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A retroviral-like metal binding motif in an aminoacyl-tRNA synthetase is important for tRNA recognition

(zinc binding site/retroviral nucleocapsid protein)

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ABSTRACT **The *gag* genes of retroviruses encode nucleocapsid proteins that package genomic RNA and are essential for viral infectivity. These RNA binding proteins have a Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys zinc binding motif that is distinct from the typical zinc-finger motif Cys-Xaa$_2$-Cys-Xaa$_{12-14}$-His-Xaa$_2$-His that is found in some transcriptional activators. *Escherichia coli* alanyl-tRNA synthetase contains a zinc-binding Cys-Xaa$_2$-Cys-Xaa$_6$-His-Xaa$_2$-His motif that resembles that of retroviral nucleic acid binding proteins. We show here that, for alanyl-tRNA synthetase, the metal bound at the retroviral-like metal binding motif is important specifically for tRNA recognition and not for amino acid activation. Moreover, the enzyme–tRNA interaction is strongly dependent on the geometry of metal coordination to the protein. These and additional experiments collectively suggest a role for the retroviral-like metal binding motif in RNA recognition and, further, raise the possibility that the protein-bound metal itself participates in an RNA interaction.**

The *gag* genes of retroviruses encode nucleocapsid (NC) proteins that package genomic RNA and are essential for viral infectivity (1, 2). The gag polypeptides contain one or two copies of the sequence motif Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys (CCHC boxes) (3, 4). This motif is distinct from the structures of DNA-binding zinc fingers exemplified by ADR1 (5), Xfin (6), and Zif268 (7) or the binuclear cluster of six cysteines such as found in GAL4 (8). The high-resolution NMR structure of the first zinc binding domain from the NC protein of human immunodeficiency virus has a tetracoordinate bound zinc surrounded by a compact structure that is devoid of any regular secondary structure (9). Mutational analysis of NCs indicates that the cysteines and histidines in the CCHC box are essential for viral function (10–12). However, because there is no biochemical activity that can be conveniently assayed, the involvement of the CCHC box in RNA recognition has not been clarified.

Escherichia coli alanyl-tRNA synthetase contains the retroviral-like Cys-Xaa$_2$-Cys-Xaa$_6$-His-Xaa$_2$-His motif that was recognized by Berg (13) as a potential metal binding site. Atomic absorption analysis established that the enzyme has 1 mol of tightly bound zinc per mol of polypeptide (14). A synthetic peptide model of the Cys-Xaa$_2$-Cys-Xaa$_6$-His-Xaa$_2$-His motif binds Zn^{2+} or Co^{2+} stoichiometrically and the visible spectrum of the Co^{2+}-bound peptide has the *d*-orbital splitting that is characteristic of tetrahedral geometry and also has a metal-to-sulfur charge transfer band (14). The peptide–Co^{2+} dissociation constant of 1 μM is similar to that for Co^{2+} complexes with the Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys-containing peptides derived from NC sequences (15). Mutational analysis established that the Cys-Xaa$_2$-Cys-Xaa$_6$-His-Xaa$_2$-His motif that starts at position 178 in alanyl-tRNA synthetase is essential for enzyme structure and activity (14). Collectively the results suggest that this motif is an essential portion of the metal binding domain. The possibility that the metal binding domain plays a role in specific tRNA recognition was explored in the present study.

MATERIALS AND METHODS

Materials. Purified samples of *E. coli* Ala-tRNA synthetase were provided by Matthew W. Davis and Karin Musier-Forsyth (Department of Biology, Massachusetts Institute of Technology). The enzyme was prepared as described by Hill and Schimmel (16). *E. coli* tRNA^{Ala} (UGC isoacceptor) and tRNA^{Met} were from Subriden RNA (Rolling Bay, WI) and [^{3}H]alanine (70 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Microhelix^{Ala} and microhelix^{His} were synthesized according to methods as described (17). Cobalt dichloride hydrate (99.999%) and zinc dichloride (99.999%) were obtained from Aldrich.

Enzyme Assays. Aminoacylation of tRNA^{Ala} by native or Co(II)-substituted alanyl-tRNA synthetase was measured as described by Schreier and Schimmel (18). Aminoacylation reactions were performed with 23.0 μM [^{3}H]alanine. The range of tRNA^{Ala} concentrations used was 0.25–4.0 μM; enzyme concentration was 20 nM. The velocity of formation of aminoacylated tRNA^{Ala} was calculated from five time points in the initial 2.5 min of reaction.

ATP–pyrophosphate exchange assays (16, 19) were carried out with 10 nM enzyme and 2.0 mM sodium [^{32}P]pyrophosphate (New England Nuclear). Each reaction proceeded for 5 min at 37°C.

Metal-Binding Studies. Atomic absorption spectroscopy was carried out at the Massachusetts Institute of Technology Central Analytical Facility using a Perkin–Elmer 703 spectrometer. Samples were prepared for atomic absorption spectroscopy as described (14). Absorption spectroscopy and spectral subtractions were performed with a Beckman DU-64 spectrophotometer and black-walled cuvettes. To generate apoenzyme, alanyl-tRNA synthetase (270 μM) in 50 mM Hepes/100 mM NaCl, pH 7.5, was treated with the zinc chelator 1,10-phenanthroline in the same buffer. This was followed by centrifugation through a Centricon-30 (Amicon) microconcentrator (20). Excess 1,10-phenanthroline was removed by washing with metal-free buffer in a Centricon-30 unit until the A_{320} of the filtrate was less than 0.01. The cobalt-substituted enzyme was prepared by addition of 5 mM $CoCl_2$ in Hepes buffer and excess cobalt was removed by centrifugation in a Centricon-30 microconcentrator. This procedure was repeated several times to give complete substitution with cobalt (total time ≈3 hr), and samples were

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Abbreviation: NC, nucleocapsid.

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removed at various time points during the preparation of the Co(II)–enzyme to record absorbance spectra and to measure enzymatic activity.

RESULTS AND DISCUSSION

Effect of Zinc Removal on the tRNA-Dependent Step of Catalysis. The overall aminoacylation of tRNA proceeds in a two-step reaction that is given by:

$$E + AA + ATP \rightleftharpoons E{\cdot}AA{\cdot}AMP + PP_i \quad [1]$$

$$E{\cdot}AA{\cdot}AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E \quad [2]$$

where E is enzyme, AA is amino acid, E·AA·AMP is the enzyme-bound aminoacyl adenylate, and AA-tRNA is aminoacyl-tRNA (21). The existence of these two reactions provides a direct means to investigate the role of zinc (bound to the retroviral-like motif) specifically in RNA recognition. Previous work showed that, after extensive dialysis of the enzyme versus 1,10-phenathroline, less than 10% of the original aminoacylation activity remains (14). In contrast, dialysis versus the nonchelating isomer 1,7-phenanthroline does not severely reduce activity. It was not determined whether reaction 1 or 2 or both are affected by the removal of Zn^{2+}. Because inhibition of either reaction will prevent overall aminoacylation, further experiments were done.

Reaction 1 was monitored by the alanine-dependent incorporation of ^{32}P from PP_i into ATP (PP_i exchange reaction) (16, 19) and overall aminoacylation was studied by the ATP-dependent incorporation of [^{3}H]alanine into tRNA (18). After dialysis against 1,10-phenanthroline, which reduced aminoacylation activity to less than 10%, alanine-dependent ATP–PP_i exchange was reduced to only 95% of its original value (data not shown). This change in ATP–PP_i exchange activity is not significant experimentally. Thus, removal of zinc specifically affects the tRNA-dependent step of aminoacylation.

Substitution of Cobalt for Zinc. Spectroscopically silent Zn^{2+} can generally be replaced in metalloproteins with Co^{2+} (22, 23). Although the coordination of the two metals is similar, bound Co^{2+} has a visible absorption spectrum that can be easily measured (22). This property has been used to study the metal-binding sites of zinc fingers (24) and of retroviral NC proteins (25, 26). Co^{2+} was added to apoalanyl-tRNA synthetase and the excess cobalt was removed by extensive washing with metal-free buffer in a centrifugal microconcentrator (20). The resulting metalloenzyme contained 0.92 mol of Co^{2+} per mol of polypeptide (established by atomic absorption analysis) and less than 0.05 mol of Zn^{2+} per mol of polypeptide. The addition of Co^{2+} to apoalanyl-tRNA synthetase gave a visible absorption spectrum consistent with formation of a Co^{2+}–alanyl-tRNA synthetase species (Fig. 1*A*). Although there is an overall blue shift, the shape of the spectrum is similar to that of Co^{2+} complexed with the synthetic peptide model of the Cys-Xaa_2-Cys-Xaa_6-His-Xaa_2-His motif from alanyl-tRNA synthetase (14). [The bound Co^{2+} absorption spectrum is also blue-shifted relative to that of Co^{2+} bound to the CCHC boxes of retroviral NC proteins (15, 25) and may reflect differences in the ligand coordination/geometry between the retroviral and synthetase systems.] The absorption spectrum between 420 and 570 nm is due to *d*-orbital splitting of bound Co^{2+} (ε_{520} = 375 $M^{-1}{\cdot}cm^{-1}$) and resembles visible absorption spectra of metalloproteins that bind Co^{2+} with distorted tetrahedral or pentacoordinate geometry (22, 27).

Aminoacylation activity is completely restored by addition of Co^{2+}. Kinetic parameters for the apoenzyme reconstituted with Co^{2+} are the same as those for apoenzyme reconstituted with Zn^{2+}. To perform this analysis, apoenzyme was generated by treatment with 1,10-phenanthroline. This sample was divided in two; half was reconstituted with Zn^{2+} and half was reconstituted with Co^{2+} in centrifugal microconcentrators. The K_m values for aminoacylation of $tRNA^{Ala}$ by the Zn(II)–enzyme and the Co(II)–enzyme are 3.87 μM and 4.06 μM, respectively, and the k_{cat} values are 0.48 sec^{-1} and 0.46 sec^{-1}, respectively. Because the adenylate synthesis activity of the enzyme (reaction 1) is not affected by removal of metal, it is reaction 2—the tRNA-dependent step of aminoacylation—that specifically requires bound Zn^{2+} or Co^{2+}. To further establish the relationship between bound metal ion and the tRNA-dependent step of aminoacylation, the time course of the reconstitution of activity was followed spectroscopically. The line drawn in Fig. 1*B* assumes that there is a one-to-one correlation between fraction of bound metal and the fractional restoration of the tRNA-dependent step. Within the accuracy of these measurements, the experimental points are consistent with this assumption.

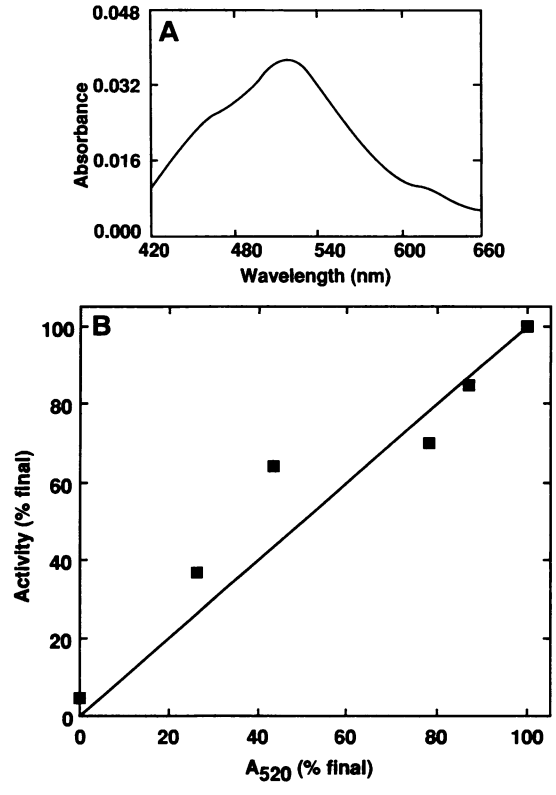

FIG. 1. Reconstitution of apoenzyme with cobalt. (*A*) Absorbance scan of the cobalt-substituted Ala-tRNA synthetase at 100 μM (per monomer). The spectrum was corrected by subtracting out the absorption spectrum of the apoenzyme. Protein concentration was determined by Bio-Rad protein assay. No metal-to-sulfur charge-transfer band was observed in the region of 330–400 nm. Because of the intensity of absorbance of high concentrations of this high molecular weight protein at lower wavelengths, the existence of a charge-transfer band in the region of 280–330 nm (cf. refs. 15, 22, and 25) was not established. (*B*) Restoration of aminoacylation activity by addition of Co^{2+}. The activity of Co(II)–enzyme was measured by enzyme-catalyzed transfer of [^{3}H]alanine to $tRNA^{Ala}$ (Subriden RNA) at pH 7.5 (18). The points shown represent data from a typical experiment. The line with a slope of 1.0 is drawn assuming that there is a 1:1 restoration of activity with binding of cobalt(II); when the experimental data is used to generate a line with the best least-squares fit, the resulting slope is 0.97.

Oxidation of Co(II) *in Situ*. The enzyme–cobalt coordination can be altered by oxidation of Co^{2+} to Co^{3+} *in situ* with hydrogen peroxide. In contrast to exchangeable, tetrahedrally coordinated Co^{2+}, Co^{3+} complexes generally have octahedral coordination and are nonexchangeable (28, 29).

Zn^{2+} cannot be oxidized by H_2O_2, and experiments with native alanyl-tRNA synthetase showed that treatment with H_2O_2 resulted in little loss of aminoacylation or of alanine-dependent ATP–PP_i exchange activity (Fig. 2). However, treatment of Co^{2+}–alanyl-tRNA synthetase with H_2O_2 dramatically reduced aminoacylation activity but had little effect on the adenylate synthesis reaction (Fig. 2). In these experiments, the oxidation of Co^{2+} was confirmed by loss of the characteristic electron paramagnetic resonance signal at g = 4.6 (data not shown). The remaining aminoacylation activity of the oxidized cobalt-containing enzyme may be due to incomplete substitution of alanine tRNA synthetase with cobalt and, additionally or alternatively, to incomplete conversion to the Co^{3+} form (28, 29). Because the native enzyme's activity is not substantially affected by treatment with H_2O_2 and because the adenylate synthesis activity of the Co^{2+}-substituted enzyme is not altered by the oxidation, the loss of aminoacylation activity by the Co^{3+}–enzyme is probably not due to a global effect of oxidation on enzyme conformation. Instead, a subtle change in the geometry/local conformation of the metal binding site is sufficient to perturb the tRNA-dependent step of aminoacylation.

Effect of RNA Binding on Co^{2+} Absorption Spectrum. The effect of bound substrates on the visible absorption spectrum of the Co^{2+}–enzyme was investigated. A small change in A_{520} was observed when alanine and ATP, alone or together, were added to the enzyme. (ATP and/or alanine in 50 mM Hepes/100 mM NaCl, pH 7.5, were added to a 100 μM solution of Co^{2+} enzyme at saturating concentrations. Addition of ATP and alanine together caused no further time-dependent increase in the A_{520}.) The effect of bound RNA on the absorption spectrum of the Co^{2+}–enzyme was also determined with tRNA and microhelix substrates. Addition of $tRNA^{Ala}$ to Co^{2+}–enzyme gives a greater than 100% increase in the intensity of the visible absorption spectrum ($\Delta\varepsilon_{520}$ = 445 $M^{-1}\cdot cm^{-1}$). (This is more than three times the effect of alanine and ATP.) The increase in A_{520} is proportional to the amount of added $tRNA^{Ala}$, up to a stoichiometric equivalence of enzyme sites (Fig. 3*A*). (An enzyme concentration of 100 μM is required to give sufficient absorption for accurate detection; under these conditions, all added $tRNA^{Ala}$ is bound to the enzyme until saturation is achieved.) With these conditions (pH 7.5 and high enzyme concentrations) a slight sigmoidicity in the binding curve for $tRNA^{Ala}$ is observed that

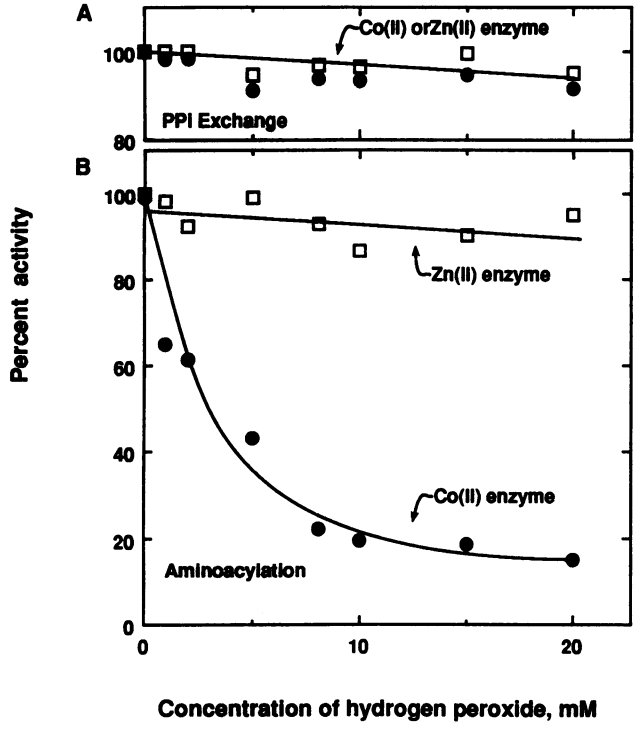

FIG. 2. Effect of hydrogen peroxide treatment on Co(II)–enzyme activity. The cobalt-substituted enzyme was prepared. Co(II)–enzyme or wild-type Zn(II)–enzyme (100 μM of subunit) were incubated with various concentrations of hydrogen peroxide for 90 sec. These reaction mixtures were diluted 1:100 with water, and samples were removed to measure aminoacylation of $tRNA^{Ala}$ (18) and PP_i exchange (16, 19). A 300-μl sample of Co(II)–enzyme was treated with 10 mM H_2O_2 under these conditions and immediately frozen in liquid nitrogen. EPR spectra of this sample and an untreated control were obtained at 5 K. (*A*) Alanine-dependent incorporation of ^{32}P from pyrophosphate into ATP (PP_i exchange reaction). Reaction mixtures contained 100 mM Tris·HCl (pH 8.0), 2 mM sodium [^{32}P]pyrophosphate, 2 mM ATP, 2 mM alanine, and 5 mM $MgCl_2$. The final enzyme concentration was 10 nM. Each enzyme reaction proceeded for 5 min at 37°C. Open squares, Zn(II)–enzyme; solid circles, Co(II)–enzyme. (*B*) Aminoacylation of $tRNA^{Ala}$. The final enzyme concentrations were 20 nM and the reactions proceeded for 5 min at 37°C.

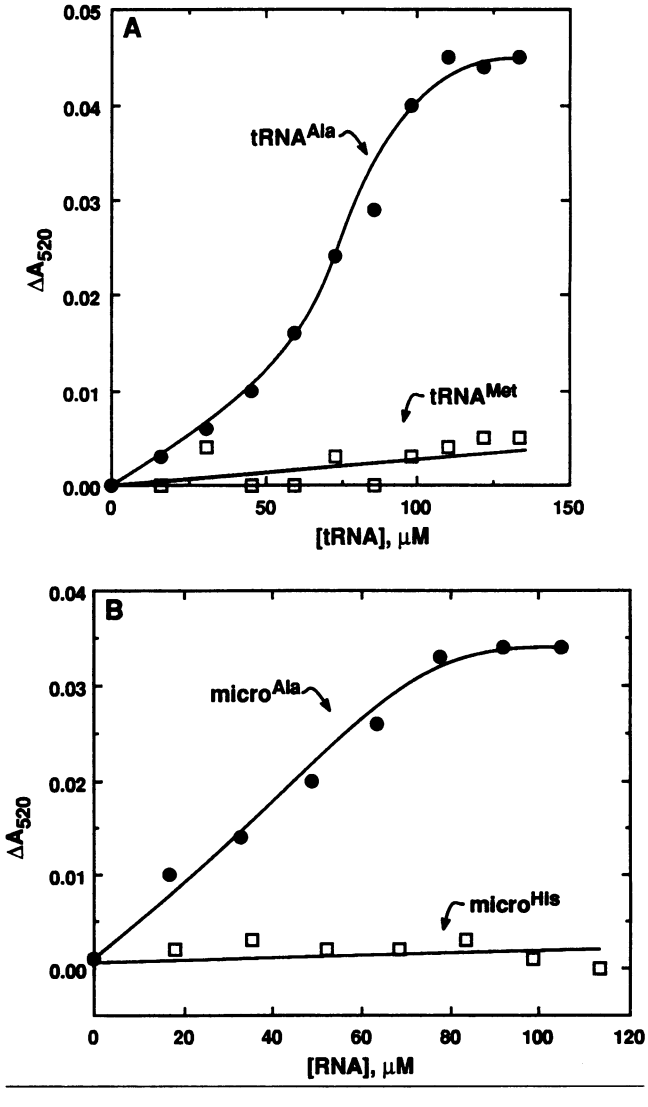

FIG. 3. Titration of cobalt-substituted Ala-tRNA synthetase with tRNAs and microhelices. Changes in A_{520} were measured in 50 mM Hepes/100 mM NaCl, pH 7.5, and were corrected for dilution. (*A*) Addition of $tRNA^{Ala}$ (solid circles) or $tRNA^{Met}$ (open squares) to 100 μM samples of Co(II)–enzyme. (*B*) Addition of $microhelix^{Ala}$ (solid circles) or $microhelix^{His}$ (open squares) to 78 μM samples of Co(II)–enzyme. Although the intensity of the spectrum increases upon binding of alanine-specific RNA substrates, there is no change in the shape of the spectrum.

is not evident at pH 5.0 and low enzyme concentrations (30). In contrast, no change in absorption is observed when the noncognate $tRNA^{Met}$ is added.

The major determinant for the identity of an alanine tRNA is a single G3·U70 base pair (31). Alteration of this base pair to G·C, A·U, U·G, etc. abolishes *in vitro* aminoacylation with alanine (31, 32). Small microhelix substrates that recapitulate the 7-base-pair alanyl-tRNA acceptor helix are also aminoacylated with alanine, provided that they contain the G3·U70 base pair (17). Within experimental error, all of the change in absorption that occurs upon binding of $tRNA^{Ala}$ is also observed with microhelix^{Ala} ($\Delta\varepsilon_{520}$ = 436 $M^{-1}\cdot cm^{-1}$) but not with the noncognate microhelix^{His}, suggesting that specific interactions with the acceptor stem are mainly responsible for the perturbation of the Co^{2+}–enzyme's absorption spectrum (Fig. 3*B*). Extensive interaction of alanyl-tRNA synthetase with the acceptor stem of $tRNA^{Ala}$ has been demonstrated previously by RNA footprinting (33).

Significant changes in the intensity of the electronic absorption spectrum of active site Co^{2+}-substituted *Bacillus aureus* β-lactamase and human sorbitol dehydrogenase occur upon substrate binding and it has been suggested that these effects are due to a substrate-induced change in the metal coordination sphere at the active site, including transient coordination of a substrate ligand to the metal (27, 34). Because nitrogen and oxygen ligands are present on the purine and pyrimidine bases of tRNA, metal ion interactions are possible either directly or indirectly through a water bridge. [One RNA ligand that is required for aminoacylation with alanine is the unpaired exocyclic 2-amino group of G3 (in the G3·U70 base pair) that projects into the minor groove of the acceptor stem helix (35).] Thus, the large spectral changes in the Co^{2+}-substituted enzyme induced by cognate, but not noncognate, RNA substrates raise the possibility that, in addition to stabilizing a structural motif, the metal itself participates in a highly specific RNA interaction.

Concluding Remarks. Prior to the identification of the Cys-His box as the site of metal binding to alanyl-tRNA synthetase, a Gly-174 → Asp mutation was isolated which is near the Cys-His box that starts at Cys-178 (36). The Gly-174 → Asp substitution enables the enzyme to aminoacylate a G3·C70 mutant $tRNA^{Ala}$ (36). This result suggests that the local conformation around the metal binding motif is important for tRNA recognition and is compatible with the observation reported here that the local conformational change caused by oxidation of Co^{2+} to Co^{3+} *in situ* impairs the tRNA interaction. The results reported here on the role of Zn^{2+} in specific RNA recognition may be relevant to the observation that Cys → Ser and other substitutions in the CCHC box of the NC from Moloney murine leukemia virus cause mispackaging of cellular RNA (11, 12) and that removal of zinc from a retroviral-like metal binding domain of T4 phage gene *32* protein affects translational autoregulation (37).

Although motifs in the two classes of aminoacyl-tRNA synthetases for recognition of tRNA may be in part idiosyncratic to each enzyme, it is worth noting that nine enzymes have potential Cys/His metal binding sites (14). Zinc has been found in the four synthetases that have been investigated (14, 38–40). Possibly, a metal binding domain is important for tRNA recognition more generally in aminoacyl-tRNA synthetases.

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