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A Tetrahedral Zinc(II)-Binding Site Introduced into a Designed Protein[†]

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ABSTRACT: The ultimate goal of protein engineering is to create novel proteins which will adopt predetermined structures, bind specified ligands, and catalyze new reactions. Here we describe the successful introduction of metal-binding activity into a model four helix bundle protein. The designed binding site is tetrahedral and is formed by two Cys and two His ligands on adjacent helices. We have introduced this site into the protein and characterized the binding activity. Using ⁶⁵Zn(II), we have shown that the protein binds Zn(II), that the sulfhydryls are essential for binding, and that binding occurs to the protein monomer. The designed protein binds metals with high affinity: we estimate the dissociation constants as 2.5×10^{-8} M for Zn(II) and 1.6×10^{-5} M for Co(II). The characteristic absorption spectrum of the Co(II)-substituted protein fully supports the model of a tetrahedral binding site comprised of two Cys and two His ligands. Circular dichroism studies indicate that no significant changes in secondary structure occur between the metal-bound and metal-free forms of the protein. However, the metal-bound form is substantially stabilized toward denaturation by GuHCl compared to the metal-free form.

The challenge of protein engineering is 2-fold: first, to design and create novel proteins which will fold to give desired structures; second, to incorporate novel activities onto these structural frameworks. In this paper we describe the successful accomplishment of an example of the second stage of the design challenge: the introduction of metal-binding activity onto a designed four helix bundle framework. The site we have introduced is a tetrahedral binding site for metal ions such as Zn(II), Cd(II), and Co(II).

Among natural proteins, tetrahedral Zn(II) sites are by far the most common, serving both structural and catalytic roles. The geometry of such sites in proteins and small molecules is well established from X-ray crystallographic studies. The creation of a tetrahedrally liganded metal-binding site, requiring the precise positioning of four amino acid side chains,

represents a very specific design challenge whose success is readily testable by solution spectroscopic techniques.

The designed protein into which the metal-binding site was introduced has been described previously (Regan & DeGrado, 1988). It is a simplified version of a motif found in several naturally occurring proteins (Weber & Salemm, 1980; Presnel & Cohen, 1989). The essential elements of the protein's structure are four α -helices which are packed nearly antiparallel, at an angle of about 20°, and which are connected by three loops. In natural four helix bundle proteins, four helices of varying length and sequence are connected by three loops which vary in length, sequence, and connectivity. In the model protein the design has been simplified to give minimal sequence complexity: four identical helices are connected by three identical loops. The X-ray crystal structure of the protein has not yet been determined; however, solution studies are consistent with the proposed model of the protein as a stable, compact bundle structure (Regan & DeGrado, 1988; Ho & DeGrado, 1987). We will refer to this model protein as wild-type α_4 , and its sequence is shown in Figure 1A.

We chose to design sites by use of combinations of His and Cys ligands. Natural examples of such tetrahedral Zn(II) sites are found in alcohol dehydrogenase (Vallee & Auld, 1990) and in the TFIIIA-like Zn-finger proteins (Daikun et al., 1986; Parraga et al., 1988; Lee et al., 1989). The site was designed

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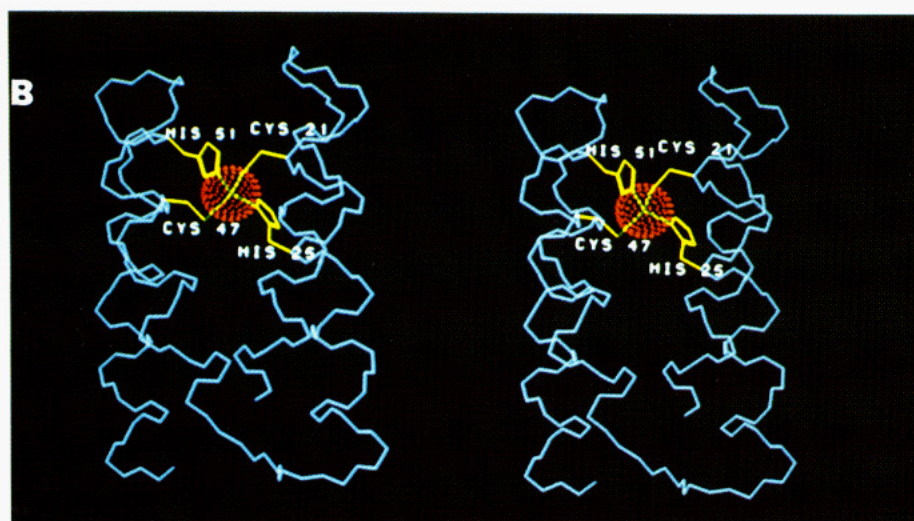
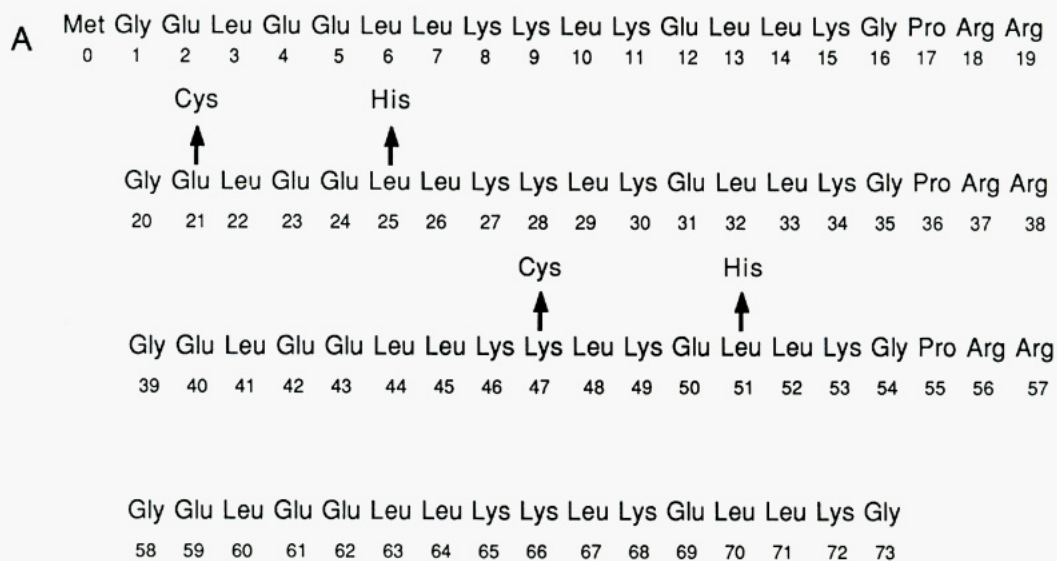


FIGURE 1: (A) Sequence of protein α_4 and positions where changes were made to introduce the metal ligands. (B) Stereo photograph of the model for the metal-binding variant. The backbone of the four helix bundle is shown in blue, the side chains of the four amino acid ligands are labeled and shown in yellow, Zn(II) is indicated by a sphere of red dots, and bonds from ligands to Zn(II) are shown by lines within the sphere. The stereo image is properly viewed by the standard "wall-eyed" or "relaxed" technique.

with the aid of a computer program which used the backbone coordinates of the model for wild-type α_4 and replaced each residue with both Cys and His to find combinations of side chains which could form a tetrahedral site. The potential site we identified is formed by ligands from two adjacent helices. Each helix contributes a Cys and a His which are separated by approximately one turn of the helix (see Figure 1). In this paper we describe the creation of the protein containing the metal-binding site and a detailed characterization of the binding activity. The results fully support the model of metal binding at a tetrahedral site formed by the four designed ligands.

EXPERIMENTAL PROCEDURES

Model Building. An initial version of the model was built by using coordinates for a model of wild-type α_4 (Wasserman and Salemme, personal communication) and replacing residues 21 and 47 with Cys and residues 25 and 51 with His. The choice of these substitutions, the specific type of His ligand (δ N or ϵ N), and the side-chain dihedral angles were obtained from a computer program we wrote for this purpose. The program searches for a combination of four residues which can be mutated to either Cys or His to create a potential

metal-binding site. For each residue the program generates a list of positions that could be occupied by a Zn(II) ion if that residue were Cys or His. The possible positions of a metal ion are dictated by the ligand involved ($S\gamma$ of Cys; $N\delta$ or $N\epsilon$ of His) and the appropriate dihedral angles for the side chain. The program uses subroutines from the PDB_PROTEUS library to generate the initial list of possible positions (Pabo & Suchanek, 1986). The sets of possible metal positions for each residue in the protein are compared to one another, and sets of four residues with potential metal sites in fairly close proximity are analyzed further. At each site the program iteratively attempts to find a consensus position for a metal, while allowing a more complete range of χ angles than was allowed in the initial screen. Finally, the site is assessed for tetrahedrality. The geometry of the initial model was improved by performing energy minimization and simulated annealing with the program X-PLOR (Brunger et al., 1987). The final model has backbone coordinates which differ little from the starting model for α_4 (RMS difference 0.16 Å), unfavorable steric contacts have been removed, and the site has good tetrahedral geometry. Details of the final Zn(II) site are as follows. Bond distances: $S\gamma(21)$ -Zn, 2.38 Å; $S\gamma(47)$ -Zn, 2.34 Å; $N\delta(25)$ -Zn, 2.04 Å; $N\epsilon(51)$ -Zn, 2.03 Å. Tetrahedral

angles: $S\gamma(21)-Zn-N\delta 1(25)$, 115.8° ; $S\gamma(21)-Zn-S\gamma(47)$, 106.9° ; $S\gamma(21)-Zn-N\epsilon 2(51)$, 106.9° ; $N\delta 1(25)-Zn-S\gamma(47)$, 109.8° ; $N\delta 1(25)-Zn-N\epsilon 2(51)$, 107.3° ; $S\gamma(47)-Zn-N\epsilon 2(51)$, 109.9° . Planarity of His-Zn: $C\beta(25)-C\gamma(25)-N\delta 1(25)-Zn$, -4.9° ; $C\gamma(51)-C\delta 2(51)-N\epsilon 2(51)-Zn$, 178.2° . Bond angles with metal: $C\beta(21)-S\gamma(21)-Zn$, 115.7° ; $C\beta(47)-S\gamma(47)-Zn$, 94.2° ; $C\gamma(25)-N\delta 1(25)-Zn$, 138° ; $C\delta 2(51)-N\epsilon 2(51)-Zn$, 128.2° .

Synthesis and Purification of the Metal-Binding Variant. The gene for the metal-binding variant was created by oligonucleotide cassette mutagenesis (Wells et al., 1985) of the gene encoding wild-type α_4 . The protein was produced in *Escherichia coli* with a T7 expression system (Rosenberg et al., 1989) and purified analogously to wild-type protein (Regan & DeGrado, 1988; L. Regan, unpublished observations).

Gel Filtration Chromatography. The protein was stored in solution with dithiothreitol (DTT) to avoid the formation of a disulfide-linked dimer which can occur in the presence of oxygen and in the absence of both metal and reducing agent. To prepare fully reduced apoprotein, the metal-binding variant was incubated with 10 mM reduced DTT in 25 mM Tris (pH 7.5). DTT was subsequently removed by gel filtration on a Sephadex G-25 column, which was equilibrated and run in argon-purged 25 mM Tris (pH 7.5). All subsequent steps were performed in argon-purged buffers. The reduced apoprotein (2×10^{-8} mol) was mixed with 20 mL of $^{65}Zn(II)$ (0.79 mg/mL, 0.5 mCi/mL, Amersham) and chromatographed on a Sephadex G-25 column (0.75 cm \times 18 cm). Fractions were collected and aliquots assayed for protein by the Bradford assay (Bio-Rad) and for $^{65}Zn(II)$ by measurement of cpm on a γ -counter. To prepare protein in which the sulfhydryls were alkylated, reduced apoprotein (2×10^{-8} mol) was incubated in the dark with 10 mM iodoacetamide and 25 mM Tris, pH 7.5. The assay for metal-binding was then performed as described above.

^{65}Zn Blots. These were performed essentially as described (Schiff et al., 1988). Approximately 1 mg of wild-type α_4 and reduced metal-binding variant were electrophoresed on an 18% SDS polyacrylamide gel and then transferred electrophoretically to nitrocellulose. The nitrocellulose filters were rinsed with 100 mM Tris (pH 7.5) and 50 mM NaCl and incubated overnight in the same buffer containing 25 mL of $^{65}Zn(II)$ (0.79 mg/mL, 0.5 mCi/mL, Amersham). The filters were rinsed with buffer alone and exposed to film. This technique was also performed with ^{109}Cd , and similar results were obtained. After autoradiography, filters were briefly stained with an aqueous solution of 0.1% (w/v) amido black and rinsed with water to detect protein bands.

Absorption Spectroscopy. Optical absorption spectra were obtained on a Phillips pu8740/UV/vis spectrophotometer. All spectral manipulations were performed with the software supplied with the instrument.

Circular Dichroism. CD spectra were recorded on a Jobin-Yvon CDVI spectropolarimeter, and spectral manipulations were performed with the software provided.

For the denaturation curves fully reduced metal-binding protein was prepared as described above. Then either DTT was added to 10 mM or $ZnCl_2$ was added to 10 mM, and these stock solutions were used to prepare samples in argon-purged 25 mM Tris (pH 7.5) plus the appropriate concentration of GuHCl. In the case of $Zn(II)$ sample we confirmed that $Zn(II)$ was bound to the protein in these conditions by performing a parallel reconstitution with 10 mM $Zn(II)$ and a trace of $^{65}Zn(II)$ and monitoring bound $Zn(II)$ as described under Gel Filtration Chromatography. As an additional

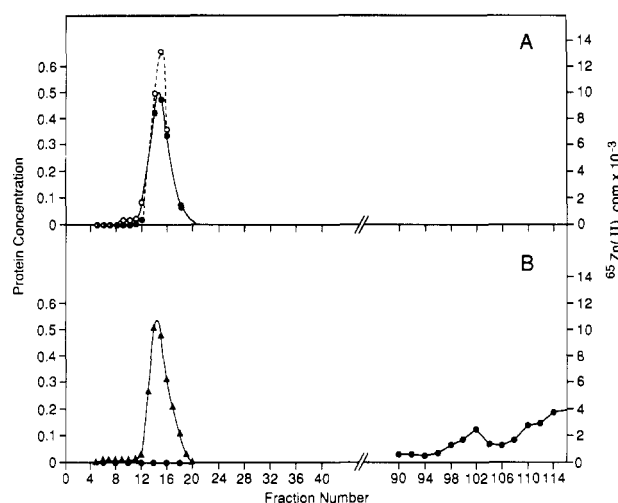


FIGURE 2: Panel A shows the gel filtration profile on a Sephadex G-25 (Pharmacia) column of the metal-binding variant after incubation with $^{65}Zn(II)$: (solid line, solid circles) protein concentration (arbitrary units); (dotted line, open circles) $^{65}Zn(II)$ counts per minute (cpm). Panel B shows the gel filtration profile of protein whose sulfhydryls were alkylated prior to incubation with $^{65}Zn(II)$: (solid line, solid triangles) protein concentration; (solid line, solid circles) $^{65}Zn(II)$ cpm.

precaution, all samples were quenched with iodoacetamide at the end of the experiment and subjected to SDS-polyacrylamide gel electrophoresis in the absence of reducing agents. This served to confirm that no disulfide dimers had formed during the course of the experiment.

RESULTS

Demonstration of Metal Binding by the Novel Protein. Gel filtration chromatography was used in an assay for metal binding. The purified metal-binding protein was incubated with $^{65}Zn(II)$ and chromatographed on a gel filtration column. Figure 2A shows the resulting elution profile. Protein and $^{65}Zn(II)$ peaks are coincident in the void volume of the column, indicating that metal is bound to the protein. Figure 2B shows the elution profile when the protein sulfhydryls were blocked by alkylation prior to incubation of the protein with $^{65}Zn(II)$. Protein is present in the void volume as expected, but no metal is bound to it. Free $^{65}Zn(II)$ ions appear in the included volume of the column. These results suggest that the metal-binding variant does bind $Zn(II)$ and that the sulfhydryls of the designed site are essential for metal binding. Identical results were obtained with $^{109}Cd(II)$.

Although the protein preparation appeared homogeneous on Coomassie Blue stained SDS-polyacrylamide gels, we wished to be certain that the metal-binding activity we observed was a property of the designed protein (and not due to a trace contaminant). For this purpose we used a $^{65}Zn(II)$ blotting technique (Schiff et al., 1988). Proteins were separated on an SDS-polyacrylamide gel and then transferred to nitrocellulose, and the filters were probed with $^{65}Zn(II)$ and exposed to film. Figure 3 shows a $^{65}Zn(II)$ blot of purified "wild type" and purified metal-binding variant. No $^{65}Zn(II)$ -binding activity is observed in the preparation of wild-type protein. For the metal-binding variant, $^{65}Zn(II)$ -binding activity is clearly evident, and this activity migrates with the purified protein. These observations confirm that the binding we observe is indeed a property of the novel protein.

It was important to verify that the protein binds metal as a monomer as there are precedents for $Zn(II)$ -mediated protein oligomerization. Examples include insulin (His ligands) (Blundell et al., 1972) and Tat, the transactivating protein of HIV (Cys ligands) (Frankel et al., 1988). We compared the

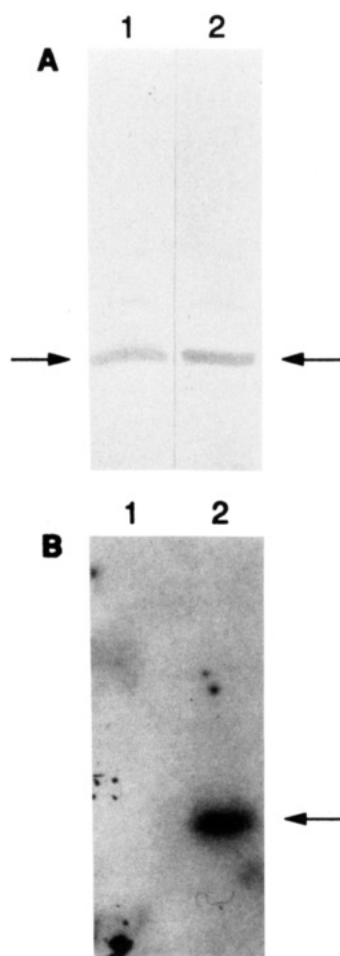


FIGURE 3: (A) Nitrocellulose filters stained for protein with amido black. In lane 1 is α_4 and in lane 2 the metal-binding variant. The arrows indicate the protein bands. (B) Autoradiographs of filters after incubation with $^{65}\text{Zn(II)}$. In lane 1 is α_4 and in lane 2 the metal-binding variant. The arrow indicates the radioactive band.

gel filtration elution profile of protein to which Zn(II) was bound with that of the fully reduced apoprotein. The two forms of the protein showed identical elution profiles and eluted at a position consistent with their monomeric molecular weight (data not shown). This result supports the model of a metal-binding site formed exclusively by ligands from one protein monomer.

Spectral Properties of the Co(II)-Substituted Protein. To obtain information on the nature of the metal-binding site, we took advantage of the spectral properties of the Co(II)-substituted protein. Studies with model compounds and metalloproteins of known structure allow the spectra of Co(II)-substituted proteins to be interpreted with respect to the nature of the metal ligands and the geometry of the binding site (Bertini & Luchinat, 1984). Figure 4 shows the absorption spectrum of the Co(II)-substituted metal-binding protein. The important features of the spectrum are the absorption envelope at long wavelength and the charge-transfer band at 300 nm. The absorption envelope at long wavelength has a maximum at 615 nm ($\epsilon = 1097 \text{ M}^{-1}\text{cm}^{-1}$) and shoulders at 557 nm ($\epsilon = 823 \text{ M}^{-1}\text{cm}^{-1}$) and at 664 nm ($\epsilon = 823 \text{ M}^{-1}\text{cm}^{-1}$). The intensity of this maximum is fully consistent with tetrahedral coordination of the Co(II) ion and rules out higher coordination numbers. The charge-transfer band, with maximum at 300 nm ($\epsilon = 7232 \text{ M}^{-1}\text{cm}^{-1}$), is clearly indicative of cysteine coordination. Measurements performed on Co(II)-substituted proteins and model compounds indicate that, as the number of thiolate ligands increases, the absorption

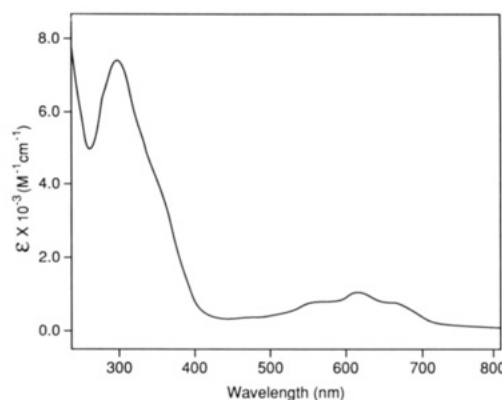


FIGURE 4: Optical absorption spectrum of the complex of metal-binding variant with Co(II) in 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5). The spectrum has been corrected by subtracting the absorption of the fully reduced apoprotein.

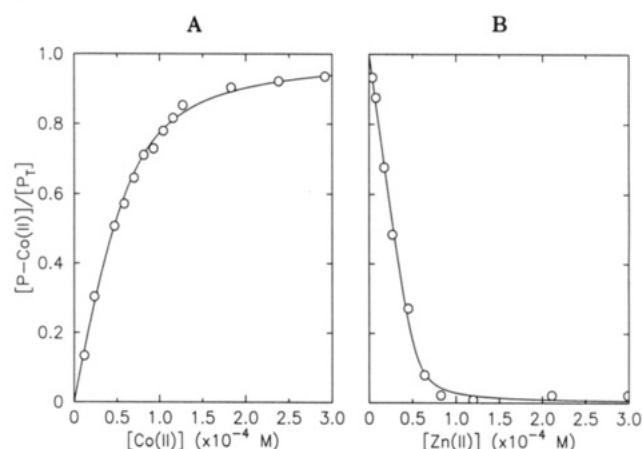


FIGURE 5: (A) Titration of the metal-binding variant with Co(II). Aliquots of CoCl_2 in 20 mM HEPES (pH 7.5) were added to a solution of reduced protein ($6 \times 10^{-5} \text{ M}$) in the same buffer, and the absorbance spectrum of the solution was monitored. The spectra were corrected for dilution and for absorbance due to the peptide alone. The concentration of complex ($[\text{P-Co(II)}]$) divided by the total protein concentration ($[\text{P}_T]$) is plotted against the total Co(II) concentration ($[\text{Co(II)}]$). The fit of the data, assuming noncooperative binding of one molecule of Co(II) to one molecule of protein, is shown by the solid line. (B) Titration of the protein-Co(II) complex with Zn(II). Aliquots of ZnCl_2 were added to a solution of metal-binding variant ($5.3 \times 10^{-5} \text{ M}$) and CoCl_2 ($9 \times 10^{-4} \text{ M}$) and the absorbance spectra recorded. $[\text{P-Co(II)}]/[\text{P}_T]$ is plotted against the total concentration of Zn(II). The solid line shows a fit of the data, assuming a simple competition between Co(II) and Zn(II) in binding to the protein, with a dissociation constant for the protein-Co(II) complex of $1.57 \times 10^{-5} \text{ M}$.

maxima of charge-transfer bands and of the absorption envelope of d-d transitions move toward longer wavelengths (Corwin et al., 1987, 1988; Swenson et al., 1978; Green & Berg, 1989). The wavelengths of the maxima that we observe are most consistent with Co(II) coordination in a (thiolate)₂(imidazole)₂ environment. These results lend strong support to the proposed model of metal coordination at a tetrahedral site formed by two His and two Cys ligands.

The Designed Protein Binds Metals with High Affinity. The characteristic absorption of the protein-Co complex was used to monitor binding in a titration of protein with Co(II). The dissociation constant for the protein-Co complex was measured directly and the dissociation constant for the protein-Zn complex by complexation. Figure 5A shows an example of a Co(II) titration, with the solid line representing a nonlinear least-squares fit of the data. From such experiments we estimate the dissociation constant for the protein-

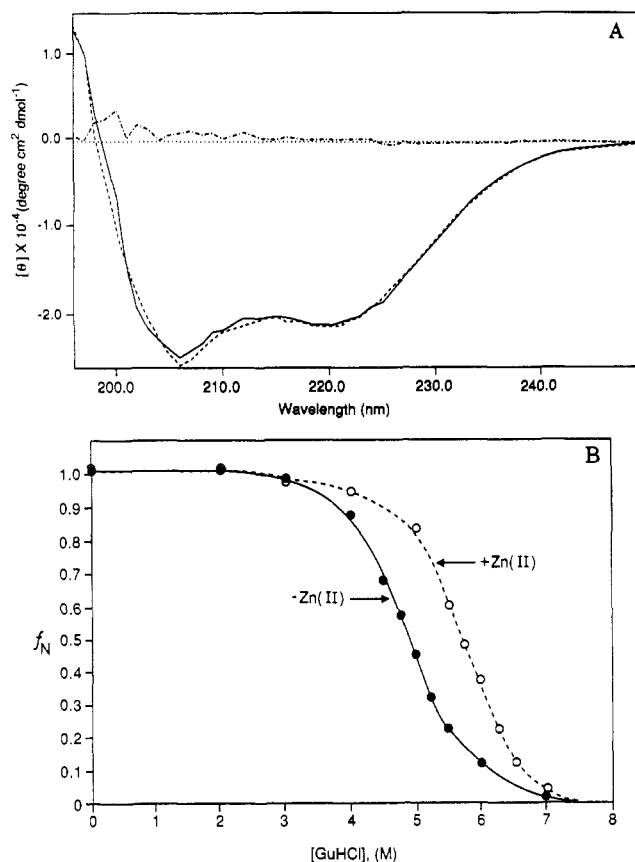


FIGURE 6: (A) CD spectra of the metal-binding protein at a concentration of 34.5 mM in 25 mM Tris (pH 7.5) in the absence of Zn(II) (dashed line) or with 10 mM Zn(II) (solid line) and the difference between the two spectra (dot dash line). (B) GuHCl denaturation curves of the metal-binding variant in the absence or presence of Zn(II). The curves are plotted as f_N (the observed mean residue ellipticity at 222 nm at a given concentration of denaturant, normalized to the observed mean residue ellipticity in the absence of denaturant) versus the concentration of denaturant.

Co(II) complex to be 1.6×10^{-5} M. To measure the relative affinity of the protein for Zn(II) we titrated the protein-Co(II) complex with Zn(II) in the presence of excess Co(II) (see Figure 5B). The solid line represents a fit of the data, giving a dissociation constant for the protein-Zn(II) complex of 2.5×10^{-8} M. The dissociation constants of the protein-Co(II) and protein-Zn(II) complexes can be compared with those reported for a (Cys)₂(His)₂ Zn-finger peptide, which are 3.8×10^{-6} and 2.8×10^{-9} M, respectively (Berg & Merkle, 1989). The lower affinity for Co(II) than for Zn(II) observed for both our model protein and for the Zn-finger peptide is consistent with binding at a tetrahedral site. Co(II) is expected to bind less tightly than Zn(II), primarily because the change from octahedral $[\text{Co}(\text{H}_2\text{O})_6^{2+}]$ to tetrahedral coordination on binding will be accompanied by a loss of ligand field stabilization energy for Co(II) but not for Zn(II). Thus the relative affinities we observe provide additional support for the model protein binding metals at the designed tetrahedral site.

Effects of Metal Binding on Protein Structure and Stability. Among natural metalloproteins the effects of metal binding on protein structure and stability are diverse. In some proteins the metal site is preformed in the apoprotein, for example, in plastocyanin and superoxide dismutase (Carrico & Deutsch, 1970; Garrett et al., 1984), whereas in others the proteins is essentially unfolded in the absence of metal, as seen with CUP2 (ACE1) and gene 32 protein (Furst et al., 1988; Buchman et al., 1989; Giedroc et al., 1986). To study the effect of metal binding, we used circular dichroism (CD) to monitor changes

in protein secondary structure. Figure 6A shows CD spectra of the apoprotein and the protein with Zn(II) bound and the difference spectrum between the two. It is evident that little, if any, change in secondary structure occurs upon metal binding.

We also compared the stabilities of the apo and holo forms of the metal-binding variant. Figure 6B shows guanidine hydrochloride induced denaturation curves of the apo and Zn(II)-bound forms. The metal-bound form of the protein is more stable toward denaturation than the metal-free form, as would be expected if metal binds preferentially to the folded protein. From these results we would argue that the helices are suitably positioned to enable the designed metal-binding site to form without large structural perturbations.

DISCUSSION

We have described a simple approach to the design of a metal-binding site in a protein. Important features of the final design include excellent tetrahedral geometry, minimal displacement of backbone atoms from the starting model, and absence of severe steric contacts.

We were able to easily implement the design by use of oligonucleotide cassette mutagenesis and to purify the protein to homogeneity with procedures developed for the wild-type protein. Binding of the protein to metal ions was demonstrated spectroscopically and by comigration of protein and metal-binding activity on both gel filtration columns and on SDS-polyacrylamide gels. Zn(II) binds tightly to the protein and stabilizes the metal-binding α_4 protein against denaturation by GuHCl, with negligible apparent effect on the secondary structure.

An important feature of the binding site design is that it is tetrahedral. Therefore, it was essential to perform the Co(II) substitution experiments to demonstrate this property. The spectrum of the Co(II)-substituted protein clearly shows an envelope of d-d transitions at long wavelength which are of an intensity definitive of a tetrahedral site and fully consistent with metal coordination in a (thiolate)₂(imidazole)₂ environment.

In summary, the characterization of binding activity presented here provides strong support for the proposed model of the metal-binding site. Having incorporated a precisely defined novel activity into a model protein, we would like to suggest that this work provides at least a hint at the potential for the future de novo design of receptors and catalysts. For instance, tetrahedral metal-binding sites with three protein ligands and a bound water molecule have an obvious potential for catalytic function.

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Registry No. Cys, 52-90-4; His, 71-00-1; Zn(II), 7440-66-6; Cd(II), 7440-43-9; Co(II), 7440-48-4.

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Articles

Induction of Phosphoenolpyruvate Carboxykinase Gene Expression by Retinoic Acid in an Adult Rat Hepatocyte Line

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ABSTRACT: Regulation of expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene was examined in an adult rat hepatocyte line, RALA255-10G, that was immortalized with an SV40 temperature-sensitive (ts) A mutant. These hepatocytes express a transformed phenotype at the permissive temperature (33 °C) but a differentiated liver phenotype at the nonpermissive temperature (40 °C). We have shown previously that RALA255-10G cells express only low levels of liver-specific genes such as albumin and tyrosine aminotransferase at 33 °C. In the present study, we demonstrated that at 33 °C, PEPCK synthesis and mRNA expression could be detected only in the simultaneous presence of dexamethasone (DEX), retinoic acid, and dibutyryl-cAMP (Bt₂cAMP). At 40 °C, PEPCK synthesis and mRNA expression were demonstrated in the presence of Bt₂cAMP alone, but not in the presence of either DEX or retinoic acid. However, at 40 °C, PEPCK gene expression was stimulated by the combination of DEX plus retinoic acid; additionally, DEX and retinoic acid potentiated the Bt₂cAMP-mediated PEPCK induction. In RALA255-10G cells, optimal PEPCK gene expression required the simultaneous presence of DEX, retinoic acid, and Bt₂cAMP; DEX had to be present at all times. Triiodothyronine (T₃) also potentiated the Bt₂cAMP-mediated PEPCK gene expression but failed to increase further the induction by DEX/retinoic acid/Bt₂cAMP. By performing nuclear runoff assays, we demonstrated that the PEPCK gene transcription rate in the absence or presence of inducing agents was closely related to the levels of the corresponding mRNAs. At 40 °C, the PEPCK gene transcription rate in the presence of DEX/retinoic acid/Bt₂cAMP was approximately 10-fold higher than that in the presence of DEX/Bt₂cAMP, retinoic acid/Bt₂cAMP, or DEX/retinoic acid. Thus, regulation of expression of the rat PEPCK gene by retinoic acid as well as cAMP and glucocorticoid hormone is primarily regulated at the transcriptional level.

The synthesis of hepatic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK), the rate-limiting enzyme for

gluconeogenesis, is under developmental and multihormonal controls [for reviews, see Hod et al. (1986) and Gluecksohn-Waelsch (1986)]. In the liver, the PEPCK gene is expressed primarily after birth, and its transcription rate is stimulated by cAMP (Lamers, et al., 1982), glucocorticoids (Magnuson et al., 1987), and thyroid hormones (Loose et al., 1985) and inhibited by insulin (Granner et al., 1983). Accordingly,

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