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Evidence for a "Cysteine-Histidine Box" Metal-Binding Site in an *Escherichia coli* Aminoacyl-tRNA Synthetase†

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ABSTRACT: *Escherichia coli* alanyl-tRNA synthetase contains the sequence Cys-X_2-Cys-X_6-His-X_2-His. This motif is distinct from the zinc fingers of DNA-binding proteins but has some similarity to the Cys-X_2-Cys-X_4-His-X_4-Cys zinc-binding motif of retroviral *gag* proteins, where it has a role in RNA packaging. In Ala-tRNA synthetase, this sequence is located in an amino-terminal domain which has the site for docking the acceptor end of the tRNA near the bound aminoacyl adenylate and is immediately adjacent in the sequence to the location of a mutation that affects the specificity of tRNA recognition. We show here that Ala-tRNA synthetase contains approximately 1 mol of zinc/mol of polypeptide and that addition of the zinc chelator 1,10-phenanthroline inhibits its aminoacylation activity. Conservative mutations of specific cysteine or histidine residues in the "Cys-His box" destabilize and inactivate the enzyme, whereas mutations of intervening amino acids do not inactivate. The possibility that this motif can bind zinc (or cobalt) was demonstrated with a synthetic 22 amino acid peptide that is based on the sequence of the alanine enzyme. The peptide–cobalt complex has the spectral characteristics of tetrahedral coordination geometry. The results establish that the Cys-His box motif of Ala-tRNA synthetase has the potential to form a specific complex with zinc (at least in the context of a synthetic peptide analogue) and suggest that this motif is important for enzyme stability/activity.

Although a wide variety of cellular and viral proteins interact with RNA, relatively few protein sequence motifs for RNA binding have been identified. An "RNP consensus" sequence has been implicated in RNA binding in many nuclear and cytoplasmic proteins which bind to mRNA, pre-mRNA, snRNA, and pre-RNA (Mattaj, 1989; Bandziulis et al., 1989). A crystal structure of the RNA-binding domain of U1 snRNP A shows that these RNP consensus sequences are contained within a four-stranded β-sheet at the presumed RNA-binding site (Nagai et al., 1990). The *gag* proteins of retroviruses are required for viral RNA packaging, and a three-dimensional structure of a member of this family has been solved (Summers et al., 1990). However, it is not known how these proteins interact with RNA. In contrast, several well-characterized DNA-binding motifs have been identified (e.g. the "leucine zipper," "helix–turn–helix", "helix–loop–helix", and "zinc finger"); three-dimensional structures of these motifs have been solved, and DNA recognition has been characterized at the molecular level in many cases (Struhl, 1989).

The most detailed information about protein–RNA recognition comes from the recently solved structure of the cocrystal of *Escherichia coli* glutaminyl-tRNA synthetase and $tRNA^{Gln}$ (Rould et al., 1989). Sequence-specific contacts with the acceptor stem of $tRNA^{Gln}$ are made by a domain that is inserted between the first and second halves of a dinucleotide (Rossmann) fold. This domain contains 110 amino acid residues and is composed of a five-stranded antiparallel β-sheet flanked by three α-helices. The structure of $tRNA^{Gln}$ is altered upon binding to this domain of Gln-tRNA synthetase; the terminal base pair is broken and the 3′-end of the tRNA is folded back on the rest of the structure.

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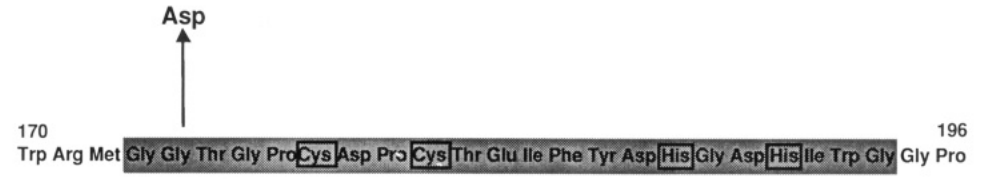

FIGURE 1: Amino acid sequence of *E. coli* Ala-tRNA synthetase in the region of the Cys-His box. The Gly174 → Asp mutation (Miller et al., 1991) which is sufficient for complementation of G3·C70 $tRNA^{Ala}$ is indicated. The shaded portion of the sequence represents the sequence of the synthetic peptide described in the text.

A series of investigations have attempted to identify the RNA-binding determinants in *E. coli* Ala-tRNA synthetase, a tetramer composed of four identical 875 amino acid subunits (Putney et al., 1981a,b). The enzyme catalyzes the attachment of alanine of $tRNA^{Ala}$ in a two-step process: in the first reaction alanine is activated with ATP to form an enzyme-bound alanyl adenylate, and in the second reaction the alanyl moiety is transferred to the 3′-end of $tRNA^{Ala}$. Gene deletion analysis has identified domains of the protein which carry out different catalytic functions (Jasin et al., 1984). An N-terminal 368 amino acid domain is sufficient to catalyze alanyl adenylate formation, and cross-linking experiments with periodate-oxidized $tRNA^{Ala}$ have identified a site within this domain (Lys73) which binds to the 3′-end of the tRNA (Hill & Schimmel, 1989). Also, the portion of the polypeptide between Arg368 and Ser385 has a role in tRNA binding (Regan et al., 1987).

Within the N-terminal domain is a cysteine- and histidine-containing motif first identified by Berg (1986) as a potential metal-binding site. The pattern of Cys and His residues in this region of Ala-tRNA synthetase (Figure 1) is reminiscent of retroviral nucleic acid binding proteins that have the sequence Cys-X_2-Cys-X_4-His-X_4-Cys. On the basis of absorption spectroscopy of Co(II)–synthetic peptides and Co(II)-substituted proteins (Green & Berg, 1989; Roberts et al., 1989), this motif has been shown to bind Zn(II). Mutagenesis demonstrated that these sequences are important for the correct packaging of viral RNA. Substitutions of the cysteine or histidine residues typically give inactive viruses (Jentoft et al., 1988; Gorelick et al, 1988). Also, Cys → Ser substitutions in the CCHC box of Moloney murine leukemia virus cause association with cellular RNA instead of viral RNA, suggesting that this motif is involved in specific RNA sequence recognition [Gorelick et al., 1988; Mêric & Goff, 1989; also cf. Shamoo et al. (1991)].

The three-dimensional structure of the HIV *gag* protein p55 has been solved by NMR techniques and shown to have zinc coordinated to three cysteines and one histidine (Summers et al., 1990). The p55 protein forms a highly compact, thermostable structure, with seven internal hydrogen bonds within the 14-residue zinc-binding domain. The structure is significantly different from the structure of zinc finger containing DNA-binding proteins, which typically have the sequence Cys-X_2-Cys-X_{12-14}-His-X_2-His. In particular, the polypeptide backbones of zinc fingers contain helices packed against two β-strands (Lee et al., 1989); in retroviral nucleic acid binding proteins such as p55, the short X_4 spacer between the second and third metal-binding residues prevents this folding pattern.

The importance of the Cys-His region in *E. coli* Ala-tRNA synthetase is suggested by the recent isolation of a mutant that has altered tRNA recognition (Miller et al., 1991). A single G3·U70 base pair in the acceptor helix of $tRNA^{Ala}$ is the major determinant for alanine identity in this system (Hou & Schimmel, 1988). Transfer of the G3·U70 base pair into other tRNAs confers the ability to be charged with alanine. Mutation of G3·U70 to G3·C70 in $tRNA^{Ala}$ abolishes recognition by wild-type Ala-tRNA synthetase in vivo and in vitro (Hou & Schimmel, 1988; Park et al., 1989). The mutant enzyme compensates for a G3·U70 mutation in $tRNA^{Ala}$, suggesting that the mutation in the protein is in a region important for tRNA recognition. A Gly174 → Asp substitution immediately proximal to the amino-terminal end of the "Cys-His box" (Figure 1) is responsible for the phenotype. Because this part of the protein is also in the same adenylate synthesis domain as the site (Lys73) for cross-linking to the 3′-end of $tRNA^{Ala}$ (Hill & Schimmel, 1989), it may be analogous to (although structurally distinct from) the aforementioned insertion in the dinucleotide fold of Gln-tRNA synthetase that binds to the acceptor helix. The experiments described here were designed to explore further the significance of this part of Ala-tRNA synthetase for structure–activity relationships and for potentially binding a metal ion.

Materials and Methods

General. Restriction enzymes and T4 DNA ligase were from New England Biolabs or Boehringer Mannheim and were used according to the instructions from the supplier. Purified $tRNA^{Ala}$ for enzyme assays was purchased from Subriden. Cobalt dichloride hydrate (99.999%) and zinc dichloride (99.999%) were obtained from Aldrich. Bovine erythrocyte carbonic anhydrase II and yeast alcohol dehydrogenase were obtained from Sigma, and bovine pancreatic ribonuclease was from Boehringer Mannheim.

Atomic Absorption Spectroscopy. These experiments were carried out at the Massachusetts Institute of Technology Central Analytical Facility using a Perkin Elmer 703 spectrometer and at Loma Linda University using an Instrumentation Laboratory 251 spectrometer. Adventitious metal ions were removed from buffers by extraction with 0.01% dithizone in carbon tetrachloride (Holmquist, 1988). All glassware employed was first soaked in 30% nitric acid and then rinsed extensively with metal-free water. Prior to analysis, the samples were either dialyzed extensively at 4 °C in metal-free dialysis tubing (Auld, 1988) against 10 mM HEPES, pH 7.4, or passed over a Chelex 100 (Bio-Rad) column (0.8 × 2.5 cm). The synthetase concentration was typically between 5 and 10 μM during this procedure (determined by Bio-Rad protein assay).

Enzyme Assays. The aminoacylation activity of *E. coli* Ala-tRNA synthetase toward purified $tRNA^{Ala}$ was assayed according to methods described by Schreier and Schimmel (1972). Enzyme assays were performed in 40 mM sodium phosphate (pH 7.5) at 37 °C and typically contained 4.0 mM ATP and 23.0 μM [^{3}H]alanine (5.0 mCi/μmol; from New England Nuclear). In the 1,10-phenanthroline inhibition studies, the enzyme assays included concentrations of 1,10-phenanthroline (or 1,7-phenanthroline) between 0.125 and 8.0 mM in the reaction mixture.

Site-Directed Mutagenesis. Site directed mutants of Ala-tRNA synthetase were constructed by using the Amersham oligonucleotide-directed mutagenesis system. The *alaS* gene (encoding wild-type *E. coli* Ala-tRNA synthetase) was cloned

into the *Eco*RI site of phage M13mp18, as described previously (Miller et al., 1991). Mutagenic oligonucleotides complementary to the coding strand of *alaS* and containing base mismatches were synthesized on an Applied Biosystems Model 380B DNA synthesizer. Mutants were screened by DNA sequencing using Sequenase (US Biochemical Co.) and were subcloned into the *Eco*RI site of plasmid pUC19 for transformation into the *alaS* null strain.

ΔalaS Null Strain. Plasmids derived from pUC19 which encoded the mutant enzymes were transformed into the *E. coli alaS* null strain W3110 *alaSΔ2*/pMJ901 (Jasin et al., 1984). This strain has a deletion of the chromosomal copy of *alaS*. Growth at 30 °C is maintained by plasmid pMJ901, which encodes the full-length Ala-tRNA synthetase and has a temperature-sensitive origin of replication. Plasmid pMJ901 is inactivated when W3110 *alaSΔ2*/pMJ901 cells are exposed to a temperature of 42 °C. Transformants encoding mutant enzymes were selected at 30 °C and the restreaked at 42 °C to test for complementation of the growth-defective phenotype.

Western blot analysis was carried out in the null strain background W3110 *alaSΔ2*/pLR461. These cells are maintained by plasmid pLR461, which encodes an active 461 amino acid N-terminal fragment of *E. coli* Ala-tRNA synthetase (Regan et al., 1987). Plasmids encoding the mutant synthetases were transformed into this strain. Cultures of these cells were grown at 30 °C, and cell extracts were made from late log phase cells using a French pressure cell press (SLM Instruments, Inc.). Aliquots of the cell extracts were electrophoresed on a 7.5% SDS–polyacrylamide gel. The bands on the gel were transferred to a poly(vinylidene difluoride) membrane by using a Millipore OM-163 electroblotting system and allowed to react with anti-*E. coli* Ala-tRNA synthetase antibodies. These bands were visualized with the ProtoBlot alkaline phosphatase system (Promega).

Peptide Synthesis and Characterization. The 22 amino acid peptide corresponding to Gly173–Gly194 of *E. coli* Ala-tRNA synthetase was synthesized in the Biopolymers Laboratory at MIT by using an Applied Biosystems Model 430A peptide synthesizer. After cleavage and extraction from the solid-phase resin, the peptide was purified on a C4 HPLC column. The crude peptide was loaded onto the column in 0.1% trifluoroacetic acid and eluted with a linear gradient to 70% acetonitrile in 0.1% trifluoroacetic acid. The major peak was collected, concentrated in vacuo, reduced by treatment with dithiothreitol, and rechromatographed on the C4 column. Amino acid analysis of the reduced and purified material was consistent with the expected amino acid composition. Peptide concentration was determined by measuring tryptophan absorbance at 280 nm (ϵ = 5600 M^{-1} cm^{-1}; Edelhoch, 1967). Free sulfhydryl concentration was measured by titration with 5,5′-dithiobis(2-nitrobenzoic acid), using a value of $\epsilon_{412} = 1.36 \times 10^4$ M^{-1} cm^{-1} for the 2-nitro-5-thiobenzoate anion (Ellman, 1959).

Metal-Binding Studies. Co(II) and Zn(II) binding by the synthetic peptide was measured in 50 mM Tris-HCl (pH 7.6) at 25 °C. Care was taken to minimize the exposure of peptide solutions to air. Absorption spectroscopy and spectral subtractions were performed with a Beckman DU-64 spectrophotometer. Co(II) titration was carried out by adding aliquots of 1.0, 10.0, or 100 mM $CoCl_2$ in the same buffer, and in Zn(II) competition experiments the peptide was preincubated with 1.0 equiv of $ZnCl_2$. The dissociation constant of the peptide–Co(II) complex was determined from the spectrophotometric data by least-squares fit to theoretical binding curves using Microsoft Excel and a Macintosh IIci computer.

Table I: Atomic Absorption Analysis for Zinc

protein	mol of Zn(II)/mol of subunit
ribonuclease A[a]	0.39[b]
	<0.05[c]
carbonic anhydrase II[d]	0.73[e]
alcohol dehydrogenase[f]	1.5[e]
Ala-tRNA synthetase[g]	0.64[e]
	0.44[e]
	0.76[h]

[a] From bovine pancreas; enzyme concentrations of 5.3 and 5.9 μM were used. [b] Upon addition of 3.0 μM Zn(II). [c] Upon addition of 3.0 μM Zn(II) and then passage over Chelex 100. [d] From bovine erythrocytes; known to bind 1 mol of Zn(II)/mol of subunit (Tashian et al., 1980). An enzyme concentration of 7.4 μM was used. [e] Following passage over Chelex 100. [f] From yeast; presumed to bind 2 mol of Zn(II)/mol of subunit (Vallee & Auld, 1990). Enzyme concentrations of 4.6 and 5.1 μM were used. [g] From *E. coli*; values from three different protein preparations are presented. Enzyme concentrations of 0.5–5.3 μM protein (per monomer) were used. [h] Following dialysis.

Fluorescence experiments were carried out in a Perkin-Elmer LS 50 luminescence spectrometer with an excitation wavelength of 281 nm and an emission wavelength of 357 nm. The peptide concentration used in the fluorescence studies was 2.8 μM, and Co(II) concentrations of 0.2–200 μM were used for fluorescence quenching.

Results

Atomic Absorption Spectroscopy of Ala-tRNA Synthetase. In order to test whether zinc was present in the highly purified enzyme, three preparations were subjected to atomic absorption analysis for determination of their Zn(II) content. Ala-tRNA synthetase was found to contain 0.44–0.76 mol of tightly bound Zn(II)/mol of polypeptide (Table I). As controls, bovine carbonic anhydrase and yeast alcohol dehydrogenase had 0.7 and 1.5 mol of Zn(II)/mol of polypeptide, versus expected values of 1.0 and 2.0 mol of Zn(II)/mol of polypeptide, respectively. Zinc is firmly complexed to the alanine enzyme because some remains bound even after dialysis or passage of the protein over a Chelex 100 column. A control experiment with ribonuclease A demonstrated that passage over Chelex 100 was effective in the removal of free Zn(II); after such treatment of RNase A, the Zn(II) content was shown to be less than 0.05 mol/mol of protein.

Inhibition of Activity with 1,10-Phenanthroline. Chelating agents such as 1,10-phenanthroline can inhibit zinc metalloenzymes by sequestering the metal from the protein (Vallee & Galdes, 1984). We tested whether 1,10-phenanthroline would inhibit aminoacylation activity of *E. coli* Ala-tRNA synthetase. The enzyme (12 nM tetramer) was assayed at 37 °C in the presence of varying concentrations of 1,10-phenanthroline or of the nonchelating regioisomer 1,7-phenanthroline. The 1,10-phenanthroline inhibits the enzyme in a concentration-dependent manner (Figure 2). Inhibition is largely attributable to the metal-chelating properties of the reagent, because the enzyme sample treated with 1,7-phenanthroline showed only a modest loss of activity, particularly at concentrations less than 2 mM. Inhibition with 1,10-phenanthroline was less than complete, possibly due to difficulty in complexing the tightly bound zinc ion. [Extended dialysis of the enzyme versus 1,10-phenanthroline results in complete inactivation (data not shown).] The chelating agent may not remove Zn(II) from the enzyme but instead may form a ternary complex analogous to the phenanthroline complex with Zn(II)-containing liver alcohol dehydrogenase (Vallee & Coombs, 1959). This might occur if one of the ligands to zinc is H_2O which is displaced by phenanthroline (see discussion below). When the data of Figure 2 are replotted as

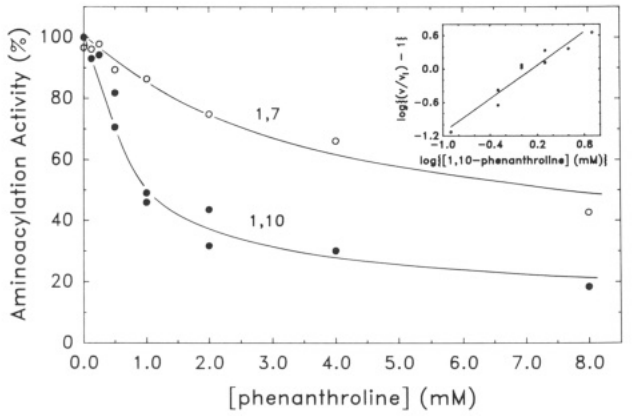

FIGURE 2: Inhibition of Ala-tRNA synthetase by 1,10-phenanthroline. Assays were performed at pH 7.5 (37 °C) as described in the text. (Inset) Linear replot of the data in the form log $[(v/v_i) - 1]$ vs log [1,10-phenanthroline], where v and v_i are reaction velocities in the absence and presence of 1,10-phenanthroline, respectively.

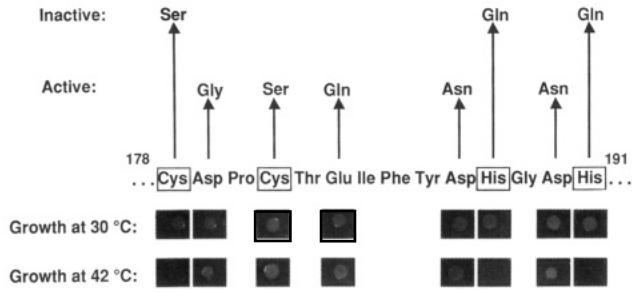

FIGURE 3: Site-directed mutagenesis of the Cys-His box. Amino acid substitutions that were introduced individually are indicated above the sequence. The mutant proteins were judged to be active or inactive on the basis of their ability to complement the growth-defective phenotype of the *alaS* deletion strain W3110 *alaSΔ2*/pMJ901. Growth of these cells at 30 °C and at 42 °C is shown below the amino acid sequence.

log $[(v/v_i) - 1]$ vs log [1,10-phenanthroline], where v and v_i are reaction velocities in the absence and presence of 1,10-phenanthroline, respectively, a straight line with slope = 1.0 results (Figure 2, inset). From this plot the apparent K_I for 1,10-phenanthroline is estimated to be approximately 0.7 mM.

Site-Directed Mutagenesis of the Cys-His Box Region. The functional importance of the region containing the putative metal-binding motif was investigated by site-directed mutagenesis (Figure 3). Eight separate amino acid substitutions were introduced into the protein via oligonucleotide-directed mutagenesis techniques so as to replace all of the amino acids in the region whose side chains were predicted to be potential ligands for Zn(II) [Cys, His, Asp, and Glu (Vallee & Auld, 1990)]. After construction of the mutants in phage M13mp18, the mutated *alaS* sequences were subcloned into plasmid pUC19 and their phenotypes were evaluated in the *E. coli alaS* deletion strain W3110 *alaSΔ2*/pMJ901 (Jasin et al., 1984). This strain is maintained by plasmid pMJ901, which encodes *alaS* and has a temperature-sensitive replicon; the plasmid is lost upon incubation at 42 °C. Thus, complementation of the growth defect at 42 °C indicates the presence of active (mutant) Ala-tRNA synthetase.

Five of the eight mutants, Cys181Ser, Asp179Gly, Glu183Gln, Asp187Asn, and Asp190Asn, complement the *alaS* null strain and show no indication of toxicity or slower cell growth than observed for the wild-type enzyme (Figure 3). In contrast, the Cys178Ser, His188Gln, and His191Gln mutants were unable to complement the null strain, indicating that each of these amino acids has an important functional and/or structural role.

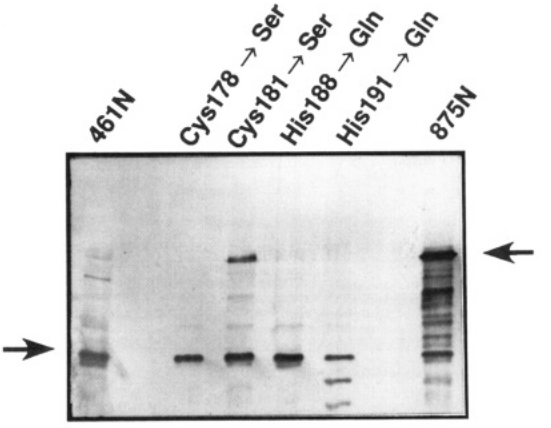

FIGURE 4: Western blot analysis of mutant proteins. Extracts of W3110 *alaSΔ2*/pLR461 cells containing the appropriate mutants were made as described previously (Hill & Schimmel, 1989), and portions were electrophoresed on a 7.5% SDS–polyacrylamide gel. The bands were electroblotted onto a poly(vinylidene difluoride) membrane and immunostained with anti-*E. coli* Ala-tRNA synthetase antibodies. Lane 1: W3110 *alaSΔ2*/pLR461, showing the position of the 461 amino acid N-terminal fragment. Lanes 2–5: The same cells harboring plasmids encoding the Cys178 → Ser mutant (lane 2), the Cys181 → Ser mutant (lane 3), the His188 → Gln mutant (lane 4), and the His191 → Gln mutant (lane 5). Lane 6: An extract of W3110 *alaSΔ2*/pLR461 containing a pUC19-derived plasmid encoding wild-type Ala-tRNA synthetase. The position of full-length Ala-tRNA synthetase (875 amino acids) is indicated with an arrow.

Western Blot Analysis of Mutant Proteins. Plasmids encoding four of the mutant enzymes, Cys178Ser, Cys181Ser, His188Gln, and His191Gln, were introduced into the strain W3110 *alaSΔ2*/pLR461. In this background, the *alaS* deletion strain is maintained by plasmid pLR461, which encodes the active 461 amino acid N-terminal fragment of Ala-tRNA synthetase (Regan et al., 1987). This protein is easily distinguished from the full-length 875 amino acid protein on SDS–polyacrylamide gels. Extracts of the transformants were made from 200 mL of late log phase cells grown at 30 °C. Aliquots of the extracts were analyzed by SDS–polyacrylamide gel electrophoresis. The proteins in the gel were transferred to a poly(vinylidene difluoride) membrane with an electoblotting apparatus, and the membrane was immunostained with anti-*E. coli* Ala-tRNA synthetase antibodies. These experiments showed that the three inactive enzymes, Cys178Ser, His188Gln, and His191Gln, are unstable, and little or no full-length protein accumulates in these cells (Figure 4). In contrast, the plasmid-encoded Cys181Ser mutant *alaS* directs synthesis of a stable protein of the expected molecular weight (Figure 4).

Metal-Binding Properties of the Synthetic Peptide. To investigate whether the Cys-His motif in Ala-tRNA synthetase has in principle the capacity to bind zinc, a 22 amino acid synthetic peptide based on the sequence of the Cys-His box region was prepared by using solid-phase methodology (see Figure 1). Solutions of the peptide were sensitive to air oxidation of the thiols; consequently, freshly prepared solutions were used for metal-binding studies. Prior to use, the sulfhydryl content of these peptide solutions was measured by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) and 2.0 mol of thiol/mol of peptide was found (data not shown).

Because the coordination of Co(II) is similar to that of Zn(II), and because of the well-characterized spectral properties of Co(II) complexes (Vallee & Galdes, 1984; Bertini & Luchinat, 1984), the reduced peptide was treated with Co(II) and metal binding was assayed by UV/visible absorption spectroscopy. Addition of Co(II) to the peptide in-

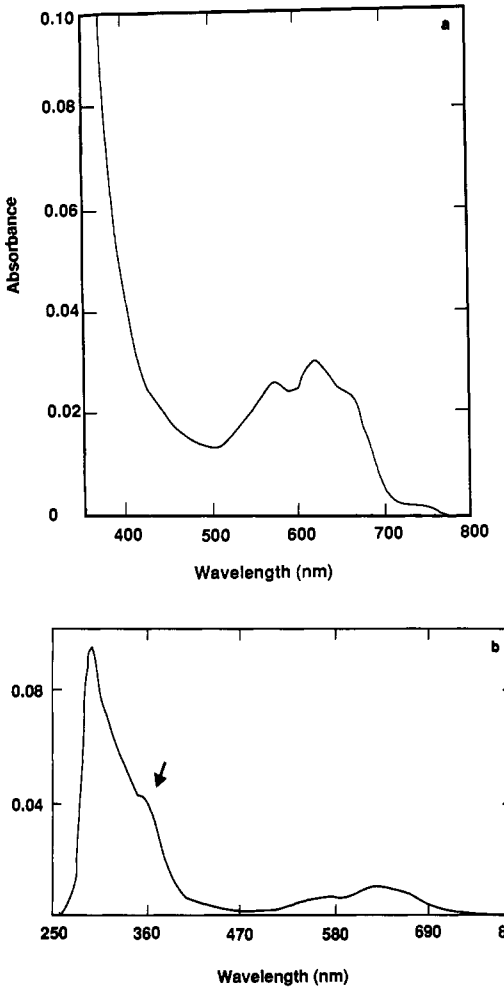

FIGURE 5: (a) Visible absorption spectrum of the Co(II)–synthetic peptide complex. A solution of the peptide (2.3×10^{-4} M) in 50 mM Tris-HCl (pH 7.6) was treated with 2.3×10^{-4} M $CoCl_2$ at 25 °C. The absorption spectrum was recorded and corrected by subtracting out the absorption spectrum of peptide alone. No additional increase in absorption was observed when the concentration of $CoCl_2$ was increased further. (b) UV/visible absorption spectrum of the Co(II)–synthetic peptide complex. A solution of 8.0×10^{-5} M peptide was treated with 1.0×10^{-4} $CoCl_2$ in 50 mM Tris-HCl (pH 7.6), and the spectrum was recorded. The absorption due to peptide alone was subtracted from this spectrum, as in (a). No increase in absorption resulted upon further addition of $CoCl_2$. The arrow at ≈360 nm indicates the presumed Cys → Co(II) charge-transfer band.

duced absorbances at 295 nm (ϵ = 1190 M^{-1} cm^{-1}), 360 nm (shoulder), and 620 nm (ϵ = 130 M^{-1} cm^{-1}), as shown in Figure 5. These extinction coefficients are comparable to those of previously studied Co(II) proteins (Vallee & Galdes, 1984). Although a pentacoordinate cobalt site cannot be excluded by these data, the shape of the absorption spectrum in the range 500–700 nm resembles the d–d transition bands of other Co(II) proteins and is characteristic of tetrahedral cobalt complexes (Figure 5a; Vallee & Galdes, 1984). The shoulder present in the absorption spectrum at ≈360 nm probably represents cysteine → Co(II) charge transfer (Vallee & Galdes, 1984). We attribute the signal at 295 nm to a change in the environment around Trp193 upon Co(II) binding that shifts λ_{max} from 280 nm (Figure 5b). Additionally or alternatively, this signal may represent a Cys → Co(II) charge-transfer band. No additional increase in absorption was observed when the concentration of Co(II) was raised above a 1:1 molar ratio with peptide. The absorbances of the peptide–Co(II) complex are eliminated by the addition of 1 equiv of $ZnCl_2$/mol of peptide, suggesting a preference of

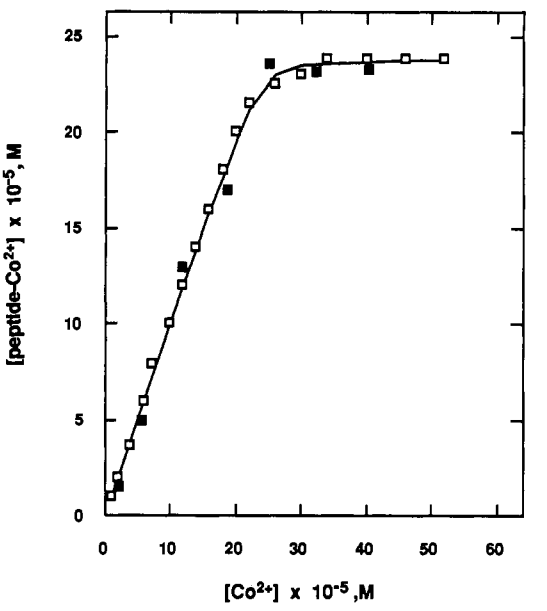

FIGURE 6: Titration of the synthetic Cys-His box peptide with Co(II). A solution of peptide (2.3×10^{-4} M in 50 mM Tris-HCl, pH 7.6) was treated with $CoCl_2$, and the absorbances at 295 and 620 nm were measured. Absorbance readings were corrected for the absorbance due to free peptide and for dilution. The absorbance data have been fit to a curve with dissociation constant = 1.0 μM, as described in the text. Open symbols: Absorbance readings at 295 nm. Closed symbols: Absorbance readings at 620 nm.

Zn(II) in the metal-binding site (data not shown).

A solution of 2.3×10^{-4} M peptide was titrated with Co(II), and absorption readings at 295 and 620 nm were recorded (Figure 6). By use of a least-squares procedure, these spectrophotometric data were best fit to a curve with dissociation constant = 1.0 μM, although values lower than 1.0 μM also gave reasonable fits to the data. Because the peptide concentration was greater than the dissociation constant, there was little free Co(II) throughout the titration so that the K_d could not be accurately determined. However, theoretical curves with progressively higher dissociation constants gave progressively larger least-squares differences between observed and calculated points in the titration. By comparison, a peptide based on the sequence of the *gag* protein of Rauscher murine leukemia virus bound Co(II) with a K_d of 1.0 μM (Green & Berg, 1989).

The values of the extinction coefficients at 295 and 620 nm were too low to carry out the absorption spectroscopy experiments at peptide concentrations near 1.0 μM. As another measure of peptide–Co(II) binding, we measured the ability of added Co(II) to quench the fluorescence of the tryptophan residue. With peptide at a concentration of 2.8 μM, fluorescence measurements were made at different Co(II) concentrations and the data were replotted according to the method of Stinson and Holbrook (1973). The K_d was determined to be in the range of 1–5 μM (data not shown); this corroborates the results obtained by absorption measurements. Because equimolar Zn(II) could displace Co(II) from the peptide, the K_d for the peptide–Zn(II) interaction is likely to be less than 1 μM.

Discussion

Aminoacyl-tRNA synthetases have recently been divided into two classes on the basis of the presence of two characteristic sets of sequence motifs (Eriani et al., 1990). In class I enzymes (such as Gln-tRNA synthetase) these sequences are in the N-terminal dinucleotide fold domain and are in-

Table II: Cys-His Sequences in Aminoacyl-tRNA Synthetases[a]

organism/enzyme	position[b]	sequence	reference
E. coli/alanine	178	CDPCTEIFYDHGDH	Putney et al., 1981a
	564	HSATHLMHAALRQVLGTH	
Bombyx mori/alanine	184	CGPCSELHYDRIGDREAAH	Chang & Dignam, 1990
E. coli/cysteine	209	CSAMNCKQLGNHFDIH	Hou et al., 1991
E. coli/glutamic acid	125	CRHSHEHHADDEPC	Breton et al., 1986
E. coli/isoleucine	177	HLHKGAKPVHWCVDC	Webster et al., 1984
	401	HSYPCCWRH	
	902	CPRCWHYTQDVGKVAEHAEICGRC[c]	
E. coli/methionine	145	CPKCKSPDQYGDNCEVC	Barker et al., 1982
E. coli/threonine	73	HSCAHLLGH	Mayaux et al., 1983
	175	HEEYVDMCRGPHVPNMRFC	
E. coli/tryptophan	36	HCIYCIVDQH	Hall et al., 1982
Bacillus stearothermophilus/tryptophan	31	HEYNCYFCIVDQH	Barstow et al., 1986
Neurospora crassa (mitochondria)/leucine	351	HDLRDHAFWKEHH	Chow & RajBhandary, 1989
Saccharomyes cerevisiae/methionine	321	CPVHNSYLADRYVEGECPKC	Walter et al., 1983
	337	CPKCHYDDARGDQCDKC	
S. cerevisiae/threonine	155	HVLGESCECHLGAHIC	Pape & Tzagoloff, 1985
	261	CGKLIDLCVGPHIPH	
S. cerevisiae (mitochondria)/phenylalanine	124	HLLRTHTSAHELEC	Koerner et al., 1987
S. cerevisiae (mitochondria)/leucine	350	CPGHDNRDFEFWQTNCPGEH	Tzagoloff et al., 1988
Saccharomyces douglassi (mitochondria)/leucine	350	CPGHDSRDFEFWQQNCPGEH	Herbert et al., 1988
Thermus thermophilus/methionine	127	CVSCERFYTEKELVEGLCPIH[d]	Nureki et al., 1991

[a] Sequences of aminoacyl-tRNA synthetases were searched for regions containing 4 or more occurrences of cysteine or histidine within a 20 amino acid window. When the size of the window was expanded to 25 amino acids, additional sequences were found. [b] Amino acid position for the first cysteine or histidine. [c] This region contains two overlapping sequences fitting the criteria of the search. [d] Contains 21 amino acids.

volved in ATP binding (Rould et al., 1989). The ATP-binding domain of Ala-tRNA synthetase, which is tentatively assigned as a member of class II, has also been localized to the N-terminal region of the protein (Jasin et al., 1983; Schimmel, 1990). Thus, the overall organization of Ala-tRNA synthetase resembles that of the class I enzymes. The "Cys-His box" may occur in an analogous place to the insertion in the dinucleotide binding fold of Gln-tRNA synthetase, which is responsible for binding the acceptor stem of $tRNA^{Gln}$.

Because relatively conservative amino acid substitutions (Cys178Ser, His188Gln, His191Gln) apparently lead to increased intracellular degradation (Figure 4), the Cys-His box region must play an important structural role in Ala-tRNA synthetase. Discrete bands of lower molecular weight were observed only for the His191 → Gln mutant, suggesting that proteolysis of the Cys178Ser and His188Gln mutant proteins is extremely rapid. These findings are similar to those for the nucleocapsid protein of Rauscher murine leukemia virus, in which it was shown that removal of Zn(II) from the protein greatly accelerated proteolysis (Green & Berg, 1990). NMR experiments on the HIV *gag* protein p55 have defined a compact three-dimensional structure containing extensive internal hydrogen bonding. In particular, hydrogen bonding between amide backbone protons and sulfur atoms in p55 induce so-called type I and type II NH–S tight turns (Summers et al., 1990). Perturbations in such a structure in Ala-tRNA synthetase might be expected to disrupt the tertiary structure enough to render the protein susceptible to proteolysis.

The protein sequence database was searched systematically by Berg (1986) for nucleic acid binding proteins which contained potential metal-binding motifs. Among the proteins identified by this search were three other aminoacyl-tRNA synthetases (*E. coli* and *Saccharomyces cerevisiae* methionyl-tRNA synthetases and *E. coli* isoleucyl-tRNA synthetase). *E. coli* Met- and Ile-tRNA synthetases have been shown to contain 1 mol of Zn(II)/mol of polypeptide chain (Posorske et al., 1979; Mayaux & Blanquet, 1981). In the recently solved crystal structure of *E. coli* Met-tRNA synthetase, the zinc atom appears to be near the active site, but not at the Cys-X_2-Cys-X_9-Cys-X_2-Cys sequence that occurs in an insertion of the nucleotide binding fold (Brunie et al., 1990).

We have searched the most recent database of aminoacyl-tRNA synthetase sequences for potential metal-binding sites where there are at least four Cys/His residues within 20 consecutive amino acids [see also Nureki et al. (1991)]. This would identify all sequences that are similar to those found in the nucleic acid binding proteins of retroviruses and to *E. coli* Ala-tRNA synthetase. Several sequence candidates from nine different synthetases were identified (Table II). Whether the cysteine/histidine-rich sequences in these enzymes have a similar structural motif or functional role to that of Ala-tRNA synthetase remains to be investigated. The specificity of each aminoacyl-tRNA synthetase for its cognate tRNA may arise in part from interactions unique to that system; consequently, the potential involvement of a metal-binding domain in tRNA recognition may be unique to the alanine system.

The amino acid sequence of the *Bombyx mori* Ala-tRNA synthetase has recently been determined (Chang & Dignam, 1990). Because cross-species aminoacylation has been demonstrated for the *E. coli* and *B. mori* Ala-tRNA synthetases (Hou & Schimmel, 1989), the features of tRNA recognition are likely to be similar in the two enzymes. The *B. mori* Ala-tRNA synthetase contains regions of significant sequence similarity with the *E. coli* enzyme, particularly in the N-terminal domain. This domain contains the sequence Cys-X_2-Cys-X_3-His-X_{10}-His in the position expected for the Cys-His box (Table II).

Because one of the cysteines (Cys181) in the Cys-His box is not essential for enzyme stability or activity, the fourth ligand to zinc may be another side chain in the protein that has not been identified in this study. Alternatively, the fourth site may be occupied by a water molecule, as observed in several other metalloproteins (Vallee & Auld, 1990). Comparisons of the sequences and structures of zinc metalloproteins support the generalization that tridentate zinc sites (with one water ligand) are catalytic sites. In contrast, tetradentate zinc sites primarily play a structural role. This raises the possibility that the structure of the Cys-His box in Ala-tRNA synthetase differs from that of p55, which has four amino acid ligands to zinc (Summers et al., 1990; South et al., 1990). One significant difference between the two sequences is the presence

of Pro180 in Ala-tRNA synthetase. Model building studies of the synthetic peptide suggest that simultaneous coordination of zinc by Cys178 and Cys181 would be difficult because of the restricted flexibility of the peptide backbone due to Pro180. The nature of the structural unit formed by the Cys-His box is currently under investigation by biophysical methods.

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