Characterization of Metal Binding by a Designed Protein: Single Ligand Substitutions at a Tetrahedral Cys_2His_2 Site†

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ABSTRACT: The tetrahedral Cys_2His_2 Zn(II)-binding site in the *de novo* designed protein $Z\alpha_4$ [Regan, L., & Clarke, N. D. (1990) *Biochemistry* 29, 10878] has been studied by independently mutating each of the metal-binding ligands to alanine. The contribution of each ligand to the geometry and affinity of metal binding has been characterized using Co(II), Zn(II), and Cd(II). The results indicate that all four ligands contribute to high-affinity metal binding in $Z\alpha_4$. Two of the four metal-site mutants retain the tetrahedral Zn(II)-binding geometry of $Z\alpha_4$, with one water molecule presumed to bind in the vacant ligand position. These mutants provide the first examples of a demonstrated *de novo* tetrahedral three-coordinate site designed into a protein and as such are a first step toward the design of catalytic rather than structural Zn(II) sites. One of the metal-site mutants binds Zn(II) with either tetrahedral four-coordinate or five-coordinate geometry, while the last ligand-to-alanine substitution abolishes tetrahedral binding. The importance of ligand type for metal-binding in $Z\alpha_4$ was investigated by characterizing two ligand-swap mutants in which a cysteine residue was replaced with a histidine. In both cases, tetrahedral metal binding was lost. Collectively, these results affirm the strategy used to design $Z\alpha_4$ by showing that all designed liganding residues are participating in metal binding, and by suggesting that the tetrahedral geometry of the binding site is perturbed when the designed side chain ligands are replaced with alternate ligands.

During the course of evolution, proteins have recruited ions and small molecules to increase the range of structures and activities accessible to them. In particular, the transition metal ion Zn(II) has been used in a wide variety of structural and catalytic roles. A number of properties make this ion well-suited for these roles: Zn(II) is a good Lewis acid, has a stable +2 oxidation state, and can expand a four-coordinate ligand geometry to a five-coordinate geometry with little energetic cost (Bertini et al., 1985). In many proteins, the large free energy of Zn(II) binding is exploited to stabilize protein structure. A dramatic example of the importance of structural Zn(II) sites is found in "zinc finger" DNA binding domains, which are unfolded in the absence of metal and require Zn(II) to stabilize the DNA-binding conformation (Frankel et al., 1987; Párraga et al., 1988). Catalytic Zn(II) sites take advantage of the electrophilicity of the Zn(II) ion to activate a nucleophile, as in carbonic anhydrase, or to stabilize the negative charge in a transition state, as in alcohol dehydrogenase (Bertini et al., 1985).

Most structural and catalytic Zn(II) sites in natural proteins have four-coordinate tetrahedral or distorted tetrahedral geometry, despite the ability of Zn(II) to adopt five- and six-coordinate geometries (Christianson, 1991). The low Zn(II)-coordination number found in metalloenzymes is of functional significance. In the enzyme carbonic anhydrase, for example, the Zn(II) ion acts as an electrophilic catalyst, lowering the pK_a of the single bound water molecule to facilitate generation of hydroxide at physiological pH (Silverman & Lindskog, 1988). The electrophilicity of Zn(II) is higher, and consequently the pK_a of the bound water is lower, with low coordination number. Furthermore, a tetrahedral binding geometry aids in metal discrimination at both catalytic and structural Zn(II) sites. Because Zn(II) has a full 3d electron shell, there is no difference in ligand field stabilization energy between octahedral and tetrahedral geometries. In contrast, ligand field stabilization energies for metals bearing incomplete d shells such as Co(II) and Ni(II) favor octahedral over tetrahedral binding (Lippard & Berg, 1994). Our overall aim, consequently, is to design Zn(II)-binding sites that reproduce the tetrahedral geometry, and hence the properties, of natural Zn(II) sites.

Several designs for Zn(II)-binding sites have been reported in the literature. Three-histidine sites have been introduced into the designed four-helix bundle α_4 (Handel & DeGrado, 1990; Handel et al., 1993), antibody light and heavy chains (Iverson et al., 1990; Wade et al., 1993) and human retinal binding protein (Müller & Skerra, 1994). A three-histidine Zn(II) site has been incorporated into a novel protein derived from an antibody, referred to as a "minibody", to verify correct folding of the designed protein (Pessi et al., 1994). Significantly, none of these novel Zn(II) sites has been demonstrated to coordinate metal with tetrahedral geometry. In two cases, tetrahedral Zn(II) binding at designed sites has been experimentally verified. The first case consists of a set of four-coordinate His_3Cys metal-binding variants of the B1 domain from *Streptococcal* immunoglobin G-binding protein G (Klemba et al., 1995). The second example is a four-coordinate His_2Cys_2 metal-binding site in the four-helix bundle protein α_4 (Regan & Clarke, 1990), which is the subject of further study in this report.

$Z\alpha_4$ is a metal-binding variant of the *de novo* designed four-helix bundle protein α_4 (Regan & DeGrado, 1988). $Z\alpha_4$ binds metal ions such as Zn(II) and Co(II) with high affinity at a tetrahedral binding site formed by two histidine and two cysteine ligands. Metal binding enhances the stability of $Z\alpha_4$ toward chemical denaturation and does not perturb the secondary structure of the protein (Regan & Clarke, 1990).

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The high stability of $Z\alpha_4$, even in the absence of metal, provides a solid framework for further manipulation of the metal site.

We have independently mutated to alanine each of the metal-binding ligands in $Z\alpha_4$. The aim of this study was twofold: first, to enable us to establish the relative importance of each metal ligand in determining the coordination geometry and affinity of metal binding; and second, to test whether novel tetrahedral Zn(II)-binding sites can be created in which the metal is coordinated by three protein ligands and a single solvent water molecule. If a single water molecule replaces the mutated ligand, the electrophilicity of the Zn(II) ion may lower the pK_a of the bound water such that the acid−base equilibrium lies in favor of Zn(II)-hydroxide near physiological pH. This effect can accelerate reactions involving hydroxide as a nucleophile. Precedent for such Zn(II)-OH based catalysis has been observed with small-molecule chelates in which Zn(II) is tetrahedrally coordinated by three nitrogen atoms and one molecule of water (Kimura et al., 1990). This study, along with others, suggests that the introduction of a catalytic tetrahedral Zn(II) into a protein is a reasonable design goal. The only attempt to create a catalytic site from a natural structural Zn(II) site in a protein, based on this principle, was made by removing a coordinating histidine residue from a zinc finger peptide; however, the resulting peptide did not catalyze aldehyde hydration or ester hydrolysis, probably because the pK_a of the metal-bound water with Cys_2His ligands was too high (Merkle et al., 1991).

The results we obtained from the single ligand-to-alanine mutants prompted us to make two other types of mutations in $Z\alpha_4$. In response to Cd(II)-binding data suggesting that one of the ligand-to-alanine mutants was forming a metal-linked dimer, two of the metal-binding residues of $Z\alpha_4$ were simultaneously mutated to alanine. This mutant also bound Cd(II) as a dimer, allowing us to propose a mode of binding for the dimer. Also, each of the two cysteine ligands has been independently replaced with histidine to investigate the plasticity of the designed metal-binding site: is the identity of the side chain at these positions crucial, or will any residue with the ability to ligand Zn(II) suffice?

EXPERIMENTAL PROCEDURES

Construction of the Mutant Genes. Mutants of $Z\alpha_4$ were constructed *via* cassette mutagenesis as follows. Oligonucleotide cassettes spanning the codon to be mutated were synthesized on an Applied Biosystems Model 392 DNA Synthesizer and were phosphorylated at the 5′ termini with T4 polynucleotide kinase. These cassettes were then ligated into the vector pT7$Z\alpha_4$ (containing the $Z\alpha_4$ gene in the T7 expression vector pET8c) that had been cut with the appropriate restriction enzymes and treated with calf intestine alkaline phosphatase. Following transformation into *Escherichia coli* strain JM101 and selection on ampicillin, positive clones were identified by dideoxy DNA sequencing. The double mutants $Z\alpha_4$C21H:C47A and $Z\alpha_4$H25A:C47H were produced in an identical manner except that the starting plasmid carried a gene containing one of the mutations. $Z\alpha_4$-C47A:H51A was derived from $Z\alpha_4$ and a single cassette containing both mutations.

Expression and Purification. Plasmids containing $Z\alpha_4$ mutants were transformed into an *E. coli* T7 expression host (Studier et al., 1990). Protein was produced by growing the cells in a 12 L fermentor or in several 4 L shaker flasks to an A_{600} of 1.0−1.2, inducing expression with 100 μM IPTG[1] and growing for an additional 2.5 h. Cell pellets were then treated in one of two ways. Early in this work protocol (i) was used to purify protein, and later the more rapid protocol (ii) was adopted.

(i) Cells from 12 L of Terrific Broth (Maniatis et al., 1982) were resuspended in 400 mL of TDE buffer (25 mM Tris-HCl, 3 mM DTT, 1 mM EDTA, pH 7.5) and lysed either by high-speed blending in a Waring blender or by sonication. The lysate was cleared by centrifuging at 13000*g* for 15 min and then was treated with 10 mL of 10% poly(ethylenimine), while stirring on ice, to precipitate nucleic acids. After removing the precipitate by centrifugation at 13000*g* for 15 min, the supernatant was diluted to 1 L with TDE and applied to a 200 mL Toyopearl-CM 650M cation exchange column (Tosohaas). The column was washed with TDE, and then the protein was eluted with a linear gradient of NaCl. $Z\alpha_4$ and the mutants typically eluted at 100−150 mM NaCl. Fractions containing protein were pooled, concentrated by ultrafiltration using a 3K-cutoff membrane (Amicon), and injected onto a Superdex 75 16/60 gel filtration column (Pharmacia) equilibrated with TDE containing 200 mM NaCl. Protein eluting from the gel filtration column was concentrated by ultrafiltration as above and determined to be greater than 95% pure by SDS−PAGE; however, we note that SDS−PAGE does not resolve the two variants of $Z\alpha_4$ observed during reverse-phase HPLC analysis (see below).

(ii) Cells from 6 L of Luria−Bertani medium were resuspended in 300 mL of 50 mM Tricine-HCl and 10 mM DTT, pH 7.5, and heated to 85 °C with rapid stirring. The suspension was held at 85 °C for 5 min, allowed to cool slowly, and centrifuged at 13000*g* for 15 min to remove the debris. $Z\alpha_4$ remained in the soluble fraction and was approximately 80% pure after this step based on Coomassie-stained SDS−PAGE. Seven milliliters of 10% poly(ethylenimine) was added slowly with stirring on ice to precipitate nucleic acids and then cleared by centrifugation at 13000*g* for 15 min. The poly(ethylenimine) supernatant was heated to 90 °C for 5 min, clarified by centrifugation at 13000*g* for 15 min, and concentrated by ultrafiltration as above to 10 mL. The protein was then loaded onto a Superdex 75 16/60 gel filtration column (Pharmacia) equilibrated with TDE containing 200 mM NaCl. Fractions containing pure protein were pooled and concentrated by ultrafiltration as above.

Protein purified by both methods (i) and (ii) was found to be a mixture of two species when analyzed on an analytical C18 reverse-phase HPLC column. The two species from an α_4 stock were purified by HPLC and analyzed as follows. Electrospray mass spectrometry indicated that the one of the species had the expected molecular mass of α_4 and that the other species had a mass 32 Da higher than α_4. One round of manual dimethylaminoazobenzene isothiocyanate protein sequencing showed that the amino terminus of the higher molecular mass species was blocked, while that of the other

[1] Abbreviations: CM, carboxymethyl; Cys, cysteine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFBA, heptafluorobutyric acid; His, histidine; HPLC, high-pressure liquid chromatography; IPTG, isopropyl β-D-galactoside; K_d, dissociation constant; LMCT, ligand-to-metal charge transfer; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TDE buffer, 25 mM Tris-HCl, 3 mM DTT, 1 mM EDTA, pH 7.5; Tris, tris(hydroxymethyl)aminomethane.

species was free (Chang, 1983). The difference in retention time was therefore ascribed to the presence (80% of the mixture) or absence (20% of the mixture) of a formyl group on the N-terminus.

HPLC purification was necessary to remove protein impurities or tightly bound, yellow-colored small molecule contaminants. The formylated species was isolated by reverse-phase HPLC on a preparative C18 column (2.2 × 25 cm, 10 μm particle size). Protein was eluted at 9.9 mL/min with a gradient of 55−70% acetonitrile in water, each containing 0.2% HFBA (Pierce), at a gradient steepness of 1% per minute. Protein pools were lyophilized to dryness. Early metal-binding experiments were carried out on the mixture of formylated and deformylated protein purified by protocol (i). In some cases ($Z\alpha_4$C21A, $Z\alpha_4$C47A, $Z\alpha_4$H51A, and $Z\alpha_4$C47A:H51A), metal-binding experiments were repeated after reverse-phase HPLC purification of the protein; in these instances, only the formylated protein was used. We have no evidence to suggest that the mixture of ± N-formylated species behaves differently from the pure formylated species in metal-binding experiments.

Metal-Binding Experiments. 20 mM Na-HEPES pH 7.5 buffer and metal stock solutions were deoxygenated by two rounds of freezing in liquid nitrogen, pumping under vacuum, and thawing. Protein was prepared by reduction with 20 mM DTT for 15 min and desalting into deoxygenated buffer over a PD-10 Sephadex G25 column (Pharmacia) in an argon-containing glove box. The protein solution and metal stocks were sealed with septa under argon. Titrations were performed at ambient temperature. Aliquots of metal were transferred from the stock flask to the cuvette with a 10 μL syringe. For Co(II) and Cd(II) titration, metal (as $CoCl_2$ or $CdCl_2$) was added directly to the apoprotein. For Zn(II) titration, the protein was first loaded with saturating amounts (0.5−5 mM) of Co(II), and then $ZnSO_4$ was titrated in. Scanning spectra were collected on a Perkin-Elmer Lambda 6 or a Hewlett Packard 8452A Diode Array spectrophotometer. The free thiol concentration was measured before and after titration with DTNB to confirm that the protein was fully reduced throughout the experiment (Riddles et al., 1983). The concentrations of stock metal solutions were determined by flame atomic absorption spectrometry on a SpectrAA-20 instrument (Varian).

The Co(II) spectra of $Z\alpha_4$, $Z\alpha_4$H25A, and $Z\alpha_4$H51A were derived from the Co(II) titrations and were obtained by subtracting a spectrum of buffer and protein alone from a spectrum of buffered protein in the presence of 0.25−1.8 mM $CoCl_2$. The Co(II) spectra of $Z\alpha_4$C47A and $Z\alpha_4$C47A:H51A were taken in the presence of 5 and 11 mM $CoCl_2$, respectively, and the difference spectra were derived by subtracting the spectrum of the same sample containing two equivalents of $ZnSO_4$. Extinction coefficients were calculated by dividing the absorbance at each wavelength by the concentration of protein as determined by DTNB assay.

Titration data were analyzed using the thiol ligand-to-metal charge transfer (LMCT) absorbance around 290 nm (Co(II)) or around 245 nm (Cd(II)). In all cases the total absorbance (buffer + protein + metal + LMCT) was less than 1.0. The absorbance values were corrected for volume changes upon metal addition. For Co(II) titrations, the LMCT absorbance at saturation was determined from a double-reciprocal plot (1/absorbance vs $1/[Co(II)]_T$). The concentration of metal-bound protein was plotted against the total concentration of metal. The points were fit to an equation representing the equilibrium for binding of one metal ion to one protein molecule using the program Kaleidagraph (Abelbeck Software). For Zn(II) and Cd(II) titrations, the LMCT absorbance was plotted against total concentration of metal. The points up to approximately 80% saturation were fit with a straight line. The concentration of metal at the saturating absorbance was calculated, and hence the stoichiometry of binding determined, by dividing by the protein concentration. The data from Cd(II) titration of $Z\alpha_4$C47A:H51A was slightly curved; for this protein, the saturating concentration of Cd(II) was taken from the first maximal LMCT absorbance. We estimate the error associated with the K_d and stoichiometry values to be ±15% of the reported values.

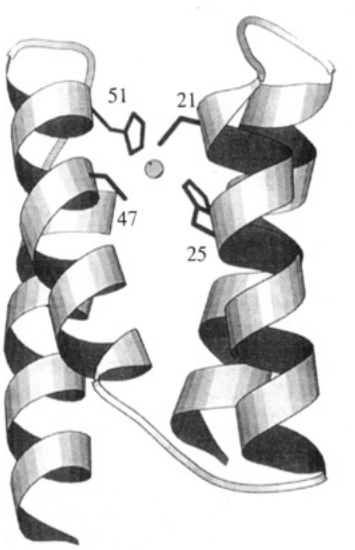

FIGURE 1: Model of $Z\alpha_4$ (Regan & Clarke, 1990). The helices are shown as ribbons and the loops as rope. The liganding side chains (clockwise from top right: Cys21, His25, Cys47, and His51) are displayed along with a metal ion. For clarity, the Zn(II) ion is not shown to scale. This figure was generated with the program Molscript (Kraulis, 1991).

The pH dependence of the Co(II) spectra was determined by desalting aliquots of the protein into the following buffers: 25 mM Tris-HCl, pH 7.5, 25 mM Tris-HCl, pH 8.5, and 25 mM $NaBO_4$/10 mM Tris, pH 9.5. Tris served as a metal carrier in the pH 8.5 and 9.5 buffers. Buffers were degassed by stirring for 30 min under vacuum followed by 10 min of vigorous argon purge. Protein was desalted into each of these buffers with a PD-10 column. Scanning spectra were taken before and after addition of Co(II). Co(II) was added as follows: 2 mM at pH 7.5 and 8.5; 0.5 mM at pH 9.5.

RESULTS

The high-affinity, tetrahedral Zn(II)-binding site in the designed protein $Z\alpha_4$ is formed by two His and two Cys side chains. A model of $Z\alpha_4$ is presented in Figure 1; the orientations of the four liganding side chains and the metal were obtained from the computer algorithm used to identify the site (Regan & Clarke, 1990). Here we report the effects of independently mutating each of these liganding side chains to alanine. The four ligand-to-alanine mutations are referred to as $Z\alpha_4$C21A, $Z\alpha_4$H25A, $Z\alpha_4$C47A, and $Z\alpha_4$H51A, for mutation of Cys21, His25, Cys47, and His51 to alanine, respectively. Several possible effects of such mutations could be anticipated: metal binding could be completely abolished; removal of a ligand might effectively "loosen" the coordination shell, allowing two or three molecules of

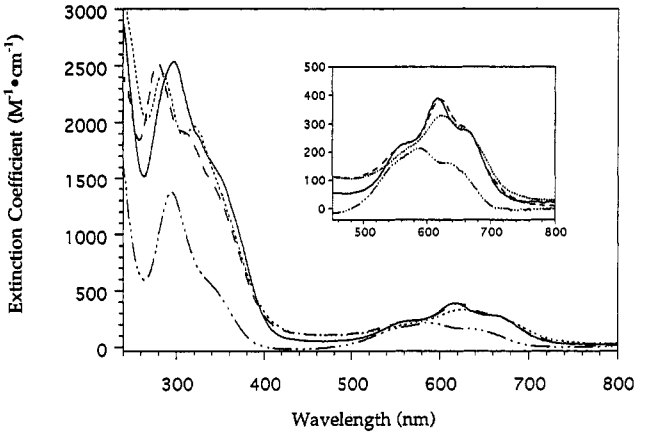

FIGURE 2: Optical absorption spectra of protein−Co(II) complexes: Zα4 (solid line), Zα4H25A (small dashes), Zα4C47A (dots and dashes), and Zα4H51A (large dashes). Absorbance of the buffer and protein has been subtracted. The inset shows an enlargement of the long-wavelength region.

Table 1: Metal-Binding Properties of Zα4 and Three Ligand-to-Alanine Mutants

	extinction maxima						
	LMCT		d−d				
protein	λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	K_d Co(II) (μm)	stoichiometry Zn(II)	stoichiometry Cd(II)
Zα4	297	2540	617	390	16[a]	0.86	0.71
Zα4H25A	285	2430	622	329	150	0.79	0.70
Zα4C47A	294	1370	592	213	530	0.83	0.5
Zα4H51A	280	2520	621	386	140	0.78	0.75

[a] From Regan and Clarke (1990).

water to bind, with an associated change in coordination geometry from four- to five- or six-coordinate; or a single molecule of solvent may bind at the vacant coordination position with retention of tetrahedral coordination. To more fully understand how designed metal-binding sites can be manipulated, we performed detailed characterizations of the metal-binding properties of the single-ligand mutants.

The effect of each mutation on metal binding was first investigated by taking advantage of the spectroscopic properties of Co(II)-substituted protein. Co(II) is a convenient probe for studying Zn(II) proteins: its ionic radius and polarizability are very similar to those of Zn(II) (Shannon, 1967; Irving & Williams, 1953), but Co(II) is optically active, and the spectral properties of a protein−Co(II) complex yield information on the nature of the first coordination sphere (Bertini & Luchinat, 1984). Optical absorption spectra of the Co(II) complexes of Zα4 and three of the four single-ligand mutants are shown in Figure 2. The spectra of these three mutant−Co(II) complexes exhibit a LMCT absorbance around 290 nm arising from thiol coordination. The intensity of the LMCT absorbance is very similar for those proteins containing two cysteine ligands (Zα4, Zα4H25A, and Zα4H51A). The LMCT intensity of Zα4C47A is approximately half that of Zα4, consistent with the removal of a single cysteine residue (see Table 1). The visible region of the Co(II) spectrum, representing d−d electronic transitions, provides information on the geometry and ligand composition of the first coordination shell: the extinction coefficient of the absorbance maximum is indicative of the coordination number, while the wavelength of the absorbance maximum is sensitive to ligand type. In the Co(II)−Zα4 visible spectrum, the maximal extinction coefficient of 390 M^{-1} cm^{-1} is in the range observed for tetrahedral coordination (greater than 200 M^{-1} cm^{-1}; Bertini & Luchinat, 1984), and the wavelength of the absorbance maximum is consistent with S_2N_2 ligands (Krizek et al., 1993; Table 1). The visible spectra of the protein−Co(II) complexes of both Zα4H25A and Zα4H51A are very similar to that of Zα4 (Figure 2, inset). This observation suggests that tetrahedral binding has been retained, with the vacant ligand position presumably occupied by a molecule of water. Zα4C47A has a maximum extinction coefficient in the visible region (213 M^{-1} cm^{-1}) that is significantly lower than that of Zα4. This reflects a perturbation in the four-coordinate geometry of Zα4 or even expansion of the first coordination shell to include five ligands. The wavelength of the absorbance maximum is lower than that of Zα4, which is expected for the replacement of a sulfur ligand with an oxygen ligand. The spectrum of Zα4C21A in the presence of Co(II) exhibits weak (60 M^{-1} cm^{-1}) d−d transitions (not shown), indicating that tetrahedral metal binding is abolished in this mutant. The loss of tetrahedral metal binding by Zα4C21A is accompanied by a substantial decrease in Zn(II) affinity as indicated by ^{65}Zn(II)−protein comigration experiments: Zα4C47A, but not Zα4C21A, coelutes with ^{65}Zn(II) from a Sephadex-G25 gel filtration column (data not shown). It is important to note that the loss of tetrahedral metal binding in Zα4C21A is not due to global destabilization of the protein; the guanidinium hydrochloride denaturation midpoint of Zα4-C21A is indistinguishable from that of Zα4C47A (data not shown).

The affinity of each protein for Co(II) was measured by monitoring the thiol LMCT absorbance as a function of Co(II) concentration. Co(II) titrations of Zα4H25A, Zα4-C47A, and Zα4H51A are shown in Figure 3. The dissociation constant (K_d) was determined by fitting the titration to the binding equilibrium as described in the figure legend. The mutants all show decreased affinities for Co(II) in comparison to Zα4 (Table 1). Removal of a single histidine ligand reduces the affinity for Co(II) by about 10-fold, whereas removal of Cys47 lowers the affinity 33-fold. Based on the relative affinities reported for designed two, three, and four coordinate Zn(II)-binding sites, the increase in K_d of roughly one order of magnitude is consistent with the removal of one ligand (Regan, 1993).

The affinity of Zα4 for Zn(II) is two to three orders of magnitude greater than its affinity for Co(II) (Regan & Clarke, 1990). The difference in binding energy is largely derived from the difference in ligand field stabilization energy between octahedral and tetrahedral coordination of the two metals: Co(II) is destabilized in a tetrahedral environment whereas Zn(II) is not (Lippard & Berg, 1994). This difference in affinity allowed us to measure the Zn(II)-binding stoichiometry of Zα4 and the three Co(II)-binding mutants by titrating out the more weakly bound Co(II). Co(II)−Zn(II) competitions with Zα4H25A, Zα4C47A, and Zα4H51A are shown in Figure 3, and Table 1 summarizes the stoichiometry data. All four proteins bind Zn(II) with essentially a 1:1 protein to metal ratio. The fact that Zn(II) affinities are two to three orders of magnitude higher than Co(II) affinities for each of the mutants provides additional evidence that these proteins coordinate both metals with tetrahedral geometry.

To more extensively characterize the metal-binding properties of Zα4 and the mutants, the stoichiometry of Cd(II) binding was determined directly by following the thiol LMCT absorbance of the protein−Cd(II) complex near 240

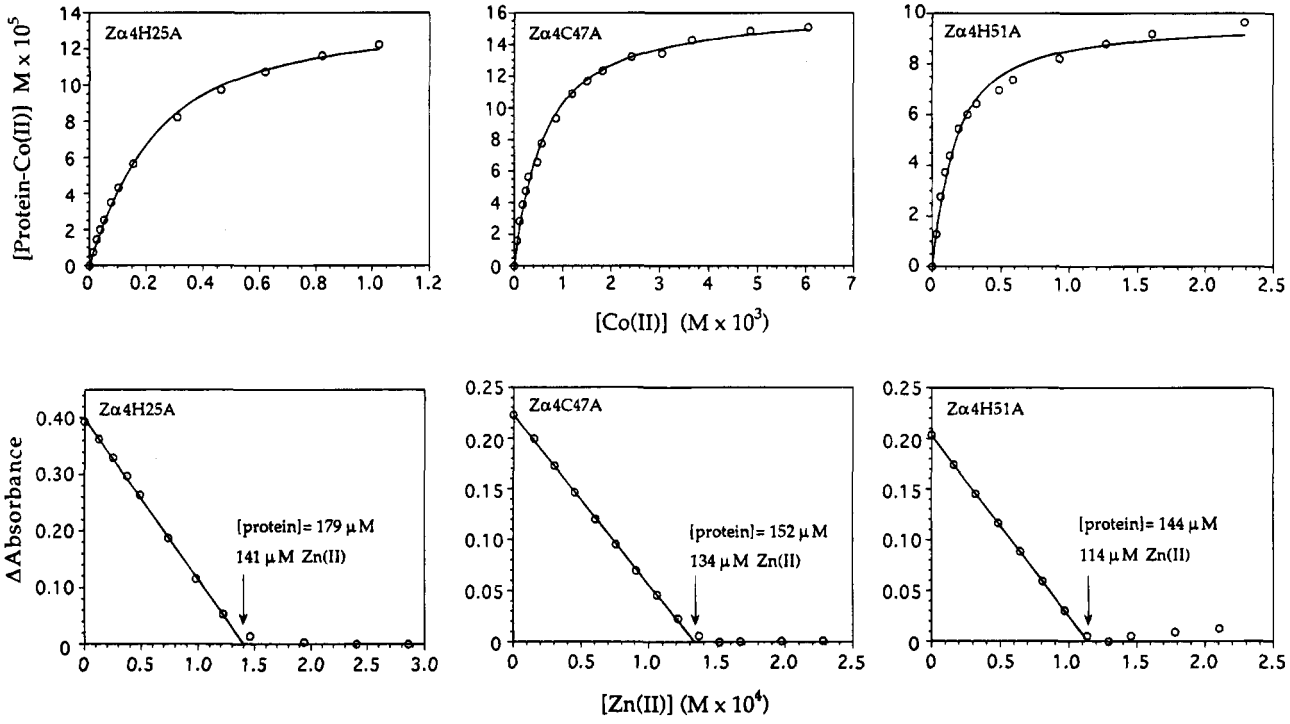

FIGURE 3: Co(II) (upper panel) and Zn(II) (lower panel) titrations of the three single-ligand mutants. The Co(II) titration is plotted as the concentration of Co(II)−protein complex vs total cobalt concentration. Open circles are data points, and the line represents a fit to the equilibrium for binding of one metal ion to one protein molecule. Both the dissociation constant and the total protein concentration were allowed to vary; however, the fitted value for the total protein concentration was always within 17% of the experimental value. The Zn(II) titration is plotted as absorbance vs total Zn(II) concentration. The linear fit to the data points was used to calculated the concentration of Zn(II) at saturation. The stoichiometry of Zn(II) binding was calculated by dividing the Zn(II) concentration at saturation by the protein concentration.

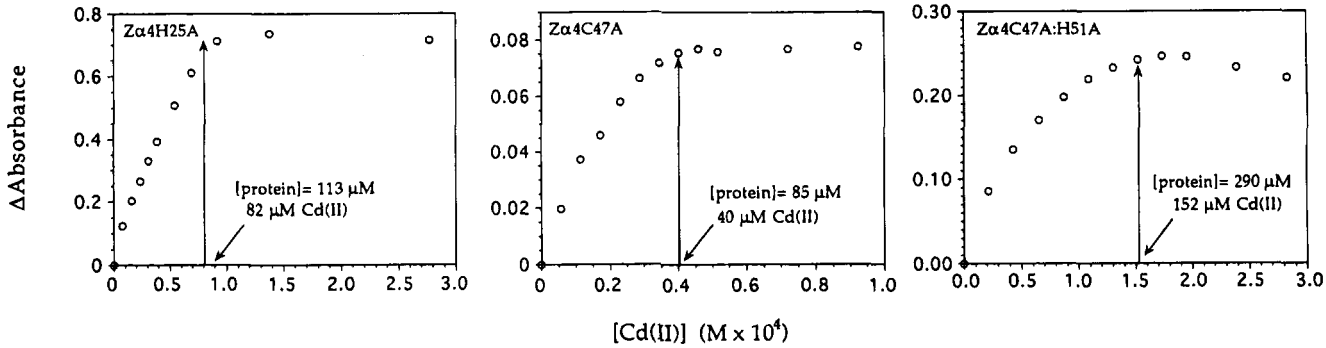

FIGURE 4: Cd(II) binding to $Z\alpha_4$H25A (left), $Z\alpha_4$C47A (center), and $Z\alpha_4$C47A:H51A (right). Titrations of $Z\alpha_4$ and $Z\alpha_4$H51A were similar to that of $Z\alpha_4$H25A. Data are plotted as LMCT absorbance vs total Cd(II) added. The protein concentration, as determined by DTNB assay, is indicated, and the Cd(II) concentration at which the absorbance plateaus is shown with an arrow. Cd(II) stoichiometries are listed in Table 1.

nm. Figure 4 shows Cd(II) titrations of $Z\alpha_4$H25A and $Z\alpha_4$C47A. $Z\alpha_4$ and both of the histidine-to-alanine mutants bind one equivalent of Cd(II) (Table 1). Interestingly, a different result was obtained when $Z\alpha_4$C47A was titrated with Cd(II). For this protein, saturation with metal occurred when the Cd(II):protein ratio was approximately 1:2. A possible explanation for this observation is that $Z\alpha_4$C47A binds Cd(II) as a metal-linked dimer, incorporating two cysteine ligands into its first coordination sphere. The driving force for dimeric binding would come from the strong preference of the soft metal Cd(II) for soft thiolate ligands over hard oxygen and nitrogen ligands (Pearson, 1966).

We thought that the ligands involved in dimeric Cd(II) binding by $Z\alpha_4$C47A were likely to be Cys21 and His25 from each of two protein molecules. These two residues are on the same α-helix; thus, dimeric coordination might require only a small relative rotation of the helices. To further investigate this possibility, the double mutant $Z\alpha_4$C47A:H51A was created, which leaves only Cys21 and His25 from the original metal site. This double mutant bound Cd(II) in a 1:2 metal/protein ratio, as observed for the single C47A mutant (Figure 4). In addition, the double mutant bound Co(II). A comparison of the double mutant−Co(II) d−d spectrum with those of the two single-ligand mutants $Z\alpha_4$C47A and $Z\alpha_4$H51A is shown in Figure 5. If dimeric binding of Co(II) is presumed, the extinction coefficients in the visible region suggests that $Z\alpha_4$C47A:H51A is coordinating Co(II) tetrahedrally. In addition, the wavelength of maximal absorbance for the $Z\alpha_4$C47A:H51A-Co(II) complex (626 nm) is much closer to that of $Z\alpha_4$H51A (621 nm) than that of $Z\alpha_4$C47A (592 nm), suggesting that the Co(II) ion is complexed by two thiolate ligands in the double mutant. The above observations suggest that the energy barrier separating different modes of metal coordina-

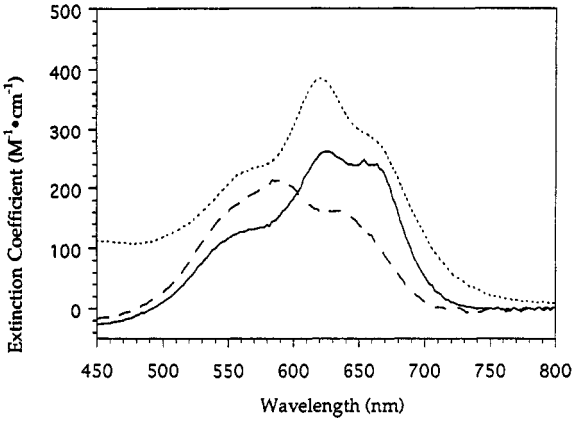

FIGURE 5: Co(II) spectrum of the double mutant Zα4C47A:H51A (solid line) and the single-ligand mutants Zα4C47A (dashed line) and Zα4H51A (dotted line). The extinction coefficient for the double mutant−Co(II) complex was calculated assuming the binding of two protein molecules per Co(II) ion.

tion by Zα4C47A is small and that the differential affinity of particular ligands for metal can influence which of these modes is adopted.

The metal-binding experiments with Zn(II) and Co(II) indicate that the single-ligand mutants Zα4H25A, Zα4H51A, and possibly Zα4C47A bind Zn(II) with retention of the tetrahedral geometry of Zα4. These results suggest that water occupies the vacant ligand position in these mutants. One consequence of water binding to the metal is that the pK_a of the bound water should be suppressed compared to that of a water molecule in bulk solvent. Because the visible spectra of Co(II) complexes of the proteins should be sensitive to changes in the protonation state of the bound water, the Co(II) complex spectrum was taken for each of the three mutants at pH 7.5, 8.5, and 9.5 (data not shown). Minor changes in the visible Co(II) spectra at pH 9.5 vs 7.5 were observed for Zα4H25A and Zα4C47A, while no changes were observed for Zα4H51A. Spectra of Co(II)−carbonic anhydrase show large pH-dependent changes (Lindskog, 1963); however, in other systems the magnitude of the changes may not be as substantial. While the small spectral changes observed with Co(II) complexes of Zα4H25A and Zα4C47A may not be definitive evidence against ionization, our current view is that the pK_a of the Co(II)−bound water is greater than 9.5 for each mutant.

One possible reason for the high pK_a of the Co(II)−bound water in Zα4H25A, Zα4C47A, and Zα4H51A is the relatively high thiolate content of the binding sites. The thiolate ligand, carrying a negative charge, will reduce the electrophilicity of the Zn(II) ion to a greater degree than a neutral histidine ligand. The significance of this difference is borne out by the observation that most catalytic Zn(II) sites in natural metalloenzymes are histidine-rich (Vallee & Auld, 1990). To reflect the preference for histidine in natural catalytic Zn(II) sites, further modifications of the designed metal-binding site were made specifically to decrease the number of thiolate ligands in the first coordination shell. At the same time, we could determine whether the metal site is flexible enough to accommodate alternate ligands. We converted a cysteine residue to a histidine in two of the single ligand-to-alanine mutants: in Zα4C47A, Cys21 was changed to histidine to yield a three-histidine site, and in Zα4H25A Cys47 was mutated to histidine to produce a $CysHis_2$ site (generating the double mutants named Zα4C21H:C47A and Zα4H25A:C47H, respectively). The Co(II) spectrum of Zα4H25A:C47H contained neither thiol LMCT nor d−d transitions, suggesting that metal binding has been abolished in this mutant. The mutant Zα4C21H:C47A has no cysteine ligands; therefore, no thiol LMCT is expected. Since the Co(II) spectrum of this mutant is devoid of d−d transitions, metal is most likely not bound although six-coordinate binding by a combination of histidine and water ligands cannot be ruled out.

DISCUSSION

We have investigated the effects of independently mutating to alanine each of the amino acid ligands in the designed metal-binding protein Zα4. The four metal-binding residues are not equal determinants of the affinity and coordination geometry. The most dramatic change results from mutation of Cys21 to alanine: tetrahedral Co(II) and Zn(II) binding is abolished. The Co(II) complex of the Zα4C47A mutant exhibits spectral properties indicating either a distortion of the tetrahedral geometry found in Zα4, with one water molecule replacing the cysteine ligand, or perhaps a five-coordinate geometry with two water molecules binding to the metal. The intensity of the d−d Co(II) transition does not allow discrimination between these two possibilities. The Co(II) affinity of this mutant is lowered by 33-fold compared with Zα4. Mutation of either His25 or His51 to alanine, however, does not perturb the tetrahedral coordination found in the parent Zα4 protein. Furthermore, there is only an approximately 10-fold reduction in Co(II) affinity upon mutation of either histidine. Taken together, these results suggest that—in the case of Zα4—the cysteine ligands are stronger determinants of Co(II)-binding than histidine ligands. One possible reason for the greater contribution of cysteine over histidine in this designed metal site is that the cysteine side chain can more readily reposition itself to adopt the optimal bond angles and distances for metal binding than can the histidine side chain.

Characterization of Zn(II) binding by competition with Co(II) indicates that Zα4, Zα4H25A, Zα4C47A, and Zα4-H51A bind one equivalent of Zn(II). Direct titration of these proteins with Cd(II), however, yielded an unexpected result: although Zα4, Zα4H25A, and Zα4H51A bind close to one equivalent of Cd(II), Zα4C47A binds one-half equivalent. These data suggest that Zα4C47A forms a metal-linked dimer in the presence of Cd(II), while the other proteins bind with a 1:1 metal-to-protein stoichiometry. The question of why only Zα4C47A forms a Cd(II)−linked dimer can be addressed in terms of hard and soft acid−base theory (Pearson, 1966). Cd(II), being a soft Lewis acid, has a significantly higher affinity for soft sulfur ligands than for hard nitrogen or oxygen ligands. The designed metal-binding site in Zα4C47A contains one cysteine ligand, while a dimeric complex could potentially provide two with one cysteine coming from each protein molecule. Presumably, the associated increase in binding energy is sufficient to offset both the conformational change required to form the dimer and the entropic cost associated with forming a ternary vs a binary complex. We thought that the simplest model for dimerization would involve the two liganding residues from helix 2 (Cys21 and His25) from each of two protein molecules. To test this hypothesis, the double mutant Zα4-C47A:H51A was made. This mutant, which contains only Cys21 and His25 ligands, dimerizes in the presence of Cd(II) and forms a tetrahedral complex with Co(II). Taken together, the data from Zα4C47A, and Zα4C47A:H51A studies

indicate that metal−ligand preferences can play a large role in determining the mode of metal binding in designed proteins. The apparent lack of Cd(II)−dimer formation with $Z\alpha_4$, $Z\alpha_4$H25A and $Z\alpha_4$H51A is likely due to the presence of two cysteine residues in the designed site; thus, the number of thiolate ligands surrounding Cd(II) cannot be increased by dimerization through Cys21 and His25. Other examples of metal-mediated dimerization have been noted following substantial mutagenesis of a Zn(II)-binding zinc finger peptide: Krizek et al. (1993) have reported the Cd(II)−induced dimerization of a Cys_2His_2 zinc finger consensus peptide, while Michael et al. (1992) have observed Co(II)−induced dimerization of a "minimalist" Cys_2His_2 zinc finger. These studies, as well as ours, point to the need for "negative design" elements that disfavor the formation of alternate structures (Michael et al., 1992).

The ligand specificity of the $Z\alpha_4$ Zn(II)-binding site was investigated by mutating the two cysteine residues independently to histidine. Neither of the cysteine-to-histidine mutants appears to bind metal in a tetrahedral fashion as judged from Co(II)-binding experiments, although other nontetrahedral metal−protein interactions have not been ruled out. Apparently, the designed metal site is not sufficiently flexible to accommodate the larger histidine side chain and cannot properly orient either of the two potentially liganding imidazole nitrogen atoms. While we have not been able to substitute cysteine ligands with the larger histidine residue, others have been successful in replacing histidine with cysteine. Krizek et al. (1993) have generated Cys_3His and Cys_4 zinc finger peptides starting from a Cys_2His_2 consensus peptide. All of the ligand mutants bind Co(II), Zn(II), and Cd(II) with high affinity. In the Zn(II) metalloenzyme carbonic anhydrase, His94 has been mutated to cysteine giving a 10^4-fold decrease in the affinity for Zn(II) (Alexander et al., 1993). In both of these cases, there is spectroscopic evidence that the introduced cysteine ligand is indeed binding to metal.

The incorporation of novel catalytic activities into proteins is one of the most ambitious goals of protein design; however, the complexity of enzymes makes this a daunting task. One way to simplify this problem is to recruit a metal ion onto the protein. The single-ligand mutants $Z\alpha_4$H25A and $Z\alpha_4$H51A represent the first designed Zn(II)-binding sites to demonstrably bind metal with one water and three protein ligands. To investigate the potential for Zn(II)−hydroxide based catalysis with $Z\alpha_4$H25A, $Z\alpha_4$C47A, and $Z\alpha_4$H51A, we studied the spectroscopically active Co(II) complexes as a function of pH. No evidence for ionization of the putative metal-bound water was detected up to pH 9.5. This is likely due to the presence of thiolate ligands, which reduce the electrophilicity of the Zn(II) ion more than neutral ligands such as histidine (Kiefer & Fierke, 1994). These results are similar to those obtained with a truncated Cys_2His zinc finger peptide, which retained tetrahedral binding of Co(II) with a water molecule as a putative fourth ligand (Merkle et al., 1991). No change in the Co(II) spectrum was observed up to pH 9.5, again suggesting that the pK_a of the metal-bound water is too high in such a thiolate-rich site. These studies serve as a guide for the design of catalytic Zn(II) sites. To maximize the electrophilicity of the Zn(II) ion, and hence suppress the pK_a of the metal-bound water to the greatest degree possible, histidine-rich Zn(II) sites should be emphasized in future designs.

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