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Probing the Metal Binding Sites of *Escherichia coli* Isoleucyl-tRNA Synthetase†

Bo Xu, Barton Trawick, George A. Krudy, Renee M. Phillips, Lei Zhou, and Paul R. Rosevear*

Department of Biochemistry and Molecular Biology, University of Texas Medical School-Houston, Medical School, Houston, Texas 77225

Received October 26, 1993; Revised Manuscript Received November 24, 1993®

ABSTRACT: The metal binding properties of isoleucyl-tRNA synthetase (IleRS) from *Escherichia coli* were studied by *in vivo* substitution of the enzyme-bound metals. Purified *E. coli* IleRS was shown to have two tightly bound zinc atoms per active site. Cobalt- and cadmium-substituted IleRS were also found to contain two tightly bound Co^{2+} and Cd^{2+} atoms per polypeptide chain, respectively. The d–d transitions in the low energy absorption spectrum of Co^{2+}-substituted IleRS were characteristic of that expected for two tetrahedrally coordinated Co^{2+} metals. Apo-IleRS was found to be inactive in both the aminoacylation of $tRNA^{Ile}$ and in the isoleucine-dependent ATP–pyrophosphate exchange reactions. Both Co^{2+}- and Cd^{2+}-substituted IleRS were found to have k_{cat}/K_m values in the isoleucine-dependent ATP–pyrophosphate exchange assay approximately 5-fold lower than the native Zn^{2+} enzyme. A single enzyme-bound Zn^{2+} or Co^{2+} atom per polypeptide chain could be removed by dialysis of Zn^{2+}- or Co^{2+}-substituted IleRS against 1,10-phenanthroline. Removal of one of the two enzyme-bound Zn^{2+} atoms per polypeptide chain with 1,10-phenanthroline was found to decrease $(k_{cat}/K_m)_{Ile}$ by approximately 130-fold. The dependence of the kinetic parameters on the identity and number of enzyme-bound metals in the isoleucine-dependent ATP–pyrophosphate exchange reaction suggests that at least one enzyme-bound metal is indirectly involved in aminoacyladenylate formation. Metal substitution or removal of one of the two enzyme-bound metals in IleRS was found to have little effect on the K_m value for $tRNA^{Ile}$ or the k_{cat} value for aminoacylation of $tRNA^{Ile}$. Thus, enzyme-bound metals in *E. coli* IleRS appear to play both structural and catalytic roles in aminoacylation activity. In contrast, the single enzyme-bound metal in the *E. coli* methionyl-tRNA synthetase, located in a four Cys zinc finger motif, appears to be structural and not directly involved in catalysis [Xu, B., Krudy, G. A., & Rosevear, P. R. (1993) *J. Biol. Chem. 268*, 16259–16264].

Aminoacyl-tRNA synthetases are a family of enzymes which are responsible for the acylation of their cognate tRNAs with amino acid via an aminoacyladenylate. A number of synthetases, including the *Escherichia coli* isoleucyl-tRNA synthetase (IleRS),[1] methionyl-tRNA synthetase (MetRS) and alanyl-tRNA synthetase (AlaRS), have been shown to bind Zn^{2+} and contain metal binding sequences similar to zinc finger motifs in transcription factors and retroviral proteins (Posorske et al., 1979; Mayaux et al., 1981; Berg, 1986; Miller et al., 1991). The methionyl-tRNA synthetase has recently been shown to tetrahedrally coordinate its single enzyme-bound metal in a C4 box zinc finger motif having the sequence Cys^{145}-X_2-Cys-X_9-Cys-X_2-Cys^{161} (Xu et al., 1993; Landro & Schimmel., 1993; Fourmy et al., 1993). Similarly, alanyl-tRNA synthetase has been shown to bind Zn^{2+} in a retroviral-like metal binding motif, Cys-X_2-Cys-X_6-His-X_2-His, that has been implicated in specific tRNA recognition (Miller & Schimmel, 1992a,b).

Previously it had been suggested that the *E. coli* IleRS contained one or more than one enzyme-bound zinc per polypeptide chain (Mayaux et al., 1981; Kohda et al., 1984), although more recent cysteine modification studies on both the *E. coli* and *Thermus thermophilus* IleRS proteins have suggested the presence of two enzyme-bound Zn^{2+} atoms per polypeptide chain (Nureki et al., 1993). However, none of these studies have correlated the number of enzyme-bound metals with the number of enzyme active sites or probed the role of the bound metals. From sequence comparisons, *E. coli* IleRS contains at least two potential zinc finger-like metal binding sites, His^{177}-X-His-X_8-Cys-X_2-Cys^{191} and Cys^{902}-X_2-Cys-X-His-X_{14}-Cys-X_2-Cys^{926} (Webster et al., 1984; Miller et al., 1991). The first proposed metal binding site is located in CP-I, a connecting polypeptide within the nucleotide binding or Rossmann fold (Shiba & Schimmel, 1992). This connecting polypeptide has been shown by deletion analysis to be important in aminoacylation activity. The second four Cys metal binding motif is located near the carboxy terminus of the protein. This motif is characteristic of that found in *E. coli* MetRS. However, this second zinc finger motif, C4 box, is not conserved in IleRS from all organisms. In *Tetrahymena thermophila*, the C4 box zinc finger is replaced by a Leu-rich sequence resembling a leucine zipper (Shiba & Schimmel, 1992).

In an attempt to better understand both the number, location, and the role of zinc in *E. coli* IleRS, we have prepared metal-substituted IleRS for both biochemical and physical characterization. Active site titrations and metal analyses have demonstrated that *E. coli* IleRS contains two tightly bound enzyme metals. One of the two enzyme-bound metals can be removed with 1,10-phenanthroline resulting in a 130-fold decrease in $(k_{cat}/K_m)_{Ile}$ for the isoleucine-dependent ATP–

† This work was supported by National Institutes of Health Grant GM41232 to P.R.R.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts*, January 1, 1994.

[1] Abbreviations: IleRS, isoleucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase, Tris, tris(hydroxymethyl)aminomethane.

0006-2960/94/0433-398$04.50/0

pyrophosphate exchange assay. The dependence of the exchange reaction kinetic parameters is further support that one of the two enzyme-bound metals is at least indirectly involved in aminoacyladenylate formation. Visible spectra and water proton relaxation studies of Co^{2+}-substituted IleRS are consistent with tetrahedral coordination of both enzyme-bound metals to four protein ligands.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]Pyrophosphate and [^{14}C]isoleucine were purchased from New England Nuclear. Crude tRNA was purchased from Boehringer Mannhein. Metal salts were obtained in ultrapure form from Johnson Matthey Electronics. Chelex-100 was obtained from Bio-Rad Laboratories. All other chemicals used in preparation and purification of the protein were of the highest purity available commercially.

Synthesis and Preparation of Zn^{2+}, Co^{2+}, and Cd^{2+} IleRS. Metal-substituted IleRS proteins were prepared *in vivo* by growing *E. coli* cells harboring the plasmid pKS21 under control of the lac promoter, gift from Paul Schimmel at Massachusetts Institute of Technology, in a Luria-Bertani medium which had been chelexed twice using Chelex 100 and supplemented with the necessary metals for cell growth as previously described (Xu et al., 1993; Williams & Rosevear, 1991). Metal-substituted IleRS proteins were purified as previously described with the exception that the ammonium sulfate fraction was dialyzed and purified by Mono Q and Sephacryl S-100 fast protein liquid chromatography (Pharmacia LKB Biotechnology Inc.). Using this procedure, Zn^{2+}-, Co^{2+}-, and Cd^{2+}-substituted and apo-IleRS were purified to homogeneity as judged by SDS–polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

Metal and Protein Analysis. Protein concentrations of the metal-substituted IleRS proteins were determined separately by active site titration (Yarus & Berg, 1970; Williams & Rosevear, 1991), Bradford assay (Bradford, 1976), the bicinchoninic acid (BCA) assay (Gornall et al., 1949; Smith et al., 1985), and calculated from the extinction coefficient, ϵ_{280}, based on the known amino acid sequence (Cantor & Schimmel, 1980; Elwell & Schellman, 1977). The concentrations of Zn^{2+}, Co^{2+}, and Cd^{2+} in the metal-substituted proteins were quantitated by flame atomic absorption with an acetylene/air flame as previously described (Xu et al., 1993).

Reaction of Zn^{2+}- and Co^{2+}-Substituted IleRS with 1, 10-Phenanthroline. Zn^{2+}- or Co^{2+}-substituted IleRS was dialyzed overnight into metal free 25 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol and 10 mM 1,10-phenanthroline at 4 °C. Samples were then dialyzed against metal free 25 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol to remove the metal chelator. Zinc or cobalt release from IleRS was determined by atomic absorption and, in addition, by UV/visible spectra for the Co^{2+}-substituted protein.

Enzyme Assays. Isoleucine-dependent ATP–pyrophosphate exchange assays were carried out as previously described (Williams & Rosevear, 1991). Kinetic parameters for isoleucine were determined using 0.1–100.0 μM isoleucine and 2 mM ATP. The kinetic parameters for ATP were determined using 0.1–5.0 mM ATP and 20 μM isoleucine. The tRNA-dependent aminoacylation reactions were performed as previously described (Shepard et al., 1992). The kinetic parameters for $tRNA^{Ile}$ were determined using 0.1–20 μM purified $tRNA^{Ile}$.

Purification of E. coli $tRNA^{Ile}$. Unfractionated *E. coli* tRNA obtained from Boehringer Mannhein was used for purification of $tRNA^{Ile}$. The major species of $tRNA^{Ile}$ was purified by successive column chromatography on DEAE-Sephadex A-50 (Nishimura & Weinstein, 1969) and reverse salt gradient chromatography on Sepharose (Holmes et al., 1975). The specific activity of purified $tRNA^{Ile}$ was 0.5 nmol/A_{260} unit. This value is in agreement with the previously published value, 0.5 nmol/A_{260} unit, for purified *E. coli* $tRNA^{Ile}$ (Airas, 1992).

Table 1: Metal, Protein, and Active Site Analyses of Native *E. coli* IleRS[a]

protein determination method	[protein] (μM)	[Zn] (μM)	[active sites] (μM)	metal/protein ratio
bicinchoninic acid	2.4	4.9	ND[b]	2.0
Bradford[c]	1.5	3.0	ND	2.0
active site titration	2.0[c]	4.2	2.2 ± 0.1	1.9
calculated[d]	3.5	6.2	ND	1.8

[a] Zinc concentrations were determined by atomic absorption. Protein concentration and active site titrations were performed as described under Experimental Procedures. [b] Not determined. [c] Protein concentration determined using the method of Bradford as described under Experimental Procedures. [d] Protein concentration calculated based on the known amino acid sequence of *E. coli* IleRS as described under Experimental Procedures.

Ultraviolet–Visible Absorption Spectra. Absorption spectra of Co^{2+}-substituted IleRS were obtained on a Hewlett-Packard 8450 UV/vis spectrophotometer. The buffer consisted of 25 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride.

Magnetic Resonance Methods. Measurement of water proton relaxation times were performed at 300 MHz using an internal deuterium lock. The longitudinal relaxation time (T_1) of the water protons was measured using the inversion–recovery method and a single transient per τ value. ^{113}Cd NMR measurements on $^{113}Cd^{2+}$-substituted IleRS were performed on 500- and 300-MHz spectrometers operating at 111 and 67 MHz for ^{113}Cd, respectively. Chemical shifts are referenced to 0.1 M $Cd(ClO_4)_2$. $^{113}Cd^{2+}$-Substituted MetRS was used as a standard for optimizing the instruments for the detection of protein bound ^{113}Cd (Xu et al., 1993).

RESULTS

Stoichiometry of Metal Binding to IleRS. Table 1 gives the protein to metal ratio for Zn^{2+}-substituted IleRS. Protein concentrations were determined by the bicinchoninic acid (Smith et al., 1985) and Bradford (1976) methods and calculated from the extinction coefficient at 280 nm, based on the known amino acid sequence of the *E. coli* IleRS, in the presence of 6 M guanidine hydrochloride (Cantor & Schimmel, 1980; Elwell & Schellman, 1977). In addition, the number of catalytically competent active sites was determined using active site titration (Williams & Rosevear, 1991). Based on these analyses, an average extinction coefficient at 280 nm for the *E. coli* IleRS was determined to be 1.75 cm^{-1} mg^{-1} mL in 25 mM Tris-HCl buffer at pH 7.5. From these data and metal analysis by atomic absorption, native *E. coli* IleRS was found contain an average of two tightly bound zinc atoms per active site. Cobalt- and cadmium-substituted IleRS proteins were prepared *in vivo* using zinc-depleted media supplemented with either 30 μM $CoCl_2$ or 15 μM $CdCl_2$ as previously described (Xu et al., 1993). Data in Table 2 show that Co^{2+}- and Cd^{2+}-substituted IleRS could be obtained with *in vivo* substitution efficiencies of 92% and 100%, respectively. Metal to protein ratios of Co^{2+}- and Cd^{2+}-substituted IleRS were consistent with two tightly bound Co^{2+} or Cd^{2+} atoms per polypeptide chain (Table 2). Thus, *E. coli* IleRS substituted

Table 2: Metal Content of *in Vivo* Synthesized IleRS[a]

metal-substituted proteins	[protein] μM	[metal] (μM) Zn	Co	Cd	metal/protein ratio	% yield[b]
Zn^{2+}-IleRS	3.1	6.1	≤0.1	≤0.05	2.0	100
Co^{2+}-IleRS	3.1	0.5	5.8	≤0.05	2.0	92
Cd^{2+}-IleRS	1.3	≤0.05	≤0.1	2.65	2.0	100

[a] Enzyme preparation and metal analysis were performed as described under Experimental Procedures. [b] % Yield is based on the amount of desired metal-substituted IleRS divided by the total IleRS isolated.

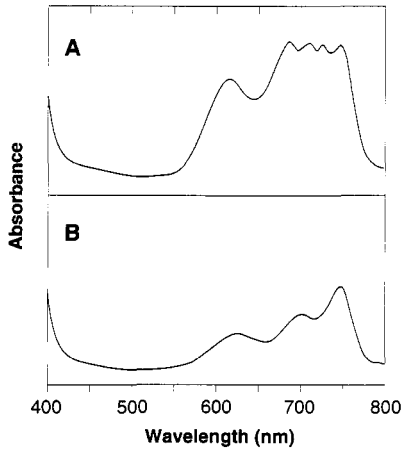

FIGURE 1: Visible absorption spectra of Co^{2+}-substituted IleRS. (A) Co^{2+}-substituted IleRS containing two enzyme-bound Co^{2+} atoms per enzyme active site. (B) [Co,0]-substituted IleRS in which one of the two enzyme-bound metals has been remonved by dialysis against 1,10-phenanthroline as described under Experimental Procedures. Spectra were obtained in 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM β-mercaptoethanol.

with either Zn^{2+}, Co^{2+}, or Cd^{2+} was shown to contain two tightly bound metals per polypeptide chain. Apo-IleRS was prepared *in vivo*, by growing the *E. coli* strain carrying the plasmid which overproduces IleRS, in zinc-depleted media as previously described for the MetRS (Xu et al., 1993). Atomic absorption was used to demonstrate that the apoenzyme was devoid of divalent metals except for residual trace amounts of zinc.

Dialysis of Zn^{2+}- or Co^{2+}-substituted IleRS against the metal chelator 1,10-phenanthroline was found to stoichiometrically remove one of the two enzyme-bound metals per polypeptide chain. Zinc- and cobalt-substituted IleRS treated with 1,10-phenanthroline, [Zn,0]-IleRS and [Co,0]-IleRS, respectively, were found to have metal/protein ratios between 0.9 and 1.1. In contrast, 1,10-phenanthroline was unable to remove either of the two enzyme-bound Cd^{2+} atoms in Cd^{2+}-substituted IleRS.

UV/vis Spectra. Five broad maxima at 616, 686, 710, 726, and 746 nm having molar absorptivities, based on the protein concentration, of 740, 1010, 1000, 990, and 990 M^{-1} cm^{-1}, respectively, were observed in the low-energy region of the absorption spectrum of Co^{2+}-substituted IleRS (Figure 1A). This spectral pattern is consistent with two tetrahedrally coordinated Co^{2+} atoms in different environments (Vallee & Galdes, 1984; Xu et al., 1993). Molar absorptivities of these bands are approximately twice as large as those measured from other single tetrahedrally coordinated cobalt-substituted proteins (Vasak et al., 1981; Bicknell et al., 1986; Roberts et al., 1989; Fitzgerald et al., 1991; Miller et al., 1991; Xu et al., 1993). A S → Co^{2+} charge transfer absorption was also observable as a shoulder at 350 nm (data not shown). The UV/vis spectrum of Co^{2+}-substituted IleRS was found not to change over the pH range of 6.0–9.0 or upon addition of either ATP or isoleucine. Below pH 6.0, IleRS becomes insoluble. No enhancement of the water proton relaxation rate was observed in the presence of up to 60 μM Co^{2+}-substituted IleRS suggesting that the protein provides all four ligands for both metals. There remains a possibility that a water ligand tightly bound to the metal would not be detected in these studies. Taken together, these studies suggest that both enzyme-bound metals are tetrahedrally coordinated to four protein ligands.

Figure 1B gives the spectrum of [Co,0]-IleRS in which one enzyme-bound Co^{2+} has been removed by dialysis against 1,-10-phenanthroline. This spectrum is typical of that for a single tetrahedrally coordinated enzyme-bound Co^{2+} having d–d transitions at 625, 700, and 750 nm with molar absorptivities of 355, 604, and 900 M^{-1} cm^{-1}, respectively. These values are approximately half of those obtained for the native Co^{2+}-substituted IleRS. By comparing the spectrum of the phenanthroline-treated enzyme, [Co^{2+},0] -substituted IleRS, (Figure 1B) with that of native Co^{2+}-substituted IleRS (Figure 1A), it is clear that native *E. coli* IleRS has two metal binding sites.

Kinetic Studies. Table 3 compares kinetic parameters for the isoleucine-dependent ATP–pyrophosphate exchange reaction catalyzed by Zn^{2+}-, Co^{2+}-, and Cd^{2+}-substituted IleRS. The K_m values for isoleucine and ATP are similar for the Zn^{2+}-, Co^{2+}-, and Cd^{2+}-substituted IleRS proteins (Table 3). However, both Co^{2+}- and Cd^{2+}-substituted IleRS were found to have k_{cat} values in the isoleucine-dependent ATP–pyrophosphate exchange assay approximately 3-fold lower than that of the native Zn^{2+} enzyme. Removal of one of the two enzyme-bound Zn^{2+} atoms per polypeptide chain with 1,10-phenanthroline was found to decrease $(k_{cat}/K_m)_{Ile}$ by approximately 130-fold for the [Zn,0]-IleRS protein. In contrast, metal substitution or removal of one of the two enzyme-bound metals in IleRS was found to have little effect on the K_m values for $tRNA^{Ile}$ or the k_{cat} values for aminoacylation of $tRNA^{Ile}$ (Table 3). Apo-IleRS, prepared *in vivo* using zinc-depleted media, was found to be inactive, with k_{cat} values ≤0.01 and ≤0.005 s^{-1}, respectively, in the aminoacylation of $tRNA^{Ile}$ and in the isoleucine-dependent ATP–pyrophosphate exchange reactions. These results demonstrate that at least one of the two enzyme-bound metals is essential for measurable enzymatic activity. Additionally, the nature of the enzyme-bound metal was found to influence catalytic efficiency in the isoleucine-dependent ATP–pyrophosphate exchange reaction. These results are in contrast to the *E. coli* methionyl-tRNA synthetase where a single enzyme-bound Zn^{2+} per polypeptide chain is located in a four Cys zinc finger motif. Kinetic parameters for the aminoacylation of $tRNA^{fmet}$ and the methionine-dependent ATP–PP exchange reactions catalyzed by the MetRS were found to be independent of the nature of the enzyme-bound metal (Xu et al., 1993).

DISCUSSION

Isoleucyl-tRNA synthetase isolated from *E. coli* is well known to contain a single catalytic active site per poylpeptide chain (Schimmel, 1987). We have demonstrated that the *E. coli* isoleucyl-tRNA synthetase is a zinc metalloprotein containing two tightly bound zinc atoms per catalytic site. This is contrast to previously published studies on the *E. coli* enzyme which suggest that it contains only a single metal binding site similar to that observed in the *E. coli* methionyl-tRNA synthetase. Our results confirm the recent suggestion based on chemical modification studies that IleRS isolated

Table 3: Kinetic Parameters for Reactions Catalyzed by Metal-Substituted IleRS[a]

	isoleucine-dependent ATP–pyrophosphate exchange				aminoacylation		
enzymes	K_m (mM) ATP	K_m (μM) Ile	k_{cat} (s^{-1}) Ile	k_{cat}/K_m (s^{-1} μM^{-1}) Ile	K_m (μM) $tRNA^{Ile}$	k_{cat} (s^{-1}) $tRNA^{Ile}$	k_{cat}/K_m (s^{-1} μM^{-1}) $tRNA^{Ile}$
Zn(II)-IleRS	0.7 ± 0.1	1.3 ± 0.2	104 ± 10	80.0	2.1 ± 0.2	3.1 ± 0.2	1.5
Co(II)-IleRS	0.7 ± 0.1	2.7 ± 0.3	40 ± 10	15.0	3.9 ± 0.3	6.3 ± 0.3	1.6
Cd(II)-IleRS	0.5 ± 0.1	2.2 ● 0.5	31 ± 10	14.0	3.1 ± 0.2	3.1 ± 0.2	1.0
[Zn,0]-IleRS[b]	0.3 ± 0.1	10.0 ± 0.5	6 ± 2	0.6	1.3 ± 0.2	1.3 ± 0.2	1.0

[a] Data listed in the table are the average value of two to four measurements for each reaction. Reactions were carried out as described under Experimental Procedures. [b] [Zn,0]-IleRS was prepared by dialysis against 1,10-phenanthroline as described under Experimental Procedures. The metal to protein ratio of the 1,10-phenanthroline-treated IleRS was determined to be 1.0.

from *E. coli* contains two enzyme-bound metals per polypeptide chain (Nureki et al., 1993). Previously, Kohda et al. (1984) had suggested that purified *T. thermophilus* IleRS contained two enzyme-bound metals per polypeptide chain. However, our results are the first conclusive demonstration that *E. coli* IleRS substituted with either Zn^{2+}, Co^{2+}-, or Cd^{2+} contains two tightly bound metals per enzyme active site (Tables 1 and 2). In addition, the metal chelator 1,10-phenanthroline was found to remove one of the two tightly bound metals in both Zn^{2+}- and Co^{2+}-substituted IleRS proteins. Previous sequence comparison studies have identified at least two putative zinc finger like motifs in *E. coli* IleRS, His^{177}-X-His-X_8-Cys-X_2-Cys^{191} and Cys^{902}-X_2-Cys-X-His-X_{14}-Cys-X_2-Cys^{926} (Miller et al., 1991; Webster et al., 1984). Zinc finger binding domains are known to play important roles in a number of nucleic acid binding proteins (Berg, 1990; South & Summers, 1990; Coleman, 1992). However, the roles of enzyme-bound metals in IleRS and in aminoacyl-tRNA synthetases in general remains unclear. To better understand the roles of the enzyme-bound metals, catalytic or structural, Zn^{2+}-, Co^{2+}-, and Cd^{2+}-substituted IleRS proteins were prepared *in vivo* and purified to homogeneity for biophysical studies.

Cobalt- and Cd^{2+}-substituted IleRS were found to have decreased k_{cat} values in the isoleucine-dependent ATP–pyrophosphate exchange assay when compared to the native Zn^{2+} enzyme while the K_m values were not significantly altered, implying that the aminoacyladenylate site remains intact in the metal-substituted proteins (Table 3). Removal of one of the two enzyme-bound Zn^{2+} atoms per polypeptide chain with 1,10-phenanthroline was found to decrease $(k_{cat}/K_m)_{Ile}$ in the isoleucine-dependent ATP–pyrophosphate exchange assay by approximately 130-fold. A decrease in $(k_{cat}/K_m)_{Ile}$ could result from an alteration in metal–substrate interactions through an intervening metal ligand. In contrast, metal substitution or removal of one of the two enzyme-bound metals in IleRS was found to have little effect on the kinetic constants in the tRNA aminoacylation assay (Table 3). The lack of any significant changes in these kinetic constants further supports the notion that the active site remains intact upon metal substitution and removal of a single enzyme-bound metal with 1,10-phenanthroline. The observation that *Staphylococcus aureus* V8 protease digestion of native Zn^{2+}- and [Zn,0]-IleRS yields identical proteolytic fragments by SDS–polyacrylamide gel electrophoresis is consistent with the notion that removal of a single enzyme-bound metal does not greatly disrupt the tertiary structure of the protein. More importantly, these results together with the fact that apo-IleRS is devoid of measurable catalytic activity demonstrate that the enzyme-bound metal resistant to 1,10-phenanthroline is essential for catalytic activity. This metal most likely plays a structural role in maintaining the native conformation of IleRS. The single enzyme-bound zinc in the methionyl-tRNA synthetase, located in a four cysteine zinc finger, was shown to be essential for catalysis although the catalytic rate was independent on the nature of the enzyme-bound metal (Xu et al., 1993).

Cobalt was substituted for the two intrinsic zinc atoms in IleRS since it has a visible absorption spectrum which can be used to study the environments of the metal centers (Vallee & Galdes, 1984). The metal binding centers of the alanyl-tRNA synthetase (Miller & Schimmel, 1992a) and methionyl-tRNA synthetase (Xu et al., 1993; Landro & Schimmel, 1993) have previously been studied using Co^{2+} substitution. The visible absorption spectrum of Co^{2+}-substituted IleRS gives d–d transition bands typical of tetrahedral coordination geometry for both Co^{2+} binding sites (Figure 1A). A charge transfer band was also observed at 350 nm indicating thiolate coordination to one or both sites. Removal of one of the two Co^{2+} atoms gives a visible absorption spectrum typical of a single tetrahedrally coordinated metal center (Figure 1B). The central absorption band from the remaining Co^{2+} atom is nearly identical with the central absorption band of Co^{2+} in the methionyl-tRNA synthetase (Xu et al., 1993), except that the splitting of the three bands is larger. This suggests that the coordination sphere in [Co^{2+},0]-substituted IleRS is similar to that in MetRS but with a stronger ligand field. Thus, the UV/vis spectrum of [Co^{2+},0]-substituted IleRS is most consistent with coordination of the remaining enzyme-bound metal to four cysteine residues. The *E. coli* IleRS is known to have a four-cysteine zinc finger like motif (C4 box), Cys^{902}-X_2-Cys-X-His-X_{14}-Cys-X_2-Cys^{926} (Miller et al., 1991; Webster et al., 194). This metal binding motif is most similar to the C4 box zinc binding domain, Cys^{145}-X_2-Cys-X_9-Cys-X_2-Cys^{161}, in *E. coli* MetRS (Xu et al., 1993; Landro & Schimmel, 1993). The second enzyme-bound metal, which can be extracted using 1,10-phenanthroline, may be coordinated to the His and Cys residues in the zinc finger like motif, His^{177}-X-His-X_8-Cys-X_2-Cys^{191}. Metal coordination to the two potential zinc finger metal binding domains is also consistent with preliminary EXAFS experiments on Zn^{2+}-substituted IleRS which suggest a majority of sulfur ligands (George Kwei, Graham George, Bo Xu, and Paul Rosevear, unpublished results). Cadmium-113 NMR at two frequencies failed to detect the ^{113}Cd chemical shifts of ^{113}Cd-substituted IleRS. This could result from ^{113}Cd line broadening as a consequence of a long correlation time or intermediate conformational exchange (Coleman, 1992). The addition of saturating concentrations of ATP or isoleucine did not facilitate detection of the ^{113}Cd resonances in ^{113}Cd-substituted IleRS. Water proton relaxation studies of Co^{2+}-substituted IleRS also failed to detect any rapidly exchanging water molecules. It has been suggested that catalytic zinc atoms are coordinated to at least one H_2O molecule, whereas structural zinc atoms are coordinated only to protein ligands (Vallee & Auld, 1990). At least one enzyme-bound metal is an absolute requirement for the aminoacylation of $tRNA^{Ile}$ and the isoleucine-dependent ATP–pyrophosphate exchange activities. The

dependence of the kinetic parameters on the nature of the enzyme-bound metals in the ATP–pyrophosphate exchange reaction is further support that an enzyme-bound metal is indirectly involved in catalysis (Table 3).

Taken together, these results indicate that at least one of the two enzyme-bound metals is necessary for catalysis and may indirectly function in aminoacyladenylate formation, possibly through a substrate-induced conformational change. It is likely that conformational changes and flexibility required for catalysis are responsible for the inability to detect enzyme-bound ^{113}Cd chemical shifts in ^{113}Cd-substituted IleRS. The precise role and location of the two enzyme-bound metals in the *E. coli* IleRS is currently under further investigation using mutagenesis and biophysical techniques.

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