mutagenic oligonucleotides, which were 16-20 nucleotides in length, contained a UAG stop codon in place of the codons for L544 (Term1), A512 (Term2), A484 (Term3) and W386 (Term4). Mutant candidates were identified by plaque hybridization (31) and then sequenced by standard dideoxy sequencing. The mutants were then recloned as *Eco*RI-*Hin*dIII fragments into pBR322.

The N-terminal deletions (NΔ4 and NΔ36) were constructed by making *Bal*31 deletions using a purified *Eag*I-linearized fragment of pXmaI (pBR322*glnS*) in order to maintain the correct transcriptional and translational start sites. The fragments were recloned into the M13Xma vector which contained only flanking *glnS* sequences and single-stranded DNA from several transformants sequenced. Two isolates NΔ4 and NΔ36 were selected and recloned as *Eco*RI-*Hin*dIII fragments into pBR322.

The internal deletions (ΔBstEII and ΔBglII) were constructed by taking advantage of internal *Bst*EII and *Bgl*II restriction enzyme sites. A pBR*glnS* clone was digested with either

*Bst*EII or *Bgl*II, treated with alkaline phosphatase and then re-ligated. The appropriate clones were selected by digestion with the above enzymes and analysis on an agarose gel.

Site-specific substitution in GlnRS. With the exception of *supE* ($tRNA^{Gln}CUA$) which was expressed from a lysogen, the constitutively expressing pGFIB clones of the amber suppressor tRNAs derived from $tRNA^{Ala}$, $tRNA^{Cys}$, $tRNA^{His}$, $tRNA^{Phe}$, and $tRNA^{Tyr}$ (29) were cotransformed into UT172 (26) along with the various Term mutants expressed on a compatible plasmid. Complementation in the presence of the amber suppressors was assayed by growth on appropriately supplemented LB plates at 44°C.

Purification of the C-terminal deletion mutant Term1. The Term1 mutant was purified by first transforming pTerm1 (a pBR322 *Eco*RI-*Hin*dIII clone of Term1) into the deletion strain X3R2 (27) and plating at 30°C on LB plates containing ampicillin. The lysogenic copy of wild-type *glnS* was induced by raising the temperature of a culture containing pTerm1 to 42°C. The loss of the lysogenic copy of wild-type *glnS* was confirmed by cross-streaking against phage λ of the same immunity and Southern analysis (32). The GlnRS Term1 mutant was then purified from an S100 by chromatography on DEAE-cellulose followed by phosphocellulose (33).

Aminoacylation assays. GlnRS activity was measured as the rate of Gln-tRNA formation at varying concentrations of $tRNA^{Gln}$, ATP or glutamine. When the kinetic constants were measured for one of the substrates, the other two were present at concentrations which would be saturating for wild-type GlnRS (16). The assays (30-50 μl in volume) contained 100 mM sodium Pipes (pH 7.0), 10 mM magnesium acetate, 2 mM ATP, 1 mM [^{14}C]glutamine (40-80 cpm/pmol) and 3 μM $tRNA^{Gln}$ and were performed at 32°C. Aliquots were removed at various time points and spotted onto Whatman glass fiber filters which were washed extensively in 5% TCA and once in 95% ethanol. The filters were then dried and counted in Optifluor. The kinetic constants were determined from Hanes plots.

Western blot. S100 extracts were prepared from *E. coli* cells by resuspending the cells to a final concentration of 1 g cells/ml in 20 mM Hepes (pH 7.9), 20 mM KCl, 2 mM DTT, 0.5 mM PMSF, 5% glycerol and disrupted by sonication, followed by centrifugation. The S100 was electrophoresed on a 10% polyacrylamide-SDS gel and transferred onto an Immobilon-P membrane (34). The membrane was blocked in TBS (50 mM Tris (pH 8.0), 150 mM NaCl) containing 5% nonfat-dry milk for 10 hr at 4°C and then incubated for 2 hr at room temperature with either anti-HAI IgG_{2b} (1:1000 dilution of raw ascites fluid in TBS containing 3% BSA) or with anti-GlnRS IgGs (2.5 ng/ml in TBS, 3% fetal calf serum, 0.1% Tween 20) which had been affinity purified over a Sepharose column on which GlnRS had been immobilized. Membranes were washed extensively in TBS containing 0.1% Tween 20

followed by several brief rinses with TBS and finally probed with secondary antibodies, i.e. horseradish peroxidase conjugates of anti-mouse IgG_{2b} or anti-rabbit IgG respectively. Immunohybridizations were developed in DMB.

Immunoprecipitation. An S100 containing HAI-tagged GlnRS (60 μg) was dialyzed against phosphate-buffered saline and then combined with 2.4 μg of protein A-Sepharose affinity-purified anti-HAI IgG_{2b} in a final volume of 20 μl. After incubation for 20 min at room temperature, the solution was mixed with 10 μg of protein A-Sepharose and incubated for another 10 min at room temperature in order to precipitate any antibody:antigen complexes. The suspension was filtered through a micropipette tip plugged with glass wool and the presence of uncomplexed antigen (HAI-GlnRS) in the filtrate was assayed for by Western blot analysis as described above using the anti-HAI IgG as the primary antibody. To evaluate the influence of denaturing agents on the formation of the antibody:antigen complex, prior to the addition of antibodies, S100 extracts were incubated for 15 min at room temperature with 0-1.5 M guanidinium hydrochloride (GuHCl).

Construction of the HAI-tagged glnS gene. In order to clone the HAI tag onto the N-terminus of the *glnS* gene, a unique *Bsp*HI site was introduced at the translational start codon by site-directed mutagenesis (30) of a pSK*glnS* clone. The *Bam*HI/*Sal*I-fragment containing the mutated *glnS* gene was isolated, dephosphorylated with calf intestinal phosphatase and then digested with *Bsp*HI. Thus, two fragments were isolated, one containing the 5'-upstream sequence and the other, the *glnS* gene from its translational start codon on. Both were ligated into a *Bam*HI/*Sal*I fragment of pBR322 in the presence of a 48 bp 5'-dephosphorylated *Bsp*HI-fragment coding for the antigenic determinant of the influenza virus hemagglutinin, i.e. the HAI epitope (28). This short fragment was obtained by denaturing equimolar amounts of two complementary synthetic 48-mers in 10 mM Tris (pH 7.5) and 250 mM NaCl for 5 min at 95°C and then annealing them together during slow cooling over a period of 30 min. The *glnS* gene in the recombinant plasmid pBR*HAIglnS* codes for a tagged *E. coli* GlnRS, yielding a AARS whose N-terminus is extended by 16 amino acids. The *HAIglnS* gene was inserted by homologous recombination into the chromosome of *E. coli* AB1157 in place of the wild-type *glnS* gene (strain Happy101). A detailed description of the construction of this strain will be published elsewhere (manuscript in preparation).

Specific activity determination. The specific activities of S100 extracts were determined from initial velocity measurements of aminoacylations performed at 37°C in 30 mM Hepes (pH 7.2), 15 mM $MgCl_2$, 20 mM KCl, 2 mM ATP, 2 mM DTT, 750 μM [U-^{3}H]-glutamine (10.7 cpm/pmol) and 16.5 μg/μl total *E. coli* tRNA. The final volume of the assays

was 60 µl. The reactions were stopped by spotting on Whatman filters which were washed once in 10% TCA, three times in 5% TCA, and once in 95% ethanol. The filters were then dried and counted in Optifluor.

RESULTS

We were interested to see which regions in GlnRS were essential for structure and function. For this reason a deletion analysis of the *glnS* gene was undertaken. This study was carried out prior to the availability of the GlnRS:$tRNA^{Gln}$ crystal structure (1, 2). The solution of the structure has aided the interpretation of the functional studies presented below.

C-terminal truncations were constructed by substituting the amber UAG codon for the codons for tryptophan 386 (W386), alanine 484 (A484), alanine 512 (A512) and leucine 544 (L544). In the absence of amber suppressor tRNAs, these mutations result in GlnRS enzymes which lack between 10 and 168 amino acids at their C-terminus compared to the wild-type (553 amino acid) protein (Table 1). In the presence of the amber suppressors, the effect is to substitute the suppressor-specific amino acid for the wild-type amino acid to create full-length enzymes. These C-terminal 'Term' mutants were then tested for their ability to complement a *glnS* deletion strain (X3R2) and strain UT172 carrying a temperature-sensitive GlnRS (T266P) (26, 27). The only C-terminal mutant able to complement either strain in the absence of amber suppressors was Term1 which produces a GlnRS lacking only 10 C-terminal amino acids (Table 1). As complementation of both the temperature-sensitive and deletion strains requires stability at elevated temperatures (42-44°C), the lack of functional complementation may be due to reduced stability of some or all of these mutants. The level of expression of the four Term mutants in the deletion strain X3R2 was determined on a Western blot probed with a polyclonal antibody against GlnRS. Only the Term1 mutant was expressed at the same level as the single, genomic copy of GlnRS, but not overproduced as would be expected from a pBR322 clone (35). Term2, Term3 and Term4 are expressed poorly if at all (35) suggesting that these mutant proteins may be unstable. Experiments using a protease deficient *lon*$^-$ strain and maxicell preparations (35) indicated that while Term4 is stable under conditions when the effects of proteases are minimal, GlnRS from Term2 and Term3 are unstable even under these conditions and thus are likely to be unstable in a standard cell cultured at elevated temperatures. Clearly, only the Term1 mutant is both functional and/or stable enough to support growth at an elevated temperature in both strains.

However, when the Term mutants are expressed in the presence of amber suppressors which promote read-through of the amber termination codons, all of the Term mutants are active regardless of the amino acid inserted. Thus, any of six amino acids (alanine, cysteine,

TABLE 1: *In vivo* phenotypes of the deletion mutants of GlnRS. Only the Term1 mutant can complement the *glnS* deletion strain X3R2 or the temperature-sensitive *glnS* strain (UT172) in the absence of an amber suppressor tRNA. However, all four of the Term mutants, which were truncated by insertion of an amber codon at the position indicated in the Table, could complement the temperature-sensitive strain in the presence of any of the amber suppressors supplied.

GLNRS	MUTATION	X3R2	UT172 Amber Suppressor						
			Sup$^-$	*Gln*	*Tyr*	*Cys*	*Phe*	*His*	*Ala*
GlnRS$^+$	Wild-type	+	+	+	+	+	+	+	+
Term1	L544(UAG) (-10 aa)	+	+	+	+	+	+	+	+
Term2	A512(UAG) (-42 aa)	–	–	+	+	+	+	+	+
Term3	A484(UAG) (-70 aa)	–	–	+	+	+	+	+	+
Term4	W386(UAG) (-168 aa)	–	–	+	+	+	+	+	+

glutamine, histidine, phenylalanine or tyrosine) which vary in their hydrophobicity and size can substitute for W386, A484, A512 and L544 to produce mutant GlnRS enzymes which are sufficiently stable and active to complement the temperature-sensitive strain and sustain growth at 42°C. This is despite the fact that lysogenically expressed *supE* (the amber suppressor derived from $tRNA^{Gln}$) is not sufficient to promote overexpression of any of the Term mutants expressed on pBR322 or on an inducible plasmid pLc28 (35, 36).

As the Term1 mutant was active and stable enough to complement the deletion strain X3R2, it was purified from the cured X3R2 strain which expressed only the Term1 mutant, and its steady-state kinetic parameters determined (Table 2). While its K_M values for ATP and glutamine were essentially the same as wild-type GlnRS, the K_M of the Term1 mutant for tRNA was reduced by almost a factor of 10. However, its V_{max} was even more dramatically affected as it is almost 30-fold lower than that of wild-type. The combined effect of the decrease in V_{max} and the increase in K_M for tRNA was a relative specificity constant (V_{max}/K_M) for tRNA of approximately $4x10^{-3}$. This reduction in the specificity constant is quite significant considering that only ten amino acids have been removed from the C-terminus of GlnRS and that these C-terminal residues are not thought to be important for tRNA recognition (2). Clearly, amino acids 544 to 553 are important for aminoacylation as their deletion affects both the affinity of GlnRS for $tRNA^{Gln}$ and catalysis.

TABLE 2: Kinetic parameters of wild-type GlnRS and the GlnRS Term1 mutant.

Enzyme	V_{max} (nmol/mg·min)	K_M (μM) ATP	K_M (μM) Glutamine	K_M (μM) $tRNA^{Gln}$	Relative V_{max}/K_M
$GlnRS^+$	3400	40	190	0.19	1.0
Term1	120	30	230	1.70	$3.9x10^{-3}$

The effects of the N-terminal deletions were even more striking as deletion of as few as four amino acids (ΔR6-N9) in the N-terminal region of GlnRS (NΔ4) is sufficient to inhibit complementation of either the temperature-sensitive or deletion strain (data not shown). The same is true for the NΔ36 (ΔR6-I41), ΔBstEII (ΔV277-M362) and ΔBglII (ΔD18-E333) deletion mutants. However, the two N-terminal and the two internal deletion mutants appear to be unstable as the corresponding GlnRS protein cannot be detected by SDS-gel electrophoresis of maxicell preparations (35). This is not surprising as the large internal deletions which have removed entire domains of the protein and as the NΔ36 mutant lacks two of the amino acids (H40 and I41) of the conserved HIGH signature sequence (37) involved in ATP binding (1, 38, 39). It is however somewhat surprising that the loss of just four amino acids which are not conserved in GlnRS from various organisms (22, 25) and which do not appear to be important in the crystal structure (1, 2) completely destabilizes the (NΔ4) enzyme.

A second type of experiment was based on our knowledge of the GlnRS crystal structure (1, 2) and on the fact that the N-terminus of GlnRS enzymes is variable as the yeast and human enzymes contain N-terminal extensions (22, 25) which in the case tested do not affect the kinetic parameters of the enzyme (23). The N-terminus of *E. coli* GlnRS was tagged with the hemagglutinin epitope (HAI) by cloning. In the crystal structure of the *E. coli* GlnRS:$tRNA^{Gln}$ complex (2), the N-terminus is not well-ordered and is exposed to solvent, a fact which is critical as the anti-HAI antibody must be able to interact with the HAI tag fused to GlnRS. Specific activity measurements were determined from S100s prepared from the *E. coli* strain Happy101 which expresses only HAI-GlnRS (manuscript in preparation) and from the parent strain AB1157 expressing wild-type GlnRS. The specific activities of the purified tagged and untagged wild-type GlnRS are the same (about 5.5 nmol/mg·min) as are the apparent levels of expression as assessed on a Western (Fig. 1) of a denaturing gel probed with a polyclonal antibody against GlnRS. The anti-HAI antibody also recognizes HAI-GlnRS (Fig. 1). However, the anti-HAI antibody is not able to

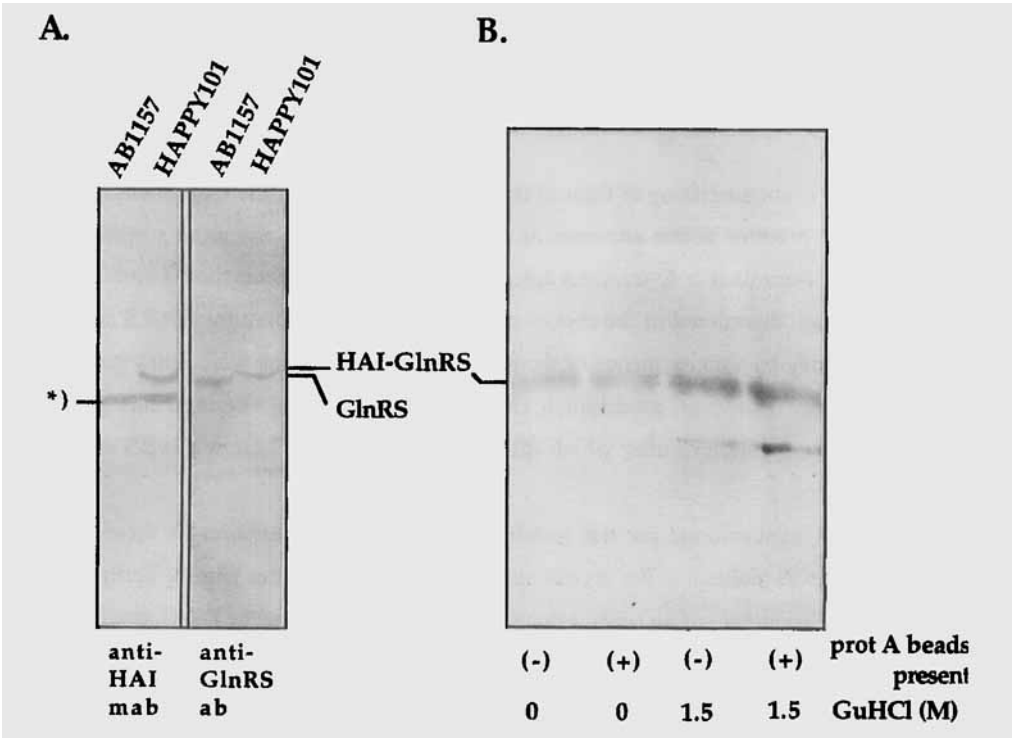

FIGURE 1: **A)** Immunohybridization of S100 extracts containing HAI-tagged and untagged GlnRS from strains AB1157 and Happy101. Each lane contains the equivalent of 40 µg of S100 protein. In addition to recognizing HAI-GlnRS, the monoclonal anti-HAI antibodies react with a 55 kDa protein (indicated by an asterisk) in all *E. coli* strains tested. **B)** Immunoprecipitation of HAI-GlnRS by anti-HAI antibodies. Western blot showing the supernatant obtained after immunoprecipitation of S100 from Happy101 with anti-HAI antibodies in the presence (+) and absence (-) of protein A beads, and before and after incubation with 1.5 M GuHCl and probing with anti-HAI antibodies. The fact that the signals are the same intensity in the + and - protein A beads lanes indicates that HAI-GlnRS is not being precipitated to any significant extent by the anti-HAI antibody.

immunoprecipitate the HAI-GlnRS even under mildly denaturing conditions as virtually all of the HAI-GlnRS is in the supernatant after immunoprecipitation with the anti-HAI antibody (Fig. 1). In addition, the HAI-GlnRS does not bind to a column packed with the anti-HAI antibody coupled to protein A-Sepharose (data not shown). The inability of the anti-HAI antibody to recognize native HAI-GlnRS (while it clearly recognizes denatured HAI-GlnRS) suggests that the HAI-tag may be buried in the HAI-GlnRS protein. This result correlates well with the fact that the HAI-GlnRS binds weakly to a column packed with polyclonal anti-GlnRS antibody coupled to protein A-Sepharose, in contrast to the tight binding to the same

column by wild-type GlnRS (data not shown). These results suggest that while the HAI-tag does not affect the activity of GlnRS, it is not accessible to solvent when fused to GlnRS.

DISCUSSION

While it is not surprising in light of the crystal structure that the large internal GlnRS deletions do not produce active enzymes or even stable proteins, it is more surprising that only one of the N-terminal or C-terminal deletions show activity. Thus, the N- and C-termini of GlnRS although disordered in the crystal structure (2) are important for GlnRS activity or stability. This may be a consequence of the small size of the enzyme which may have fewer dispensable parts. However, information from the GlnRS crystal structure has permitted rather large internal deletions after which the resulting proteins still show GlnRS activity *in vivo* (40).

Potential explanations for the instability and/or loss of function of these mutants become apparent in looking at the crystal structure (Fig. 2C). In the GlnRS Term1 protein lacking the 10 C-terminal amino acids, truncation occurs at the end of the last β-strand located in the proximal β-barrel; the deleted amino acids are disordered and lie outside the β-barrel. The C-terminal amino acids of GlnRS are not thought to be critical for tRNA recognition; although they may pack on top of guanine 36, this nucleotide is already specifically recognized by amino acids 398-402 which form a guanine-specific binding pocket (1, 2). However, the specificity constant (V_{max}/K_M) of the purified Term1 protein is reduced by more than 250-fold compared to wild-type (Table 2). The observed 10-fold increase in K_M (Table 2) is similar to that observed for the G36A mutation in $tRNA^{Gln}$ (17) and indicates a reduced affinity for the tRNA (the K_M values for glutamine and ATP are unaffected). In addition to its effect on K_M, the 10 amino acid deletion also decreases V_{max} by almost 30-fold (Table 2); however, the magnitude of the effect on V_{max} is less than that observed for either the G36A or G36U $tRNA^{Gln}$ mutants (17). The effect of a C-terminal deletion on catalysis of the Term1 enzyme and thus on the active site (located >35Å away) is not surprising, as GlnRS requires $tRNA^{Gln}$ for amino acid activation (15, 16) and specifically interacts with both the anticodon and the acceptor stem (for review, see 41). The ten residues deleted in Term1 are disordered in the crystal structure (2) suggesting that they may be highly mobile. They could form a floppy lid for the G36 binding pocket and participate in both specific recognition of the tRNA and transmission of this recognition to the active site by contacting a part of the protein domain which connects the anticodon binding domain to the dinucleotide binding domain (2).

The Term2 mutant is truncated in the middle of a long loop which connects two β-strands and results in the deletion of 3 strands of the proximal β-barrel (Fig. 2C) and in so

FIGURE 2. (A - Upper left) Internal deletion mutants. The start and end points in the mutants ΔBstEII (V277-M362) and ΔBglII (D18-E333) are marked on a three-dimensional ribbon diagram which is based on the refined crystal structure of the GlnRS:tRNA^{Gln} complex (2). The tRNA is drawn using dotted lines while the GlnRS is a ribbon. The ΔBstEII deletion is drawn in light-colored ribbon except where it overlaps with the ΔBglII deletion which is drawn in black. **(B - Upper right)** The N-terminal deletion mutants. The sites of truncation are indicated for NΔ4 (N9) and NΔ36 (I41) on the same view of the structure depicted in (A). The deletion start point at Arg6 is not marked as this region of the crystal structure is disordered (2). The black strand indicates the part of GlnRS that would be deleted in the NΔ36 mutant. **(C - Lower middle)** The C-terminal truncation mutants. The mutation sites (to an amber UAG codon) in Term mutants are marked in the GlnRS structure which has been rotated by 180° compared to (A) and (B). The black ribbon indicates the region which would be deleted in the largest deletion, Term4 (UAG codon in place of the W386 codon). Amber codons replaced the codons for L544, A512 and A484 in the Term1, Term2 and Term3 respectively.

doing, probably destabilizes the remainder of GlnRS. Functionally, deletion of the C-terminal 42 amino acids which includes the U35 binding pocket (2) would be expected to lead to loss of function, as U35 is the single most important determinant for GlnRS-tRNA^{Gln} recognition (for reviews, see 41, 42). The Term3 truncation (Fig. 2C) occurs in the long loop which connects the proximal β-barrel to the active site by packing against the outside of the dinucleotide binding domain (2), and thus is likely to be critical for both the structure and function of GlnRS. The structural importance of this loop is confirmed by the instability of the Term3 mutant even when produced in maxicells. Termination in the Term4 mutant occurs in the N-terminal end of a short β-strand (Fig. 2C) which is critical to formation of the distal β-barrel and deletes most of the anticodon binding domain. While the loss of all interaction with the anticodon is likely to lead to inactivity, the greater impact is probably structural as an important globular domain has been deleted leaving the α-helices, which form the anticodon - acceptor binding domain connection, exposed to solvent.

The other result of our Term mutant strategy was to define functional amino acid replacements in positions now known to be located either at the end of β-strands (L544 and W386) or in loops (A512 and A484) (Fig. 2C). These parts of the structure may be more flexible and permit variations in size (from alanine to phenylalanine) and hydrophobicity (from glutamine to phenylalanine) and thus account for the tolerance of these positions to substitutions by the amino acids tested (Table 1).

In the case of the N-terminal and internal deletions, it is not surprising that the NΔ36, ΔBstEII and ΔBglII GlnRS's are unstable and/or inactive (Figures 2B and C). In the latter two, entire domains of the protein are deleted (the dinucleotide binding domain and the connectivity domain, respectively). In order for such mutants to be stable and active *in vitro* and *in vivo*, it may be necessary to either coexpress the deleted domains (43) or to introduce hydrophilic amino acids by mutagenesis.

We were surprised that a short deletion in the N-terminal domain (R6-N9) renders the NΔ4 mutant inactive and unstable (Fig. 2B). Two of deleted amino acids, R6 and P7, are disordered in the crystal structure and are exposed to solvent (2). Possibly the N-terminal part of GlnRS packs against one of the nearby helices and loss of this interaction destabilizes the enzyme. Recently, a P7S mutation has been isolated (M.J. Rogers, personal communication) which may have reduced activity *in vivo*. Possibly a proline is required at position 7, so that a turn can be made which will allow the N-terminal amino acids to interact with and stabilize one or more of the nearby α-helices.

Addition of an N-terminal extension to GlnRS in the form of a hemagglutinin (HAI) tag also supports this interpretation. While the addition of the HAI-tag does not affect the

specific activity of GlnRS, the HAI-tag is not accessible (evidenced by the inability of the anti-HAI antibody to immunoprecipitate the HAI-GlnRS when it is in its native conformation and the failure of the HAI-GlnRS to bind to an anti-HAI Sepharose column). It is unclear whether the N-terminus is buried in the wild-type GlnRS or whether this occurs due to the addition of the HAI tag, particularly since polyclonal anti-GlnRS antibodies only weakly recognize the HAI-GlnRS (Fig. 1). Both experiments, N-terminal deletion and insertion, indicate that the N-terminus is important for the stability of GlnRS.

While these deletion studies are not comprehensive, they suggest that in the case of GlnRS, which requires tRNA for glutamine activation and recognizes both the anticodon and acceptor stem of its tRNA substrate, most of the protein is necessary for adequate activity.

ACKNOWLEDGEMENTS

We thank Alex Szewczak for help in preparing Figure 2. This work was supported by a grant from NIH.

REFERENCES

1. Rould, M.A., Perona, J.J., Söll, D. & Steitz, T.A. (1989) *Science 246,* 1135-1142.
2. Rould, M.A., Perona, J.J. & Steitz, T.A. (1991) *Nature 352,* 213-218.
3. Cassio, D. & Waller, J.P. (1971) *Eur. J. Biochem. 20,* 283-300.
4. Koch, G.L.E. & Bruton, C.J. (1974) *FEBS Lett. 40,* 180-182.
5. Jasin, M., Regan, L. & Schimmel, P. (1985) *J. Biol. Chem. 260,* 2226-2230.
6. Starzyk, R.M., Webster, T.A. & Schimmel, P. (1987) *Science 237,* 1614-1617.
7. Lorber, B., Mejhoub, H., Reinbolt, J., Boulanger, Y. & Giege, R. (1988) *Eur. J. Biochem. 174,* 155-161.
8. Mellot, P., Mechulam, Y., LeCorre, D., Blanquet, S. & Fayat, G. (1989) *J. Mol. Biol. 208,* 429-443.
9. Walter, P., Weygand-Durasevic, I. Sanni, A., Ebel, J.P. & Fasiolo, F. (1989) *J. Biol. Chem. 264,* 17126-17130.
10. Toth, M.J. & Schimmel, P. (1990) *J. Biol. Chem. 265,* 1000-1004
11. Schimmel, P. (1987) *Annu. Rev. Biochem. 56,* 125-158.
12. Schimmel P.R. & Söll, D. (1979) *Annu. Rev. Biochem. 48,* 601-648.
13. Kern, D. & Lapointe, J. (1981) *Eur. J. Biochem. 115,* 29-38.
14. Carter, C.J. Jr. (1993) *Annu. Rev. Biochem. 62,* 715-748.
15. Folk, W.R. (1971) *Biochemistry 10,* 1728-1732.

16. Kern, D., Potier, S., Lapointe, J. & Boulanger, Y. (1980) *Biochim. Biophys. Acta 607,* 65-80.
17. Jahn, M., Rogers, M.J. & Söll, D. (1991) *Nature 352,* 258-260.
18. Hayase, Y., Jahn, M., Rogers, M.J., Sylvers, L.A., Koizumi, M., Inoue, H., Ohtsuka, E. & Söll, D. (1992) *EMBO J. 11,* 4159-4165.
19. Rogers, M.J., Adachi, T., Inokuchi, H. & Söll, D. (1992) *Proc. Natl. Acad. Sci. USA 89,* 3463-3467.
20. Sherman, J.M., Rogers, K., Rogers, M.J. & Söll, D. (1992) *J. Mol. Biol. 228,* 1055-1062.
21. Lapointe, J. & Giege, R. (1992) In *Translation in Eukaryotes* (Ed. H. Trachsel, CRC Press, Boca Raton), pp. 35-69.
22. Ludmerer, S.W. & Schimmel, P. (1987) *J. Biol. Chem. 262,* 10801-10806.
23. Ludmerer, S.W., Wright, D.J. & Schimmel, P. (1993) *J. Biol. Chem. 268,* 5519-5523.
24. Ludmerer, S.W. & Schimmel, P. (1987) *J. Biol. Chem. 262,* 10807-10813.
25. Fett, R. & Knippers, R. (1991) *J. Biol. Chem. 266,* 1448-1455.
26. Englisch-Peters, S., Conley, J, Plumbridge, J., Leptak, C., Söll, D. & Rogers, M.J. (1991) *Biochimie 73,* 1501-1508.
27. Uemura, H., Rogers, M.J., Swanson, R., Watson, L. & Söll, D. (1988) *Protein Engineering 2,* 293-296.
28. Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.I. & Lerner, R.A. (1984) *Cell 37,* 767-778.
28. Brick, P., Bhat, T.N. & Blow, D.M. (1989) *J. Mol. Biol. 208,* 83-98.
29. Normanly, J., Masson, J.-M., Kleina, L.G., Abelson, J. & Miller, J.H. (1986) *Proc. Natl. Acad. Sci. USA 83,* 6548-6552.
30. Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA 82,* 488-492.
31. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) In *Molecular Cloning: A laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
32. Swanson, R.N. (1988) Ph.D. Thesis, Yale University.
33. Hoben, P. & Söll, D. (1985) *Methods Enzymol. 113,* 55-59.
34. Matsudaira, P. (1987) *J. Biol. Chem. 262,* 10035-10038.
35. Conley, J.G. (1988) Ph.D. Thesis, Yale University.
36. Perona, J.J., Swanson, R., Steitz, T.A. & Söll, D. (1988) *J. Mol. Biol. 202,* 121-126.
37. Webster, T., Tsai, H., Kula, M., Mackie, G.A. & Schimmel, P. (1984) *Science 226,* 1315-1317.
39. Brunie, S., Zelwer, C. & Risler, J.-L. (1990) *J. Mol. Biol. 216,* 411-424.
40. Schwob, E. & Söll, D. (1993) *EMBO J. 12,* 5201-5208.

41. Rogers, M.J., Weygand-Durasevic, I., Schwob, E., Sherman, J.M., Rogers, K.C., Adachi, T., Inokuchi, H. & Söll, D. (1993) *Biochimie* (in press).
42. Normanly, J. & Abelson, J. (1989) *Annu. Rev. Biochem. 58,* 1029-1049.
43. Burbaum, J.J. & Schimmel, P. (1991) *Biochemistry 30,* 319-324.
44. Perona, J.J., Rould, M.A. & Steitz, T.A. (1993) *Biochemistry 32,* 8758-8771.

Received 12/25/93
Accepted 1/31/94