C-Terminal Zinc-Containing Peptide Required for RNA Recognition by a Class I tRNA Synthetase†

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Received November 22, 1995; Revised Manuscript Received January 30, 1996⊗

ABSTRACT: *Escherichia coli* isoleucyl-tRNA synthetase is one of five closely related class I tRNA synthetases. The active site of the 939 amino acid polypeptide is in an N-terminal domain which contains an insertion believed essential for interactions with the tRNA acceptor helix. The enzyme was shown previously to contain an essential (for function *in vivo*) zinc bound to a Cys_4 cluster at the C-terminal end of the polypeptide. The specific function of this zinc has been unknown. We show here that aminoacylation activity can be reconstituted *in vitro* by combining a 53 amino acid zinc-containing C-terminal peptide with a protein consisting of the remaining 886 amino acids. Reconstitution of aminoacylation is zinc-dependent. In contrast, the zinc-containing peptide is dispensable for synthesis of isoleucyl adenylate. Affinity coelectrophoresis showed that the 53 amino acid C-terminal peptide is required specifically for tRNA binding. We propose that the zinc-containing peptide curls back to the active site to make contact with the acceptor helix of bound tRNA, but not with isoleucine or ATP. It is the first example of a zinc-containing peptide in a class I tRNA synthetase that is essential for tRNA binding interactions. The design of this enzyme may be part of a more general scheme for class I tRNA synthetases to acquire acceptor helix binding elements during the development of the genetic code.

In this work, we demonstrate a role in RNA recognition for a zinc-containing small peptide motif appended to the end of the second domain of a class I aminoacyl-tRNA synthetase. This location for a tRNA binding element which contains zinc has not previously been seen in other class I enzymes. In general, tRNA synthetases are roughly comprised of two major domains which divide interactions between the two separate domains of the L-shaped tRNA molecule (Moras, 1992; Buechter & Schimmel, 1993; Carter, 1993). One synthetase domain contains the active site whose structure is either of two basic types, known as class I and class II. Insertions into the active site domain enable a synthetase to interact with the acceptor stem-containing domain of the bound tRNA (Rould et al., 1989; Steitz, 1991). These insertions tend to be idiosyncratic to the enzyme and possibly were added after development of the core catalytic site which was used for amino acid activation.

The second domain of a synthetase is highly variable and is not conserved even among enzymes which share the same class of active site (Cusack et al., 1991; Hou et al., 1991; Moras, 1992; Carter, 1993; Schimmel & Ribas de Pouplana, 1995). In cases where anticodon contacts are made by a bound synthetase, this domain provides for those interactions (Brunie et al., 1990; Schulman, 1991; Steitz, 1991; Cavarelli et al., 1993). These interactions are typically near the central part of the sequence of the second domain, far removed from the end of the structure.

For class I enzymes, a nucleotide binding fold of alternating α-helices and β-strands forms the active site (Bhat et al., 1982; Zelwer et al., 1982; Webster et al., 1984; Ludmerer & Schimmel, 1987; Schimmel, 1987; Carter, 1993). This domain is typically located in the amino-terminal halves of the proteins. An insertion known as connective polypeptide 1 (CP1)[1] subdivides the nucleotide binding fold (Starzyk et al., 1987). This insertion is idiosyncratic to each class I enzyme in both size and sequence (Hou et al., 1991). In the cocrystal of the class I *Escherichia coli* glutaminyl-tRNA synthetase with $tRNA^{Gln}$, the CP1 insertion serves as an acceptor helix binding domain (Rould et al., 1989). Modeling of the complex of *E. coli* $tRNA^{Met}$ with the class I methionyl-tRNA synthetase suggests that the CP1 has a similar role in tRNA recognition (Perona et al., 1991). These and additional results have led to the idea that CP1 is commonly used by class I enzymes for acceptor helix interactions. In contrast, interactions with the second domain of a tRNA molecule occur with a separate, second domain of the synthetase. The second tRNA domain contains the anticodon, and for many enzymes, it is the critical site for binding interactions. Like CP1, however, the anticodon binding domain of a tRNA synthetase varies considerable from enzyme to enzyme, even within the same class (Brunie et al., 1990; Perona et al., 1991; Steitz, 1991).

Variations superimposed on this basic theme (of two synthetase domains interacting separately with two tRNA domains) are now emerging. We are particularly interested in these variations because they may give better insight into the general mechanism of assembly of this canonical group

† This work was supported by Grant GM15539 from the National Institutes of Health. E.G. is an NIH postdoctoral fellow.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: CP1, connective polypeptide 1; IleRS, isoleucyl-tRNA synthetase; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ACE, affinity coelectrophoresis; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)phenylsulfonate.

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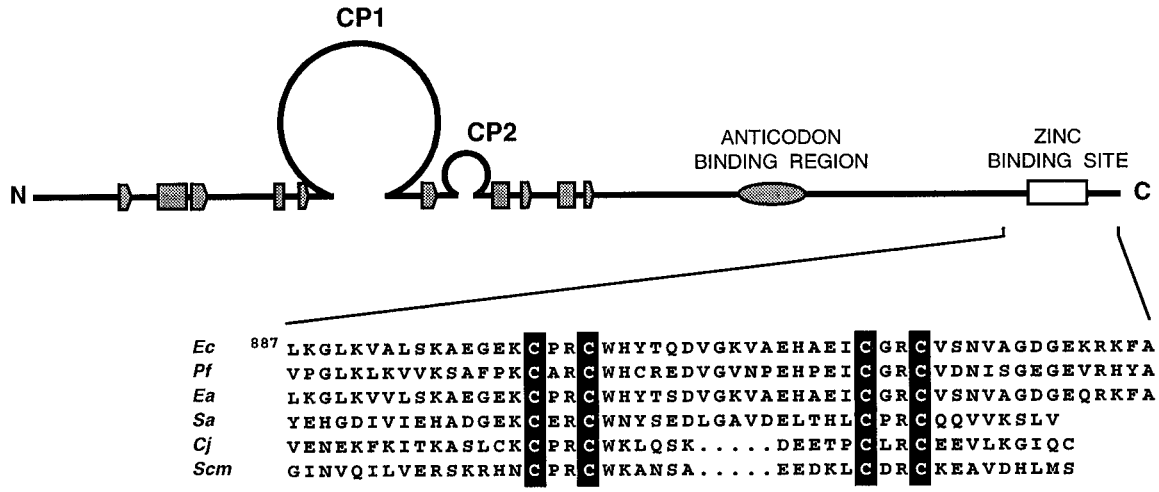

FIGURE 1: Schematic illustration of *E. coli* isoleucyl-tRNA synthetase and alignment of the sequences of the C-terminal zinc binding domain from various organisms. Filled arrows and rectangles designate β-strands and α-helices, respectively. The location of the zinc binding site examined here is indicated. The isoleucyl-tRNA synthetase sequences containing a C-terminal Cys_4 site were aligned as described by Shiba et al. (1994). The sequences are from *Escherichia coli* (*Ec*) (Webster et al., 1984), *Pseudomonas fluorescens* (*Pf*) (Isaki et al., 1990a), *Enterobacter aerogenes* (*Ea*) (Isaki et al., 1990b), *Staphylococcus aureus* (*Sa*) (Chalker et al., 1994), *Campylobacter jejuni* (*Cj*) (GenBank Accession no. U15295), and *Saccharomyces cerevisiae* mitochondria (*Scm*) (GenBank Accession No. L38957).

of proteins. In particular, the crystal structure of an active monomeric fragment of *E. coli* methionyl-tRNA synthetase showed that the C-terminal end (in the second domain) curls back to the active site (Brunie et al., 1990). Consistent with this observation, deletion analysis established that the C-terminal 15 amino acids from the monomeric fragment were essential for amino acid activation (Mellot et al., 1989). This result was surprising because all of the elements needed for amino acid activation seemed to be present in the N-terminal domain. In further work, mutational and biochemical analysis provided evidence that this C-terminal peptide appendix also interacted with the acceptor helix of bound $tRNA^{Met}$ (Kim et al., 1993a). This result, too, was unexpected because no such interactions with the acceptor helix are seen in the cocrystal of glutaminyl-tRNA synthetase with $tRNA^{Gln}$ (Rould et al., 1989).

Because the sequence of isoleucyl-tRNA synthetase (especially of the N-terminal domain) is closely related to that of methionyl-tRNA synthetase, the structure of the latter has been used to model the active site of isoleucyl-tRNA synthetase (Starzyk et al., 1987; Shiba & Schimmel, 1992). In addition, a functional and likely structural relationship between the C-terminal domains of the two enzymes was established by mutational analysis. For example, using a designed peptide which was transplanted into the anticodon binding domain of each enzyme, anticodon recognition was switched [between CAU ($tRNA^{Met}$) and GAU ($tRNA^{Ile}$)] by a single amino acid substitution in the special peptide element (Auld & Schimmel, 1995). These and other results suggested that the relationship between the two enzymes extended to their C-terminal anticodon binding domains.

With these considerations in mind, we were interested in a feature of isoleucyl-tRNA synthetase not found in methionyl-tRNA synthetase. In particular, while both enzymes have a tightly bound zinc in the CP1 insertion of their N-terminal domains, only isoleucyl-tRNA synthetase has a second zinc bound to a Cys_4 cluster at the C-terminal end (Figure 1) (Fourmy et al., 1993; Landro & Schimmel, 1993, 1994; Landro et al., 1994). This cluster extends from C902 to C925. Using an *ileS* null strain, an essential role for the zinc-containing Cys_4 cluster was established in two ways. First, a truncated protein containing the N-terminal 886 amino acids was shown to be inactive for complementation of the null allele, even though the N-terminal fragment itself was expressed and accumulated *in vivo* (Shiba & Schimmel, 1992). Second, unlike cells harboring the wild-type allele, cells containing the C905S or C925S mutant alleles cannot grow in the absence of exogenous zinc added to the growth media. However, growth of cells containing either of the two mutant alleles is rescued by added zinc (Landro & Schimmel, 1994). These results collectively showed that the C-terminal peptide element was essential and that zinc was required for its function.

In this study, we investigated the specific role for zinc in the C-terminus of *E. coli* isoleucyl-tRNA synthetase. To achieve this objective, we prepared a C-terminal peptide containing the Cys_4 cluster and the complementary large N-terminal fragment which constituted the rest of the protein. The zinc-containing peptide and the apo-peptide, and the large protein fragment, were investigated *in vitro*, taking advantage of assays for synthesis of isoleucyl adenylate, aminoacylation, and binding of $tRNA^{Ile}$. These studies enabled us to show that the zinc-containing peptide had a critical role in tRNA binding, but had no apparent role in amino acid activation. This result was surprising because, by comparisons with known X-ray structures and by direct experimental analyses, the major determinants for tRNA binding (such as anticodon binding) had been previously located to other parts of the protein. In addition, we were not aware of any other example where a C-terminal zinc-containing peptide element plays an essential role in tRNA recognition.

EXPERIMENTAL PROCEDURES

Construction and Purification of Fusion Proteins. Mutagenesis of single-stranded DNA isolated from phagemid pKS21 was performed using the Amersham oligonucleotide mutagenesis system (Amersham, Arlington Heights, IL). A unique *Bam*HI restriction site within the isoleucyl-tRNA synthetase (IleRS) gene was removed by a silent mutation of the codon for I444, and a new *Bam*HI site was introduced

on the 5′ side of the IleRS coding sequence to give plasmid pJL01. A *Hin*dIII site was also introduced at the 3′ end of the sequence (pJL03). The gene for the truncated IleRS (coding for amino acids 1−886, referred herein as 886N) was engineered by introducing a stop codon in the DNA sequence at the position corresponding to the codon for L887 (pJL04). The gene for the maltose binding protein fused to the N-terminus of 886N was prepared by digesting pJL04 with *Bam*HI and *Hin*dIII and subcloning the fragment into the similarly digested pMAL-c2 plasmid (New England Biolabs, Beverly, MA) to give pJL22.

The plasmid pJL22 was transformed into the *E. coli* strain MI1 [the chromosomal gene for IleRS contains a mutation which produces an enzyme with an elevated K_m for isoleucine and with undetectable activity under the conditions used in the standard assay (Schmidt & Schimmel, 1993)]. MI1 cells harboring the pJL22 plasmid were grown at 37 °C and induced with 1 mM isopropyl β-D-thiogalactopyranoside in mid-logarithmic phase for 4 h. Cells were harvested by centrifugation and lysed in 25 mM Tris·HCl (pH 7.5), 0.2 M NaCl, 20 mM 2-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride. After clarification of the lysate by centrifugation for 20 min at 11000*g*, poly(ethylenimine) was added to 0.2% and the precipitate pelleted by centrifugation at 11000*g* for 20 min. The supernatant was loaded onto an amylose resin column (New England Biolabs), washed with 10 bed volumes of 25 mM Tris·HCl (pH 7.5), 0.2 M NaCl, and 1 mM 2-mercaptoethanol. The fusion protein was eluted in the same buffer with 10 mM maltose. The 886N protein was cleaved from the fusion by incubation with factor Xa (400:1 w/w) at 4 °C for 18 h and purified to homogeneity on Mono Q (Pharmacia Biotech Inc., Uppsala, Sweden). Due to the choice of restriction sites in making the gene for the fusion protein, after cleavage of 886N from the fusion protein, the N-terminus begins with the sequence Ile-Ser-Glu-Gly-Ser followed by the IleRS sequence.

The coding sequence for the 53 amino acid C-terminal peptide (L887 to A939) was generated by introducing a *Bam*HI site into pKS21 at the codons for amino acids 885 and 886. The plasmid was digested with *Bam*HI and *Eco*RI and subcloned into the plasmid pGEX-KT [which encodes glutathione *S*-transferase (GST), a polyglycine linker, and a thrombin cleavage site on the 5′ side of the introduced sequence (Guan & Dixon, 1991)] to produce plasmid pJL35. The *E. coli* K12 strain MV1184 [*ara*, Δ(*lac-proAB*), *rspL*, *thi*, (*ϕ*80 *lacZ*ΔM15), Δ(*srl-recA*), 306::Tn*10*(*tet*^{r})/F′(*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)] was used as the host for expression of pJL35 and pKS21.

The glutathione *S*-transferase (GST) fusion of the C-terminal 53 amino acids of IleRS (referred to herein as peptide-53C) was expressed and purified from an MV1184 strain harboring the plasmid pJL35 following literature procedures (Smith & Johnson, 1988). The peptide was cleaved from the fusion protein by incubation for 1 h at 22−24 °C with thrombin (1000:1, w/w) in 50 mM Hepes (pH 8), 0.1 M NaCl, 2.5 mM $CaCl_2$, and 1 mM 2-mercaptoethanol. The peptide was separated from GST by passing the sample through glutathione−Sepharose (Pharmacia) equilibrated with 50 mM Hepes (pH 8) and 1 mM 2-mercaptoethanol. The fractions containing peptide were determined by measuring the absorbance at 280 nm and then heated to 70 °C for 5 min to precipitate any remaining impurities. These fractions were then pooled and concentrated by forced ultrafiltration using an Amicon (Beverly, MA) YM-3 membrane in an Amicon Stirred Cell. This procedure yielded essentially pure peptide as judged by SDS−PAGE using the Schägger and von Jagow method (Schägger & von Jagow, 1987). The peptide released retained the two amino acids from the thrombin cleavage site so that its predicted sequence is GSLKGLKVALSKAEGEKCPRCWHYTQDVGKVAEHAEICGRCVSNVAGDGEKRKFA. The sequence of the first five amino acids was confirmed through five cycles of analysis on an Applied Biosystems Model 477A Protein Sequencer with an on-line Model 120 PTH Amino Acid Analyzer. Concentrations were determined using the theoretical extinction coefficient of 6970 M^{-1} cm^{-1} for a peptide with one tryptophan and one tyrosine (Gill & von Hippel, 1989).

Native *E. coli* IleRS was overexpressed from the phagemid pKS21 in MV1184 cells and purified as described previously (Schmidt & Schimmel, 1993).

Enzyme Activity Assays. Formation of the isoleucyl adenylate was measured at 37 °C in 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM KF, 2 mM ATP, 2 mM [^{32}P]pyrophosphate, and 1 mM isoleucine as described previously (Shepard et al., 1992). The aminoacylation reaction was assayed as described (Shepard et al., 1992). The $tRNA^{Ile}$ (major isoacceptor) was isolated from *E. coli* strain MV1184 containing a plasmid overexpressing $tRNA^{Ile}$ from a gene under control of the *lac*-inducible *tac* promoter (Schmidt, 1995). The tRNA was purified by acid−phenol extraction of *E. coli* cells (Varshney et al., 1991), anion exchange on a Machery-Nagel (Düren, Germany) Nucleobond AX-500 cartridge, and reverse phase HPLC on a Vydac (Hesperia, CA) C4 column (Schmidt, 1995). The specific activity of the $tRNA^{Ile}$ used here was 1200 pmol/A_{260} unit. The tRNA was treated with calf intestine phosphatase and 5′-end-labeled with ^{32}P using T4 polynucleotide kinase (Park & Schimmel, 1988).

Reduction of Peptide and Incorporation of Zinc. To ensure complete reduction of the peptide-53C cysteine sulfhydryls, the peptide (20 μM to 3 mM in a total volume of 100 μL) was heated to 80 °C for 30 min with 0.2 M dithiothreitol and 10 mM EDTA. All further manipulations were done under an argon atmosphere at 4 °C. Buffers were treated with immobilized iminodiacetic acid (Sigma, St. Louis, MO) in order to remove endogenous metals. The peptide was separated from reductant and EDTA by passage through a 1.8 mL Sephadex G25 column (0.4 × 3.5 cm) equilibrated in argon-purged 20 mM Hepes (pH 7). For preparation of the zinc-peptide for use in the assays, 100 μL of the apopeptide eluted from the column (100−200 μM) was made 1 mM in $ZnCl_2$. Unbound zinc was removed by gel chromatography with a second 1.8 mL Sephadex G25 column. Zinc content was determined by detecting 4-(2-pyridylazo)-resorcinol (PAR) binding of zinc at 500 nm in the presence of *p*-(hydroxymercuri)phenylsulfonate (PMPS) (Hunt et al., 1984). The zinc concentration was extrapolated from a standard curve prepared using a 1000 ppm stock solution of zinc (Aldrich, Milwaukee, WI).

Spectroscopic Methods. Circular dichroism spectra were accumulated on an AVIV Model 62DS CD spectrometer with an AVIV Model W5TE-159-S thermoelectric temperature-controlled cuvette holder. Peptide samples were placed in a 0.5 mm path length cuvette. Spectra were averaged over five scans. Fluorescence experiments were done with a

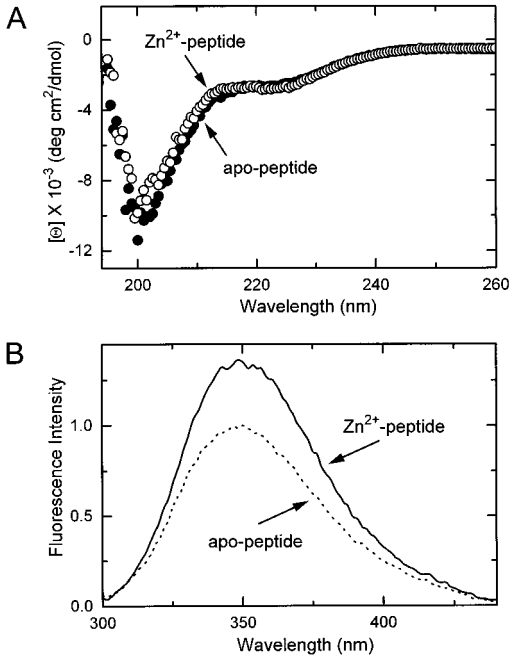

FIGURE 2: Spectral changes of 53C upon addition of zinc. (A) Circular dichroism spectra of 53C with and without added zinc. The peptide concentration was 200 μM in 10 mM Tris·HCl (pH 7). Zinc was added to the peptide to a concentration of 360 μM. Samples were at 5 °C. Similar spectra were recorded at ambient temperature (~23 °C). (B) Fluorescence spectra of 53C at ambient temperature [2 μM peptide in 20 mM HEPES (pH 7)]. The zinc-peptide spectrum was accumulated after addition of 1 equiv of $ZnCl_2$.

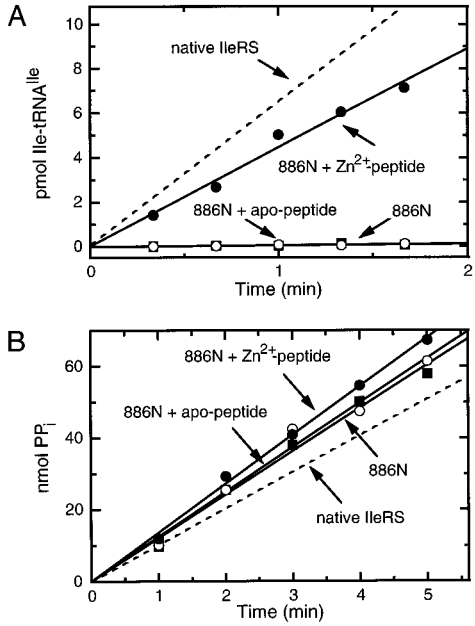

FIGURE 3: (A) Aminoacylation of $tRNA^{Ile}$ catalyzed by 886N, 886N with apo-53C, and 886N with Zn^{2+}-53C compared to the aminoacylation of $tRNA^{Ile}$ by wild-type IleRS (dashed line). Enzyme concentrations were 2 nM, peptides were 2 μM, and $tRNA^{Ile}$ was 1 μM. (B) Isoleucine-dependent ATP-pyrophosphate exchange catalyzed by 886N, 886N with apo-53C, and 886N with Zn^{2+}-53C. Native IleRS is also shown for comparison (dashed line). Enzyme concentrations were 10 nM and peptides were 2 μM. All kinetic experiments were done at pH 7.5, 37 °C.

Perkin Elmer LS 50 Luminescence Spectrometer with 5 × 5 mm path length cuvettes.

Affinity Coelectrophoresis. The preparation of agarose gels for affinity coelectrophoresis was as described previously (Gale & Schimmel, 1995).

RESULTS

C-Terminal Peptide. A peptide encompassing the C-terminal zinc binding region (peptide-53C) was isolated for further characterization of the functional significance of the bound zinc. Purification of the C-terminal peptide was achieved by expression of 53C as a fusion with glutathione *S*-transferase having an intervening polyglycine linker and a thrombin cleavage site. Following affinity chromatography of the fusion protein, cleavage of the peptide was achieved with thrombin, and essentially pure peptide could be isolated.

The metal-free form of the peptide was generated by reduction with dithiothreitol followed by gel chromatography (see Experimental Procedures). After treatment of the peptide with PMPS, no free zinc could be detected with the zinc chelator PAR. To ensure complete reconstitution of the peptide with zinc, excess zinc was added to apo-53C under anaerobic conditions, immediately after removal of the reductant dithiothreitol. Excess unbound zinc was removed via a second gel chromatography step. Assays with PMPS and PAR of the zinc content of Zn^{2+}-53C indicated that one zinc was bound per peptide.

CD and Fluorescence Spectroscopy of 53C. The CD spectrum of apo-53C is shown in Figure 2A. The large negative ellipticity at 200 nm is indicative of a mostly disordered structure (Johnson, 1990). Addition of zinc to the peptide induced very little change in the appearance of the CD spectrum, with only a slight increase in the negative ellipticity centered at 222 nm. The spectrum was independent of peptide concentration over the range of 20−200 μM (data not shown).

On the other hand, the fluorescence spectrum of the peptide was sensitive to zinc binding (Figure 2B). Peptide-53C contains a single tryptophan (W906) adjacent to C905. (The numbering reflects the amino acid position in full-length *E. coli* IleRS rather than in the peptide.) Upon addition of zinc, the fluorescence of the tryptophan is enhanced. This enhancement demonstrates that, although the overall structure of 53C is not greatly altered by binding of zinc (Figure 2A), local changes near the bound metal can be detected.

Fragment 886N. To help elucidate the contribution of 53C to the activities of IleRS, fragment 886N was purified. This fragment lacks the last 53 amino acids of IleRS. Fragment 886N precipitated with the insoluble fraction of the cell lysate and was, therefore, expressed and purified as a fusion with the maltose binding protein (MBP) to improve solubility. Cleavage of the fusion protein with factor Xa released 886N, which was then purified to homogeneity by anion exchange chromatography. Because 886N does not complement the *ileS* null strain, it was necessary to express and purify the fusion protein from *E. coli* strain MI1. The chromosomally expressed IleRS of MI1 [with a F570S point mutation (Schmidt & Schimmel, 1994)] has a particularly high K_m for isoleucine which renders its activity undetectable under normal assay conditions (Iaccarino & Berg, 1971).

We found that fragment 886N had no detectable aminoacylation activity (Figure 3A). In contrast, deletion of 53C from IleRS does not diminish the capacity of the enzyme to synthesize isoleucyl adenylate, as measured by the isoleucine-dependent ATP-pyrophosphate exchange activity (Figure 3B). Kinetic constants for isoleucine and ATP shown in

Table 1: Kinetic Parameters of Amino Acid Activation by Wild-Type IleRS and the N-Terminal Fragment 886N at pH 7.5 and 37 °C

enzyme	k_{cat} (s^{-1})[a]	K_m(Ile) (μM)	K_m(ATP) (mM)
WT	80.4	3.6	0.28
886N	81.3	2.5	0.25

[a] The k_{cat} values are the averaged values determined for K_m(Ile) (with 3 mM ATP) and for K_m(ATP) (with 500 μM Ile).

Table 1 are essentially the same for 886N as for the full-length enzyme. These results suggest that peptide-53C contributes specificity to the tRNA-dependent step of aminoacylation.

Reconstitution of 886N with 53C. Experiments performed *in vivo* have shown previously that, while the N-terminal fragment of IleRS cannot complement the *ileS* null strain, coexpression of 53C as a separate peptide rescued viability (Shiba & Schimmel, 1992). Here, the analogous experiment was done *in vitro*. The Zn^{2+}-53C was prepared and added in excess to fragment 886N. As expected, the peptide had little effect on the adenylate synthesis activity when added to 886N (Figure 3B). The fragment has at least as much activity as wild-type enzyme, and the slight increase in activity noted upon addition of the peptide might arise from an increase in the stability of 886N. In contrast, addition of Zn^{2+}-53C to 886N had a dramatic effect on the aminoacylation activity (Figure 3A). Nearly 70% of native IleRS aminoacylation activity was recovered. This result confirms that peptide 53C was specifically needed for a tRNA interaction.

To investigate the significance of zinc bound to 53C, the apo-53C was also added to 886N. To avoid incorporation of adventitious metals by the peptide, the sample was made 1 mM in 2-mercaptoethanol and 1 mM in EDTA prior to addition of 886N. This treatment had no effect on the adenylate synthesis activity of fragment 886N. In addition, the apo-peptide was unable to restore any aminoacylation activity (Figure 3A). This result suggests that the zinc bound to 53C is necessary to maintain the structure of the C-terminal peptide needed for a tRNA-dependent reaction.

Affinity Coelectrophoresis. In order to determine whether the lack of aminoacylation activity by 886N is derived from a reduced affinity for $tRNA^{Ile}$, the dissociation constant of the 886N−$tRNA^{Ile}$ complex was analyzed using affinity coelectrophoresis (ACE). Previous studies showed that this method is suitable for measuring dissociation constants for protein−RNA interactions of up to about 250 μM (Kim et al., 1993a). With this method, labeled RNA is electrophoresed vertically through a horizontal concentration gradient of protein. Bound RNA is detected as a shift relative to free RNA. Because of the protein concentration gradient, the shift takes the form of a titration curve from which a K_d can be determined (Gale & Schimmel, 1995).

Analysis of $tRNA^{Ile}$ with full-length enzyme indicated a readily detected complex with a K_d of 14 nM (Figure 4A). In contrast, binding of $tRNA^{Ile}$ by fragment 886N could not be detected. Increasing concentrations of 886N did not produce a shift in the migration of the tRNA. Instead, only a smearing of the tRNA band was observed (Figure 4B). From this result, we estimate that the dissociation constant for tRNA is increased by at least 100-fold upon deletion of 53C.

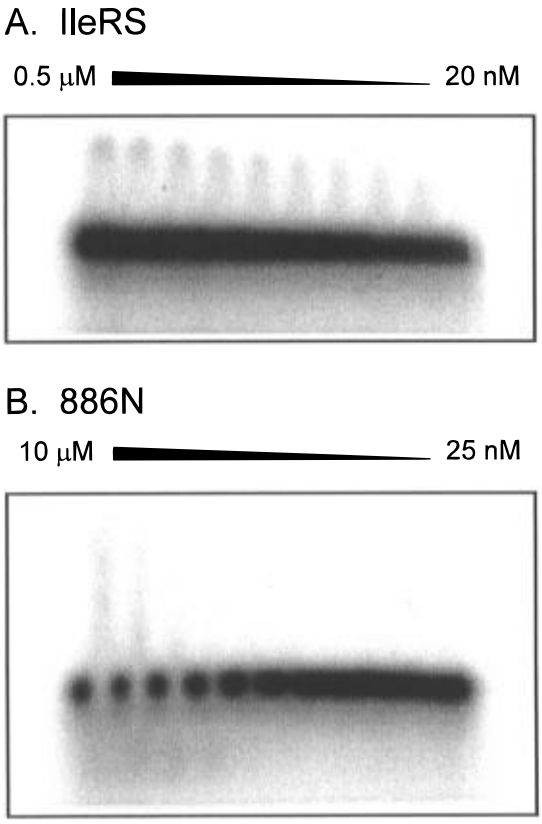

FIGURE 4: Affinity coelectrophoresis determination of the dissociation constant of $tRNA^{Ile}$ with (A) wild-type IleRS (over a concentration range of 20−500 nM) and (B) 886N (over a concentration range of 25 nM−10 μM). The concentration-dependent shift of the tRNA is apparent in (A). Both gels were run at pH 7.5, 25 °C.

DISCUSSION

We did not anticipate that deletion of the C-terminal zinc-containing peptide of isoleucyl-tRNA synthetase would result in substantial loss of tRNA binding. Major interactions between isoleucyl-tRNA synthetase and its cognate tRNA occur through association with the anticodon triplet, and these interactions are believed not to involve the C-terminal end of the protein (Leon & Schulman, 1987; Meinnel et al., 1990, 1991; Schulman, 1991; Kim & Schimmel, 1992; Kim et al., 1993b). The critical locus for the anticodon interactions is centered around K732 of isoleucyl-tRNA synthetase (Shepard et al., 1992). Although a K732R mutant is sufficiently active to complement a null allele of *ileS*, any of 15 other replacements which were tested resulted either in failure to complement or, at best, in weak complementation. Further analysis *in vitro* of the weakly complementing K732T mutant enzyme showed that the K_m for $tRNA^{Ile}$ was elevated over 200-fold. In addition, anticodon recognition specificity can be manipulated by exploitation of the K732 region of isoleucyl-tRNA synthetase and its counterpart in methionyl-tRNA synthetase (Auld & Schimmel, 1995).

The crystal structure of methionyl-tRNA synthetase has provided a working model for the structure of the isoleucine enzyme due to the close relationship between the two enzymes (Starzyk et al., 1987). In the methionyl-tRNA synthetase structure, W461 is thought to be in the location analogous to K732 of isoleucyl-tRNA synthetase (Shepard et al., 1992; Auld & Schimmel, 1995). [The numbering of amino acids in the two proteins does not correspond because

the isoleucine polypeptide is larger, in part because of the greater size of CP1 and in part because it also has an extended N-terminal region not found in methionyl-tRNA synthetase (Starzyk et al., 1987).] The C-terminal end of an active fragment of methionyl-tRNA synthetase is far removed (55 Å) from the location of W461 and the anticodon binding site (Brunie et al., 1990). Thus, we imagined that deletion of the C-terminal end of the related isoleucyl-tRNA synthetase would not affect anticodon interactions and that these interactions, together with those between CP1 and the acceptor stem, would be sufficient to allow binding of $tRNA^{Ile}$ in the affinity coelectrophoresis assay shown in Figure 4.

A tightly bound zinc occurs in the CP1 domain of *E. coli* glutamyl-tRNA synthetase (Liu et al., 1995) and *T. thermophilus* and *E. coli* methionyl-tRNA synthetases (Nureki et al., 1991; Fourmy et al., 1993; Landro & Schimmel, 1993). In glutamyl-tRNA synthetase, binding of tRNA is maintained upon removal of zinc, even though aminoacylation activity is lost (Liu et al., 1993). Site-directed mutagenesis of individual zinc-coordinating cysteines of methionyl-tRNA synthetase also leads to a decrease or complete loss of activity (Nureki et al., 1991; Fourmy et al., 1993; Landro & Schimmel, 1993). As with glutamyl-tRNA synthetase, however, fluorescence titration experiments with *E. coli* methionyl-tRNA synthetase demonstrated that tRNA binding is not affected (Fourmy et al., 1993). In the case of isoleucyl-tRNA synthetase, a zinc bound to the CP1 domain appears important for the formation of the isoleucine binding pocket (Landro et al., 1994). Thus, among the zinc-containing class I enzymes investigated so far, *E. coli* isoleucyl-tRNA synthetase is the only one for which a role for zinc in tRNA binding is clearly demonstrated.

By analogy with methionyl-tRNA synthetase, we imagine that the C-terminal peptide of isoleucyl-tRNA synthetase curls back to the active site. In the monomeric fragment of methionyl-tRNA synthetase, the C-terminal end is essential for adenylate synthesis and also provides important contacts with the acceptor domain of $tRNA^{Met}$ (Mellot et al., 1989; Kim et al., 1993a). In contrast, the C-terminal zinc-containing peptide of isoleucyl-tRNA synthetase is dispensable for adenylate synthesis and is only needed for the tRNA-dependent step of aminoacylation (Figure 3). The loss of detectable $tRNA^{Ile}$ binding upon deletion of the zinc-containing peptide (Figure 4) suggests that a major part of the synthetase-$tRNA^{Ile}$ interaction free energy is with the acceptor domain through a zinc−peptide-mediated interaction.

This conclusion is consistent with results of Nureki et al., who showed that an RNA microhelix composed of the acceptor stem of $tRNA^{Ile}$ could be aminoacylated with a K_m comparable to that of the full tRNA, thus also suggesting a major role for acceptor helix interactions (Nureki et al., 1994). Aminoacylation of the microhelix and of $tRNA^{Ile}$ is sensitive to the A73 "discriminator" base and the C4:G69 base pair. The zinc-containing peptide investigated here may be important for contacts with some or all of these particular bases in $tRNA^{Ile}$.

The C-terminal Cys_4 motif is present in other prokaryote and *S. cerevisiae* mitochondrial isoleucyl-tRNA synthetases (Figure 1), and we would expect that it plays the same role in these enzymes as in *E. coli* isoleucyl-tRNA synthetase. However, the C-terminal Cys_4 motif is absent in eukaryote cytoplasmic isoleucyl-tRNA synthetases such as the human (Shiba et al., 1994) and yeast (Englisch et al., 1987) enzymes. In the latter case, a C-terminal deletion of as little as 10 amino acids disrupts aminoacylation activity without an effect on adenylate synthesis (Cramer et al., 1991). This result raises the possibility that the C-terminal ends of the eukaryote proteins are also needed for tRNA binding and that this binding does not require zinc. If so, then the metal in the prokaryote enzymes may be solely for stabilizing a particular RNA binding structure rather than for a direct contact with the bound nucleic acid.

It is possible that the eukaryote sequence is able to form the same structure, but without the need for a metal ion. In a similar vein, the zinc-containing CP1 region of *E. coli* glutamyl-tRNA synthetase makes an interesting comparison with that of the *T. thermophilus* glutamate enzyme which lacks a zinc binding site (Liu et al., 1995; Nureki et al., 1995). This comparison suggests that similar structures are formed in both instances, thus providing an example of how sequence variations can bypass the need for a bound metal.

The results presented here emphasize the seemingly idiosyncratic way in which acceptor helix RNA binding elements are joined to tRNA synthetases. However, the apparent idiosyncratic variability may obscure a common theme which has been superficially altered in specific instances. The acceptor helix-containing domain was probably the earliest part of an emerging system of aminoacylation (Weiner & Maizels, 1987; Maizels & Weiner, 1993; Noller, 1993) whereby specific RNA structures/sequences constituted an operational RNA code for amino acids (Schimmel et al., 1993). The earliest synthetases are thought to have consisted of the class-defining catalytic unit for adenylate synthesis. This unit was interrupted with novel sequences which enable docking of an acceptor stem helix adjacent to the activated amino acid. The CP1 insertion of class I enzymes is well positioned in the structure for docking the acceptor helix into the active site, as it divides the nucleotide binding fold into two halves. Possibly, during the events in evolution that added the anticodon-containing domain of the tRNA and the corresponding second domain of the synthetase, portions of CP1 were translocated to the C-terminus of the newly added domain (Kim et al., 1993a). This translocation would segregate more of the tRNA binding elements to the C-terminal domain. This segregation may have been necessitated by the development of an additional role for CP1 in isoleucyl-tRNA synthetase. In particular, this enzyme has a potent tRNA-dependent editing activity, and recent experiments suggest that a portion of CP1 is important for that activity (Schmidt & Schimmel, 1995).

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BI9527810