NMR Spectroscopy of ^{113}Cd(II)-Substituted Gene 32 Protein†

David P. Giedroc,‡ Bruce A. Johnson,§ Ian M. Armitage, and Joseph E. Coleman*

Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT: Gene 32 protein (g32P), the single-stranded DNA binding protein from bacteriophage T4, contains 1 mol of Zn(II)/mol of protein. This intrinsic zinc is retained within the DNA-binding core fragment, g32P-(A+B) (residues 22–253), obtained by limited proteolysis of the intact protein. Ultraviolet circular dichroism provides evidence that Zn(II) binding causes significant changes in the conformation of the peptide chain coupled with alterations in the microenvironments of tryptophan and tyrosine side chains. NMR spectroscopy of the ^{113}Cd(II) derivative of g32P-(A+B) at both 44.4 and 110.9 MHz shows a single ^{113}Cd resonance, δ 637, a chemical shift consistent with coordination to three of the four sulfhydryl groups in the protein. In vitro mutagenesis of Cys^{166} to Ser^{166} creates a mutant g32P that still contains 1 Zn(II)/molecule. This mutant protein when substituted with ^{113}Cd(II) shows a ^{113}Cd signal with a δ and a line width the same as those observed for the wild-type protein. Thus, the S^- ligands to the metal ion appear to be contributed by Cys^{77}, Cys^{87}, and Cys^{90}. Relaxation data suggest that chemical shift anisotropy is the dominant, but not exclusive, mechanism of relaxation of the ^{113}Cd nucleus in g32P, since a dipolar modulation from ligand protons is observed at 44.4 MHz but not at 110.9 MHz. Complexation of core ^{113}Cd g32P with $d(pA)_6$ or Co(II) g32P with poly(dT) shows only minor perturbation of the NMR signal or d–d electronic transitions, respectively, suggesting that the metal ion in g32P does not add a ligand from the bound DNA. The visible absorption spectra of the Co(II) derivative of g32P and its Ser^{166} mutant suggest an approximately tetrahedral ligand field around the Co(II) ion in g32P. ^{35}Cl NMR shows that the Zn(II) site is not accessible to anions from solution, which suggests that a fourth ligand from the protein completes the tetrahedral complex. The best candidate is an imidazole nitrogen from His^{81}. A simple molecular modeling procedure shows that a series of peptide turns compatible with peptide backbone angles found in proteins can accommodate the formation of a tetrahedral Zn(II) complex with Cys^{77}, His^{81}, Cys^{87}, and Cys^{90} as ligands.

Gene 32 protein from bacteriophage T4 binds single-stranded nucleic acids regardless of sequence and functions in T4 DNA replication by binding cooperatively to single-stranded DNA (ssDNA) transiently formed just upstream of the replication fork [for review see Chase and Williams (1986)]. Proteolytic removal of two small amino- and carboxy-terminal domains yields the protease-resistant DNA-binding core domain of g32P[1] [residues 22–253, g32P-(A+B)] (Moise & Hosoda, 1976; Williams & Konigsberg, 1978). This fragment binds noncooperatively to polynucleotide lattices, while the determinants for binding to a single-site DNA lattice remain essentially unchanged (Spicer et al., 1979). The binding of core g32P to small oligonucleotides has been probed by proton NMR and provides evidence for the partial intercalation of nucleic acid bases with the side chains of five Tyr and two Phe residues (Prigodich et al., 1984, 1986). The primary structure of g32P shows six Tyr residues to be relatively regularly distributed from residue 72 to residue 116 (Williams et al., 1981). Site-directed mutagenesis shows two of these to contribute $\sim$1 kcal mol^{-1} each to the overall free energy of polynucleotide binding (Shamoo et al., 1987).

At the extreme amino-terminal end of this tyrosine array is found the sequence Cys^{77}-X_3-His^{81}-X_5-Cys^{87}-X_2-Cys^{90}, believed to contribute the ligands to the single Zn(II) ion tightly incorporated into g32P (Giedroc et al., 1986; Coleman & Giedroc, 1988). Physicochemical studies of the Zn(II) and Co(II) g32Ps show the metal ion to be coordinated to at least three S^- donors in a tetrahedral ligand arrangement (Giedroc et al., 1986, 1987). A fourth ligand is currently proposed to be an imidazole nitrogen atom of His^{81} (Giedroc et al., 1986). Oligo- and polynucleotide binding studies, partial proteolysis, and differential scanning microcalorimetry show that zinc provides structural stabilization to the molecule and induces a conformation such that monomer–monomer contacts can form on cooperative binding to ssDNA (Giedroc et al., 1987; Keating et al., 1987, 1988).

The intrinsic Zn(II) of g32P can be substituted with other divalent metal ions including Co(II) and Cd(II) with minimal perturbation of the protein structure and affinity of the molecule for ssDNA (Giedroc et al., 1986, 1987). Previous NMR experiments with both model ^{113}Cd complexes and various metalloproteins substituted with ^{113}Cd(II) have shown that the ^{113}Cd chemical shift and relaxation parameters are sensitive to both the nature and number of metal ligands as well as the geometry of the binding site [for review see Armitage and Otvos (1982)]. In this paper, we use NMR spectroscopy of the ^{113}Cd-substituted wild-type and mutant g32Ps to further define the structural features of the metal complex and its relationship to the bound nucleotide. Circular

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* To whom correspondence should be addressed.

‡ Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

§ Merck, Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065.

[1] Abbreviations: CD, circular dichroism; CSA, chemical shift anisotropy; g32P, gene 32 protein; g32P-(A+B), g32P core protein, g32P*, g32P residues 22–253; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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dichroism has been used to detect conformational changes induced by metal ion binding.

Materials and Methods

Materials. Gene 32 protein was purified from T4-infected *Escherichia coli* through the DNA–cellulose step according to the method of Bittner et al. (1979). Partial proteolysis of intact g32P to yield g32P core fragment was carried out by incubation of 0.5 mg of trypsin-TPCK (CalBiochem)/μmol of g32P for 60–90 min, 16 °C, followed by addition of 5 μL of undiluted diisopropyl fluorophosphate (Sigma) and rechromatography on ssDNA–cellulose. Homogeneous g32P core fragment was eluted with a step to 0.5 M NaCl in the standard buffer (Bittner et al., 1979) and pooled. ^{113}Cd(II) substitution of the intrinsic zinc was accomplished by addition of excess $^{113}CdCl_2$ (96% enriched, buffered at pH 8) directly to the Zn(II) protein, which induces metal ion exchange, or to the metal-free apoprotein, followed in both cases by exhaustive dialysis against the chelexed NMR buffer, 50 mM sodium phosphate and 30 mM NaCl, pH 8.0, and concentration by ultrafiltration. Atomic absorption analysis of the all ^{113}Cd(II) g32P preparations showed greater than 90% substitution of the zinc sites by Cd(II). NMR samples contained 2 mL of g32P [0.5–0.9 mM in ^{113}Cd(II)] in the above indicated NMR buffer containing 10–20% v/v D_2O-exchanged buffer as lock. In performing the chloride titration experiment, the protein was dialyzed against 50 mM sodium phosphate, pH 8, and reconcentrated. Small amounts of a chelexed stock solution of 4 M NaCl were then added directly to the NMR tube to achieve the desired final [Cl$^-$]. Co(II) g32P was prepared as previously described (Giedroc et al., 1986).

Site-Directed Mutagenesis. Standard cloning manipulations were carried out according to the method of Maniatis et al. (1982). The single-stranded form of M13mp8 with the gene 32 protein structural gene cloned between the *Eco*RI and *Bam*HI sites at its 5′ and 3′ boundaries, respectively, was used as template for oligonucleotide-directed mutagenesis (Shamoo et al., 1986). A synthetic 19-mer oligonucleotide was designed to change codon 166 from TGT (Cys) to AGT (Ser) with the concomitant introduction of a unique *Spe*I restriction site used for screening. This oligo was used as primer for Klenow in initiating mutagenesis according to the method of Taylor et al. (1985). Expression of the mutant C166S g32P gene was accomplished by conversion of the 5′ *Eco*RI site to a *Xba*I site with synthetic linkers, cutting at the 3′-terminus with *Bam*HI, and cloning the so-generated *Xba*I–*Bam*HI full-length g32P gene between the unique *Nhe*I and *Bam*HI sites of pTL18xw (T. Lin, unpublished observations) under the transcriptional control of the λ P_L promoter ($pP_Lg32.C166S$). This plasmid contains a copy of the gene encoding the λ cI repressor t_s mutant cI847 repressor. C166S g32P was purified as described above from 10-L cultures of $pP_Lg32.C166S$/HB101 induced at A_{600} = 0.9 by shifting from 30 to 41 °C and continuing growth for 2 h. Cysteine content was determined for both the wild-type and C166S g32Ps by DTNB titration in 4 M urea (4.0 and 2.7 mol/mol of protein, respectively) and by amino acid analysis of hydrolyzed samples subjected to performic acid oxidation (4.3 and 2.9 mol/mol, respectively). C166S g32P contained stoichiometric Zn(II) (0.91 mol/mol).

Circular Dichroism. CD spectra were collected on an Aviv 60 spectropolarimeter operating at 25 ± 0.2 °C in a 2-mm path length cell (volume, 2.4 mL). All spectra were recorded in duplicate and were base-line corrected by averaged scan derived from duplicate buffer only scans (deviation between duplicate scans ≤2.0 mdeg). For far-UV CD measurements, spectra were recorded with a time constant of 1 s and a spectral band width of 1.5 nm; data points were taken every 0.5 nm. For CD measurements in the region of the aromatic chromophores, the time constant was increased to 5 s. Difference spectra were obtained by microprocessor-controlled subtraction of the appropriate scans. All spectra are presented in units of molar ellipticity $[\theta]_M$ (deg cm^2 dmol^{-1}).

NMR Methods. ^{113}Cd NMR spectra were recorded at 298 K on either a Bruker MSL-200 spectrometer (4.7 T, 44.4 MHz for ^{113}Cd) or a Bruker AM-500 spectrometer (11.75 T, 110.9 MHz for ^{113}Cd), both equipped with a broadband tunable probe that accommodates 10-mm (o.d.) NMR tubes. Spin–lattice relaxation times (T_1) were estimated for g32P-(A+B) samples at 44.4 MHz by using a progressive saturation method and at 110.9 MHz by using an inversion recovery method with a composite pulse inversion employing four or five τ values. Data were fit with a nonlinear least-squares method to the two-parameter inversion recovery equation (Ernst et al., 1987). NOE (η + 1) values were determined at both magnetic field strengths by dividing the integrated area of the signal in the presence of continuous proton decoupling by the area obtained without proton decoupling. Chemical shifts are reported relative to the resonance position of 0.1 M $Cd(ClO_4)_2$. ^{35}Cl NMR employed the broadband probe used for the ^{113}Cd NMR on the Bruker MSL-200 operating at a frequency of 19.59 MHz. Samples consisted of 1.8 mL of buffer (50 mM sodium phosphate, 0.2 M NaCl, pH 8) alone or in the presence of 0.3 mM Zn(II) or apo-g32P in an NMR tube (10-mm o.d.) fitted with an external capillary containing D_2O as lock. A 15° pulse and a sweep width of 2000 Hz (digital resolution, 0.5 Hz/point) were used for data acquisition.

Optical Spectroscopy. Visible spectra of Co(II)-substituted g32P [66.5 μM in 10 mM Tris-HCl, pH 8, 0.2 M NaCl, 5% (v/v) glycerol] were recorded on a Cary 219 spectrophotometer at 25 °C. Small aliquots of poly(dT) were added to give the indicated ratio of phosphate/g32P. Identical manipulations were carried out on the Zn(II) protein in the reference cell. Spectra were digitized manually (10-nm resolution) and base-lined subtracted, and the absorbance was converted to extinction coefficient per mole of Co(II). Under identical buffer conditions, the binding of poly(dT) to Co(II) g32P was also assessed by monitoring the quenching of the intrinsic protein fluorescence upon addition of polynucleotide as described previously (Giedroc et al., 1987).

Distance Geometry. Distance geometry calculations were executed on a Sun 4/280S computer using the program Dspace (Hare Research Inc.). The residue data set provided with the program was used to provide the distance constraints implicit in the primary structure of the metal-binding region of g32P, i.e., Thr^{76}–Gln^{91}. Upper and lower bounds were added to the distance matrix to ensure that ligand–metal distances reflected known bond lengths of model compounds as follows: Cd(II)–S (2.45–2.55 Å), Cd(II)–N$^{\epsilon}$ (2.30 Å) (Corwin et al., 1987). Upper and lower bounds were set on ligand–ligand (S–S, 3.85–4.20 Å; S–N, 3.65–4.00 Å) distances to ensure a tetrahedral geometry as suggested by the spectroscopic data. Ten trial structures were generated from the distance matrices containing distances randomly interpolated between the upper and lower bounds. These were refined with the Dspace minimizer until the penalty function was below a value of 0.07. Every 64 steps of refinement the atomic coordinates were randomly displaced by an amount that was decreased at each cycle of randomization. This method of annealing the coordinates provides an escape from local minima (of the penalty function) and was found to converge to the desired minimum without manual intervention. The resulting structures were

compared by using the molecular graphics program MSURF (Johnson, 1987), running on the Sun 4/280S color graphics display.

RESULTS

Circular Dichroism of Apo Gene 32 Protein Reconstituted with Zn(II) and Cd(II). Metal-free apo-g32P shows a significant reduction in the cooperative mode of binding to polynucleotides, and the binding constant, K_a, is reduced by approximately 3 orders of magnitude upon removal of the Zn(II) (Giedroc et al., 1987). In addition, the apoprotein is destabilized relative to the native Zn(II) protein, a destabilization that has been quantitatively analyzed by differential scanning calorimetry (DSC) (Keating et al., 1988). The Zn(II) protein undergoes a temperature-induced unfolding characterized by a cooperative transition with a T_m of 55 °C, while the apoprotein unfolds over a somewhat broader transition with a T_m of 49 °C.

Quantitative DSC reveals denaturational enthalpy, ΔH_d, values of 138 and 89 kcal mol^{-1} for the Zn(II) and apoproteins, respectively (Keating et al., 1988). Such a large $\delta\Delta H_d$, 49 kcal mol^{-1}, contributed by the binding of Zn(II) to g32P is similar to the $\delta\Delta H_d$ between the apo and Zn(II) forms of other Zn(II) metalloproteins that have been studied by DSC (Keating et al., 1988). On the other hand, most Zn(II) proteins are thermally stabilized to a much greater extent by Zn(II) binding, as shown by a 20–30°C rise in T_m in each case (Keating et al., 1988). The finding of a relatively small thermal stabilization of g32P, ΔT_m = 6 °C, induced by Zn(II) binding suggests that Zn(II) may induce some additional conformational changes in the folded form of the apo-g32P that result in a reasonably large unfavorable entropy term being associated with formation of the Zn(II) complex (Keating et al., 1988). Hence, the large $\delta\Delta H_d$ characterizing the formation of the Zn(II) complex in g32P is not associated with the expected large increase in T_m.

If conformational changes induced by Zn(II) binding are relatively large, they may be reflected in the circular dichroism (CD) of the peptide bond chromophores. The far-ultraviolet CD spectra of apo-g32P and the apo-g32P reconstituted with stoichiometric Zn(II) are shown in Figure 1A. While both proteins show grossly similar CD profiles in the far-ultraviolet, indicating no major unfolding of the polypeptide to accompany Zn(II) removal, there are a number of small changes in the CD profile accompanying Zn(II) addition to the apoprotein. These changes are best illustrated by the difference CD spectrum between apo-g32P-(A+B) reconstituted with stoichiometric Zn(II) and that of the starting apo-g32P-(A+B) (Figure 1B). The two most prominent difference bands occur in the region 230–200 nm coincident with the major n–π^* and π–π^* transitions characteristic of α-helical structure. The Zn(II)-reconstituted protein shows a maximum $\delta[\theta]_M$ relative to the apoprotein of -2×10^5 deg cm^2 dmol^{-1} at 222 nm and a further negative difference that extends to the region below 200 nm. Converting to units of mean residue ellipticity and assuming for the sake of illustration that an α-helix is the secondary structure involved, the indicated $\delta[\theta]_{mwr} = -8 \times 10^{-2}$ deg cm^2 dmol^{-1} would correspond to $\sim 7 \pm 1$ residues induced to form helix by Zn(II) binding (Chang et al., 1978; Giedroc et al., 1983). Thus, small but significant changes in backbone folding appear to accompany metal ion binding to g32P.

Since the Zn(II) in g32P is coordinated to S^- ligands, the complex should have intense ligand to metal charge-transfer bands centered near 210 nm and extending out as far as 230 nm (Kagi & Vallee, 1961). In disymmetric protein environments, such charge-transfer bands may acquire relatively intense optical activity as is best illustrated by the analogous $S^- \rightarrow$ Cd(II) charge-transfer bands centered near 250 nm in metallothionein, bands that can more easily be separated from the peptide chromophores (Kagi & Vallee, 1961; Vasak et al., 1987). These have been observed to have ellipticity values as large as $[\theta]_M = 5 \times 10^4$ deg cm^2 dmol^{-1}/Cd(II) ion (Vasak et al., 1987). Thus, some of the additional negative ellipticity of the Zn(II) g32P present at 222 nm could be due to the superposition of optically active $S^- \rightarrow$ Zn(II) charge-transfer bands. That this does not appear to be the case is shown by the difference CD spectrum formed by adding Cd(II) instead of Zn(II) to the apoprotein, which is identical to that produced by adding Zn(II) (Figure 1B).

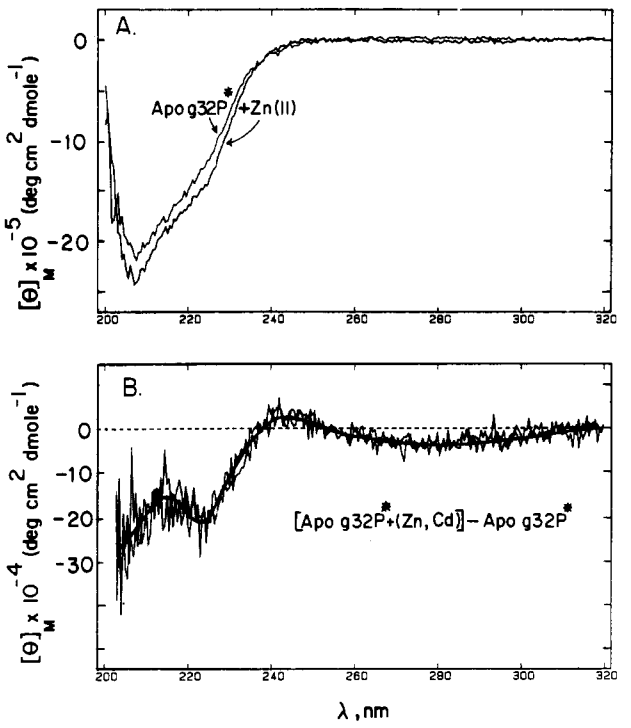

FIGURE 1: Effect of Zn(II) or Cd(II) reconstitution on the far-UV circular dichroism (CD) of apo gene 32 protein: (A) Far-UV CD of 10 μM metal-free apo-g32P-(A+B) (g32P*) prepared by the PMPS method (Giedroc et al., 1986) and the same reconstituted with stoichiometric Zn(II); (B) difference far-UV CD ($\delta[\theta]_M$ in deg cm^2 dmol^{-1}) of apo-g32P-(A+B) plus Zn(II) [or Cd(II)] less that of apoprotein. Conditions: 50 mM sodium phosphate, 30 mM NaCl, 1 mM β-mercaptoethanol, pH 8. The actual CD tracings are shown with a smooth line drawn through the average position for the Zn(II) and Cd(II) difference spectra shown in (B).

The Environment of the Aromatic Chromophores in Gene 32 Protein Is Altered by Zn(II) Binding. The difference CD spectra in Figure 1B show small but significant changes in ellipticity extending from 240 to 300 nm to accompany the addition of metal ion to the apoprotein. These changes reflect a change in the optical activity of the aromatic chromophores of g32P. The fine structure of these transitions before and after Zn(II) reconstitution to the apoprotein is shown in Figure 2A. One or more of the five tryptophan chromophores in the molecule have relatively intense Cotton effects. The two small bands positioned at 291 and 281 nm assignable to the indole chromophore (Anderson & Coleman, 1975) are located in a region of general negative ellipticity in the Zn(II) protein ($\sim -2 \times 10^4$ deg cm^2 dmol^{-1}) (Figure 2A). In the CD spectrum of the apoprotein, the ellipticity of these transitions is unchanged, but they now overlie an area of positive ellipticity (Figure 2A). Thus, relatively large changes ($\delta[\theta]_M = -4.5 \times 10^{-4}$ deg cm^2 dmol^{-1}) in the magnitude of broad ellipticity bands from 300 to 260 nm associated with the aromatic chromophores are

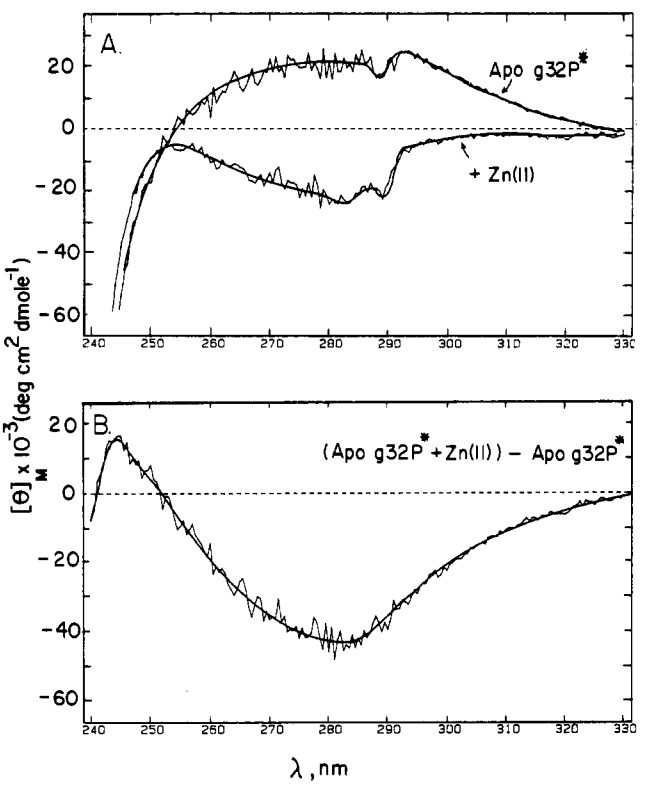

FIGURE 2: Effect of Zn(II) reconstitution on the near-UV CD of apo gene 32 protein: (A) Aromatic CD spectra of 250 μM apo-g32P-(A+B) and the same reconstituted with a slight excess of Zn(II); (B) difference near-UV CD of apo-g32P-(A+B) plus Zn(II) less that of the starting apoprotein. Conditions: 10 mM Tris-HCl, 0.1 M NaCl, 5% (v/v) glycerol, 1 mM β-mercaptoethanol, pH 8. The direct CD tracings are shown with a smooth line drawn through the average position of the pen. These spectra have a digitized resolution of 0.5 nm.

induced by Zn(II) binding (Figure 2B).

Since gene 32 protein contains eight tyrosyl residues, the observed aromatic optical activity extending from 260 to 230 nm may in fact superimpose contributions from the phenolic and indole chromophores. Thus, the prominent positive difference peak centered at 244 nm ($[\theta]_M \sim 1.4 \times 10^{-4}$ deg cm^2 dmol^{-1}) in the difference CD of the aromatic region may be assignable to changes in either Tyr or Trp chromophores or both (McFarland & Coleman, 1972) (Figure 2B).

^{113}Cd NMR of Cd(II) Gene 32 Protein and Cd(II) Gene 32 Protein-(A+B). The ^{113}Cd NMR spectrum of ^{113}Cd(II) g32P is shown in Figure 3A, while that of ^{113}Cd(II) g32P-(A+B) (residues 22–253) is shown in Figure 3B. Both proteins show a single ^{113}Cd resonance at 637 ± 0.3 ppm. The ^{113}Cd line width for the native form of the protein is ~180 Hz at 110 MHz, and that of g32P-(A+B) is ~110 Hz at the same field. These line widths are compatible with the difference in the rotational correlation times, τ_r, expected for these two species. Removal of the A and B domains results in formation of a "core" dimer as shown by small-angle X-ray scattering (Tao Pan and Coleman, unpublished results), while the holoprotein is extensively oligomerized [cf. Prigodich et al. (1984)].

The chemical shifts of ^{113}Cd(II) substituted in the metal-binding sites of a number of metalloproteins have allowed a general semiempirical correlation to be made between observed chemical shift and the nature of the ligand donor atoms, O, N, or S [see Armitage and Otvos (1982)]. Relatively symmetrical sites composed exclusively of oxygen ligands [usually octahedral, e.g., Ca(II)-binding sites] show chemical shifts ranging from 0 to −125 ppm. Sites composed of nitrogen

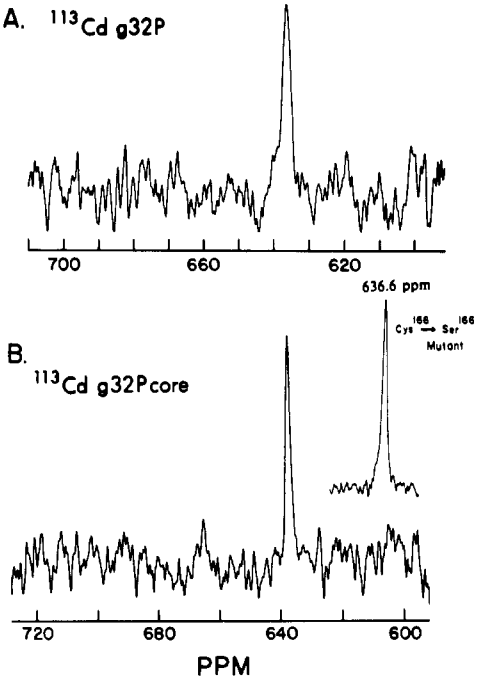

FIGURE 3: ^{113}Cd NMR spectra at 110.9 MHz of (A) ^{113}Cd g32P (0.67 mM in ^{113}Cd) and (B) ^{113}Cd g32P-(A+B) protein (0.60 mM in ^{113}Cd). A pulse angle of 45°, a sweep width of 15000 Hz, and a recycle delay time of 1–1.5 s were used to acquire these and similar spectra; 8100 transients were collected in (A), while 7300 transients were acquired in (B). [Inset to (B)] ^{113}Cd NMR spectrum (110.9 MHz, 36000 transients) of ^{113}Cd C166S g32P-(A+B)(0.90 mM in ^{113}Cd). A pulse angle of 45°, a sweep width of 15000 Hz, and a recycle delay time of 1 s were used to acquire this spectrum.

Table I: ^{113}Cd Chemical Shifts of ^{113}Cd(II)-Substituted Proteins Containing Sulfur Donor Atoms

protein	δ	donor set[a]	ref
azurin, *Pseudomonas aeruginosa*	372	N_2S[b]	Engeseth et al. (1984)
plastocyanin	432	N_2SS^*	Engeseth et al. (1984)
alcohol dehydrogenase catalytic sites	483	ONS_2	Bobsein and Myers (1980)
alcohol dehydrogenase catalytic sites plus imidazole	519	N_2S_2	Bobsein and Myers (1980)
T4 gene 32 protein	637	NS_3[c]	this work
alcohol dehydrogenase structural sites	751	S_4	Bobsein and Myers (1980)

[a] S = Cys–S$^-$; S* = Met-S–CH$_3$. [b] In contrast to the Cu(II) derivatives, Cd(II) does not appear to coordinate the methionine (Engeseth et al., 1984). [c] N is postulated to be an imidazole nitrogen atom of His^{81}.

ligands or a mixture of nitrogen and oxygen ligands induce ^{113}Cd chemical shifts from 50 to 250 ppm. Sulfur coordination induces the most deshielding, and sites containing one to four sulfur atoms as donors have ^{113}Cd chemical shifts from 350 to 750 ppm, respectively.

A correlation can be made between the number of isolated sulfur atoms present as donors and increasing downfield chemical shifts shown by the ^{113}Cd(II). This correlation is illustrated in Table I by a tabulation of the ^{113}Cd chemical shifts available for ^{113}Cd(II) substituted in protein metal-binding sites of known donor atom composition as determined by X-ray crystallography and containing at least one sulfur atom as a ligand. The chemical shift of 637 ppm for ^{113}Cd(II) g32P falls midway between the range observed for isolated complexes containing two sulfur donors and those containing four (Table I). Thus, the ^{113}Cd chemical shift shown by ^{113}Cd(II) g32P is compatible with the postulate that three of the four Cys residues in g32P participate in metal ion binding,

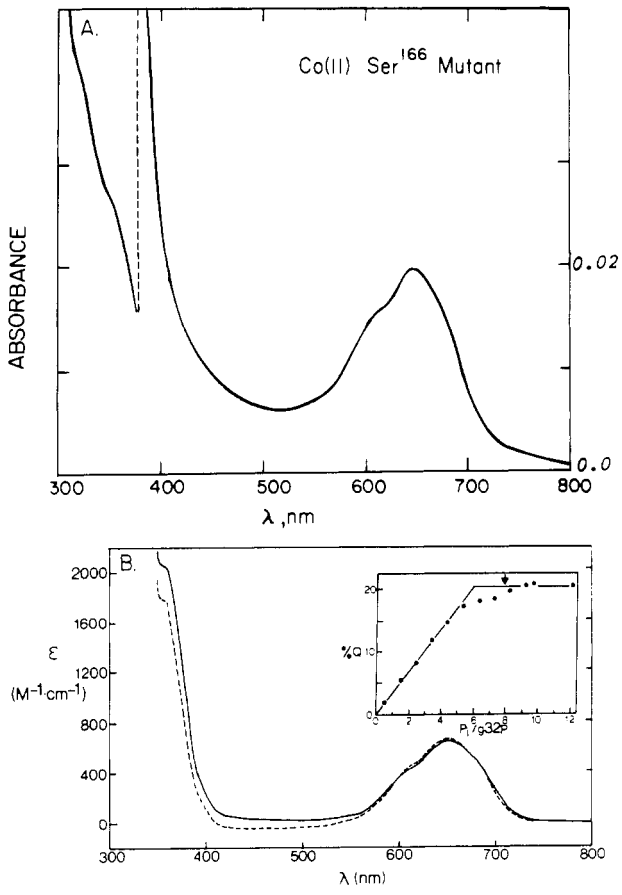

FIGURE 4: (A) Visible absorption spectrum of Co(II)-substituted C166S g32P, pH 8; (B) comparison of the visible absorption spectra of free native Co(II) g32P (—) with that of the fully saturated Co(II) g32P–poly(dT) complex (---). Conditions: 66.5 μM protein in 10 mM Tris-HCl, pH 8, 0.2 M NaCl, 5% (v/v) glycerol. (Inset) Titration of Co(II) g32P [1.0 μM protein, 2.0 mL, 10 mM Tris-HCl, pH 8, 0.2 M NaCl, 5% (v/v) glycerol] with poly(dT) normalized to equivalents of P_i added as monitored by the degree of quenching (%Q) of the intrinsic protein fluorescence. The arrow indicates the P_i:g32P ratio of the Co(II) g32P–poly(dT) complex visible absorption spectrum shown in the main body of the figure.

as suggested by the earlier mercurial titrations and spectroscopic data (Giedroc et al., 1986, 1987). The chemical shifts of ^{113}Cd(II) 4 $-S^-$ cluster sites found in mammalian metallothioneins, which fall between 620 and 690 ppm (Otvos & Armitage, 1980a), have not been included in Table I since these sites contain bridged as well as nonbridging (isolated) $-S^-$ ligands.

^{113}Cd NMR of Cys^{166} to Ser^{166} Mutant Gene 32 Protein-(A+B). Gene 32 protein contains four Cys residues at positions 77, 87, 90, and 166 in the primary structure. To define which three Cys side chains participate in metal ion binding, the Cys residue outside the proposed metal domain, Cys^{166}, was changed to Ser (C166S g32P) via oligonucleotide-directed mutagenesis. Amino acid analysis of the mutant showed 3 mol of cysteic acid/mol of protein. Purified C166S contains stoichiometric Zn(II) and can be cleanly converted to C166S g32P-(A+B) via limited proteolysis analogous to the wild-type protein (data not shown). ^{113}Cd(II)-substituted C166S g32P core shows a single ^{113}Cd NMR resonance at 637 ppm, identical with that of native g32P (inset to Figure 3B). Likewise, the optical absorption spectrum of the Co(II)-substituted C166S g32P is superimposable on the spectrum of native g32P (Figure 4). These data provide strong support for the participation of Cys^{77}, Cys^{87}, and Cys^{90} as the ligands to the intrinsic metal ion in g32P coupled with a fourth donor atom of relatively lower ^{113}Cd deshielding potential, i.e., nitrogen or oxygen.

Table II: ^{113}Cd Chemical Shifts, T_1, and NOE Values for ^{113}Cd(II)-Substituted Gene 32 Protein-(A+B)

field (MHz)	δ	line width[a] (Hz)	T_1[b]	NOE (η + 1)
44.4	637	60	0.5	0.77
110.9	637	110	1.1	1.00

[a] Reflects the line width measured at half-peak-height line, the line broadening artificially introduced into the transformed spectra. These values are ±10%. [b] These values have an estimated uncertainty of ±20%.

Exogenous Cl^- Ion as a Probe of Solvent Access to the Metal Site. The chemical shift of intact ^{113}Cd g32P is unaffected (636.0 ± 0.4 ppm) by increasing concentrations of Cl^- ion present in the buffer to 0.5 M. If Cl^- exchange were occurring within the first coordination sphere of the ^{113}Cd(II) ion, the chemical shift would be expected to change by as much as 20 ppm (Schoot-Uiterkamp et al., 1980; Gettins & Coleman, 1984; Gettins & Cunningham, 1986).

Another more direct method to assess chloride access to the inner coordination sphere of the metal ion is to measure the width of the $^{35}Cl^-$ (spin = $^3/_2$) resonance in th presence of the native zinc metalloprotein. The NMR line width of Cl^- ions bound in the first coordination sphere will be subjected to enhanced relaxation resulting from a large asymmetry in the electric field at the quadrupolar ^{35}Cl nucleus induced by binding to the protein-bound Zn(II) ion. Rapid exchange of these bound ions with the ions in solution will detectably broaden the line width of the bulk $^{35}Cl^-$ NMR resonance (Ward, 1969). Since any nonspecific and weak interaction of $^{35}Cl^-$ with a protein macromolecule will induce some broadening, the excess line width in the presence of the apoprotein must be used as the control. The excess line widths induced by addition of native Zn(II) g32P (9.3 ± 1.0 Hz) and apo-g32P (8.0 ± 1.0 Hz) to Cl^--containing solutions are essentially identical. If an exchanging Cl^- were present in the first coordination sphere of the Zn(II) g32P, the observed line width would be much greater, since quadrupolar coupling constants on the order of 15 MHz are expected for a zinc-bound Cl^- ion (Irion et al., 1978, Gettins & Cunningham, 1986). Thus, both experiments that assess anion access to the first coordination sphere of the metal ion in g32P indicate that monodentate ligands from solution do not have such access in g32P.

Spin Relaxation Properties of ^{113}Cd(II) Bound to Gene 32 Protein-(A+B). The ^{113}Cd relaxation parameters characterizing core g32P were collected at two magnetic field strengths and are compiled in Table II. The T_1 at 44.4 MHz is 0.5 s and is significantly less than that at 110.9 MHz, 1.1 s. A small NOE (η + 1 = 0.77) is observed at 44.4 MHz, while no NOE can be detected at 110.9 MHz.

Effect of $d(pA)_6$ Binding on the Metal-Site Structure of ^{113}Cd(II) g32P. When the 1:1 complex of ^{113}Cd g32P-(A+B) with $d(pA)_6$ is formed, the ^{113}Cd chemical shift of the complex is at 639.5 ppm (Figure 5, resonance B), only 2.2 ppm downfield of the chemical shift of free g32P-(A+B) (637.3 ± 0.2 ppm) (Figure 5, resonance A). When a 0.5 molar equiv of $d(pA)_6$/mol of ^{113}Cd g32P-(A+B) is added, the entire ^{113}Cd resonance shifts to an intermediate chemical shift of 638.8 ppm (Figure 5, resonance C). The line widths in all three cases are the same, and there is only a single resonance at a nucleotide:protein ratio of 0.5, indicating that the oligonucleotide–protein complex and the remaining free protein are in fast exchange on the NMR time scale (see Discussion).

Effect of Poly(dT) Binding on the Metal-Site Structure of

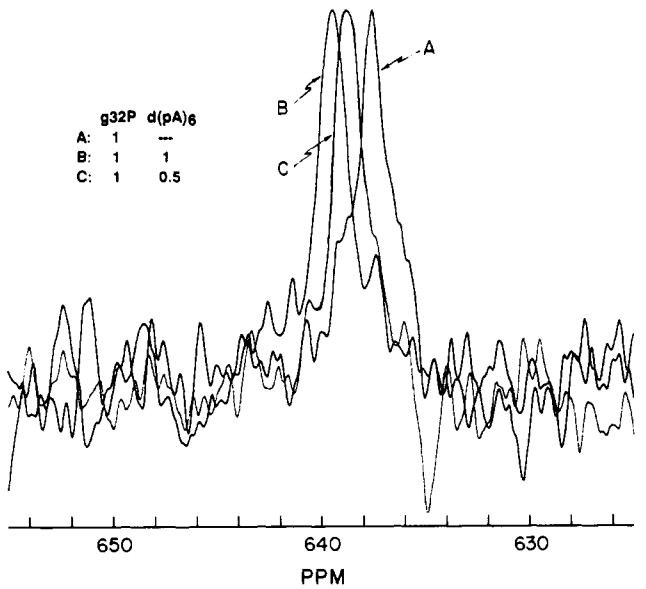

FIGURE 5: Effect of oligodeoxyribonucleotide $p(dA)_6$ on the chemical shift of ^{113}Cd g32P-(A+B) at 110.9 MHz. The resonances labeled A–C represent free core protein (0.69 mM), a 1:1 complex (0.34 mM g32P), and a 0.5:1 complex (0.51 mM g32P) of protein and DNA, respectively. The 1:1 stoichiometry case should represent a nearly fully bound protein–DNA complex, given a reasonable estimate of the equilibrium dissociation constant between the two species of $\sim 10^{-5}$ M [cf. Spicer et al. (1979)]. A pulse angle of 45°, a sweep width of 15 000 Hz, and a recycle delay time of 1.5 s were used.

Co(II) g32P. The metal ion in g32P is essential for high-affinity, cooperative binding to DNA lattices of "infinite length" such as poly(dT) (Giedroc et al., 1987). The d–d visible transitions of Co(II) g32P found in the 600–700-nm range and assigned to the $^4A_2 \rightarrow {}^4T_1(P)$ manifold (Coleman & Giedroc, 1988) are sensitive to changes in the nature, number, and geometry of the ligands and should provide a reasonably sensitive probe of changes occurring in the metal-site structure when nucleotides bind to g32P. The optical spectrum of the free Co(II) g32P is compared with that of the Co(II) g32P complexed to poly(dT) in Figure 4B. There is little change in the d–d absorption bands of the complex compared to the unliganded protein. A titration of intact Co(II) g32P with poly(dT) shows only small changes in the d–d absorption bands throughout the course of the titration to the point of fully saturated binding as assayed by fluorescence quenching (Figure 4B, inset).

Distance Geometry Calculations. Titration of g32P with an organic mercurial has previously shown that Zn(II) is linearly displaced from the protein accompanying the reaction of three of the four SH groups in the molecule (Giedroc et al., 1986). The properties of the C166S mutant described above limit the three S^- ligands to those of Cys^{77}, Cys^{87}, and Cys^{90}. The d–d electronic transitions of the Co(II) g32P show an approximately tetrahedral complex of Co(II) to be present (Giedroc et al., 1986). Since the site does not appear to offer solvent access, the imidazole nitrogen of His^{81} in the midst of the Cys cluster is the best candidate for the fourth ligand. Can the amino acid sequence of g32P from Cys^{77} to Cys^{90} fold to form a tetrahedral Zn(II) complex by using the above ligands without violating accepted peptide conformations? This was tested by using the computer program Dspace described under Materials and Methods that allows the folding of the polypeptide to meet additional structural constraints while maintaing the ϕ and ψ angles found in known protein structures. In the present case the additional structural constraints were acceptable Cd(II)–S and Cd(II)–N^ϵ bond lengths and the presence of tetrahedral geometry around the Cd(II) ion. The section of the peptide chain chosen was Thr^{76}–Gln^{91}. The "refinement" of the structure as described under Materials and Methods refers to the annealing process whereby the structure with the above minimal requirements can converge to an acceptable structure meeting all the constraints without falling into secondary minima or violating bond angles or van der Waals contacts. Ten closely related structures were generated.

It was readily possible to refine each structure to a point where distance violations did not exceed 0.1 Å. These structures were found to fall into two families that differed according to the chirality of the ligands around the metal ion. Each family of structures was superimposed to minimize the RMS deviation. Within each family of structures the RMS deviation was found to be approximately 2.1 ± 0.5 Å, whereas between the two families it was 4.1 ± 0.6 Å. A stereo drawing of a representative member of each family is illustrated in Figure 6.

Discussion

The original study of the Zn(II) in g32P showed that the metal ion was released by reaction of an organic mercurial with three of the four SH groups in the protein (Giedroc et al., 1986). Removal of the Zn(II) ion and studies of the DNA binding properties of the apo-g32P showed that the metal ion was essential for the cooperative binding of g32P to ssDNA and contributes 3 orders of magnitude to the affinity constant for DNA binding (Giedroc et al., 1987; Keating et al., 1988). Co(II) and Cd(II) can both substitute for the native Zn(II) ion in reestablishing tight cooperative binding of g32P to ssDNA (Giedroc et al., 1987). The Co(II) g32P shows a visible absorption spectrum typical of a Co(II) ion in a tetrahedral ligand field (Figure 4) (Giedroc et al., 1986). The chemical shift of the ^{113}Cd NMR signal from the ^{113}Cd(II) g32P supports the presence of three $-S^-$ ligands (Figure 3, Table I). Since the C166S mutant g32P shows the same ^{113}Cd NMR spectrum (Figure 3) and the same Co(II) absorption spectrum as the native g32P (Figure 4), the three $-S^-$ ligands must be contributed by Cys^{77}, Cys^{87}, and Cys^{90}. Site-directed mutagenesis of the ligand residues themselves is complicated by the fact that other single-site mutations of residues near the putative Zn(II)-binding sequence have been observed to result in conformational changes that disrupt Zn(II) binding. For example, the Y73S mutant does not bind Zn(II) nor does it bind to ssDNA (Shamoo et al., 1987). Thus, loss or modification of Zn(II) binding accompanying single-site mutations of the putative metal-binding residues cannot be used to prove unequivocally that a given residue participates in direct ligation.

Both the ^{113}Cd of the ^{113}Cd g32P and ^{35}Cl NMR in the presence of Zn(II) g32P suggest that anionic ligands from solution do not have access to the metal ion of g32P. Thus, it seems likely that a fourth protein ligand completes the tetrahedral ligand field. A fourth side chain that could complete the tetrahedron is one of the imidazole nitrogen atoms of His^{81}. Such a S_3N site would be compatible with the energies of the visible d–d transitions of the Co(II) g32P, as they match almost exactly the energies of these same transitions observed in alcohol dehydrogenase when Co(II) is substituted for the active-site Zn(II) ion and ^-SH replaces the solvent H_2O ligand in generating a S_3N tetrahedral complex (Maret et al., 1979; Coleman & Giedroc, 1988).

The modeling of the polypeptide chain from residue 76 to residue 91 by the simple distance geometry method shows that it is possible to form a tetrahedral metal ion complex with the proposed three Cys and one His ligand without violating ac-

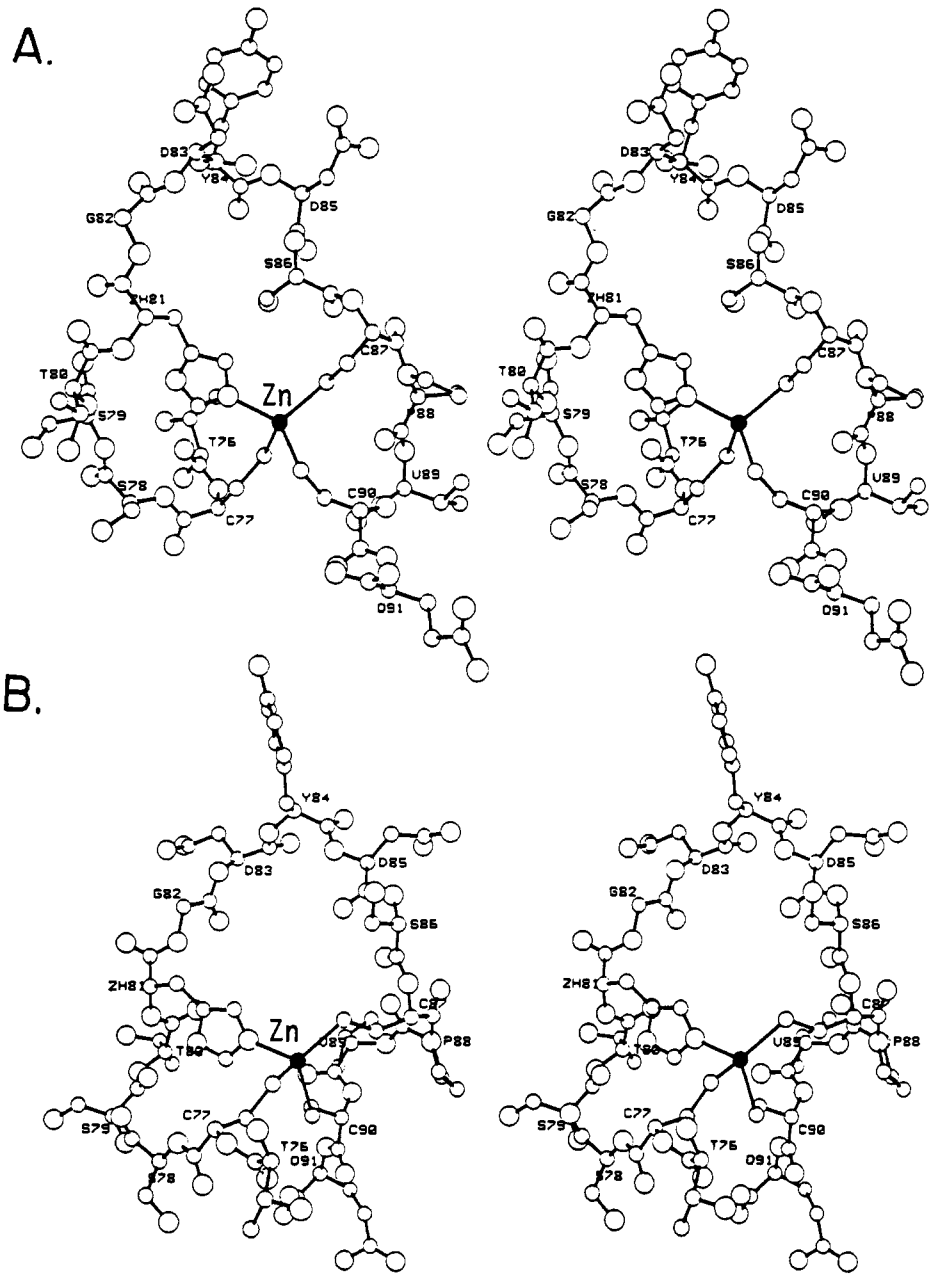

FIGURE 6: Stereo diagrams of one representative member of each family of structures generated by distance geometry methods. As can be seen, the two structures differ in their chirality of ligands about the Zn(II) ion (●). In both structures, the imidazole side chain of His^{81} has been modeled as being ligated to the metal ion through the ϵ nitrogen atom.

ceptable peptide conformations. When analyzed by algorithms that predict secondary structure including β-turns, this portion of the amino acid sequence is predicted to contain a loop or extended turn between residues 77 and 86 flanked on either side by β-sheet (Williams et al., 1981; Coleman & Giedroc, 1988). This prediction is not incompatible with the simple models generated by distance geometry (Figure 6).

Physicochemical studies of g32P from bacteriophage T4 provide a reasonably complete picture of how formation of the zinc chelate modulates the structure and stability of the molecule and how this ultimately affects function (Giedroc et al., 1987; Keating et al., 1988). As documented by the far-ultraviolet CD experiment in Figure 1, zinc appears to induce the formation of some specific secondary structure, while also providing thermodynamic stabilization to g32P (Keating et al., 1988). Such stabilization is imparted to a region of the molecule whose major role is in promoting cooperative interactions between contiguous g32P monomers on a DNA lattice rather than being essential for establishing fundamental protein–DNA contacts (Giedroc et al., 1987). Thus, the metal chelate must couple to a major surface involved in monomer–monomer interaction on the ssDNA lattice. Even so, the geometry of the metal site is essentially unchanged as a result of polynucleotide binding (Figure 4B), supporting a purely structural role for the metal ion in greatly enhancing the thermodynamic stability of the g32P–polynucleotide complex (Keating et al., 1988).

While two Trp residues, 72 and 116, are located at each end of the Zn(II)-binding domain and the aromatic CD bands in the region 275–300 nm appear easily assignable to the indole chromophore, careful inspection of the difference spectra suggests that most of the alterations of the CD in the aromatic region on Zn(II) binding may be associated with a positive CD difference band near 244 nm (Figure 2B). The phenolic chromophore of tyrosyl side chains can contribute optical activity in this region. Thus, the aromatic CD changes could also reflect alterations in the conformations of the side chains of Tyr^{73}, Tyr^{84}, Tyr^{94}, Tyr^{99}, Tyr^{106}, and Tyr^{115}, all located in

the immediate vicinity of the Zn(II)-binding domain. Tyr^{73} is a particularly prominent candidate in this regard, since mutagenesis of this residue to Ser destroys the ability of the protein to incorporate Zn(II) (Shamoo et al., 1987).

The small change in ^{113}Cd chemical shift when g32P binds an oligonucleotide, $d(pA)_6$, which fills the nucleotide binding site on a single monomer, suggests that if any conformational changes occur in g32P as a result of ligand binding, they do not substantially distort the metal-site geometry (Lacelle et al., 1984). Although the metal ion complex is required for high-affinity cooperative binding of g32P to ssDNA, it seems unlikely that the oligonucleotide contributes an additional ligand to the metal ion or causes a large change in geometry of the coordination complex. As seen by the shift of the entire ^{113}Cd NMR line induced by a 0.5:1 nucleotide:protein ratio (Figure 6), the oligonucleotide is in fast exchange between two or more protein binding sites. Since nucleotide binding is causing the ^{113}Cd chemical shift, all g32P molecules are sampling the nucleotide lattice on the NMR time scale, even though only enough $d(pA)_6$ is present to saturate half the binding sites on the g32P molecules. The frequency difference of 244 Hz betwen ^{113}Cd in the free and complexed forms of g32P represents the minimum dissociation rate; hence, k_{off} for $d(pA)_6$ from the Cd(II) protein is $\geq 240\ s^{-1}$. Previous determinations of dissociation rates of g32P from poly(dA) measured at extremely low binding densities (i.e., effectively isolated sites), reveal k_{off} values $\geq 100\ s^{-1}$ (Kowalczykowski et al., 1980). Thus, our k_{off} is in the expected range estimated for the dissociation rate of oligonucleotides from g32P. Thus, individual g32P molecules may move with ease among contiguous sites on a long polynucleotide lattice until the cooperative binding mode is established.

It is not surprising that chemical shift anisotropy (CSA) is a prominent relaxation mechanism for ^{113}Cd in ^{113}Cd-(II)-substituted g32P. That CSA is a dominant relaxation mechanism has been documented for other $^{113}Cd(II)$ proteins (Otvos & Armitage, 1980b; Bailey et al., 1980; Armitage & Otvos, 1982). T_1 values for $^{113}Cd(II)$ in metalloproteins range from 0.1 to 5 s. Since protons on ligands to Cd(II) typically found in proteins are >3.5 Å distant, T_1 would be quite long, >10 s, if dipolar relaxation were the only mechanism operating. Protons of coordinated water are closer, ~2.9 Å, but still would not account for the short T_1s found for many ^{113}Cd-(II)-substituted proteins. The magnitude of the CSA in $^{113}Cd(II)$ proteins is difficult to estimate, since solid-state spectra have not been collected on most of the examples that would permit measurement of the true anisotropy of the chemical shift. On the other hand, if one assumes that the T_1 of 1.1 s for $^{113}Cd(II)$ g32P-(A+B) at 11.75 T reflects primarily CSA, then a CSA of ~200 ppm is required to generate a T_1 of ~1 s for ^{113}Cd in a molecule with a τ_r of $\sim 10^{-8}$ s (the latter calculated from the Stoke's radius for the 26-kDa core g32P).

Theoretical plots of T_1 vs τ_r at two magnetic fields, 4.7 and 11.75 T, can be constructed by incorporating moderate CSA ($\Delta\delta$ = 200 ppm) with or without one proton within 2.5 Å of the Cd(II) ion. Because of the opposite effects of the H_0^2 and $\omega^2\tau_c^2$ terms, the CSA contribution to relaxation is approximately the same at 4.7 and 11.75 T (Farrar & Becker, 1971). As a consequence of more efficient dipolar modulation at the lower field, the T_1, in the presence of CSA of 200 ppm, is predicted to be smaller at the lower field (1.0 s) than at the high field (1.6 s). This is qualitatively in accord with the relaxation data for ^{113}Cd in g32P, since a detectable NOE is present at 4.7 T but not at 11.75 (Table II). The β-CH_2s of Cys and the protons of the His ring may account for some dipolar contribution; however, these protons are not close enough to the coordinated $^{113}Cd(II)$ to account for the T_1 of 0.5 s observed at 4.7 T (Table II). Crystal structures are available on several proteins containing cysteine sulfhydryls as ligands to metal ions, and in a number of cases the liganded $-S^-$ also makes a hydrogen bond with a NH group of the peptide backbone. If such hydrogen bonds are present in g32P, then the protons in these bonds may be much closer to the $^{113}Cd(II)$ and might account for the significant dipolar interaction present for $^{113}Cd(II)$ bound to g32P.

In summary, NMR of ^{113}Cd-substituted g32P has permitted probing of the structural characteristics of the metal-site geometry and represents the first example of a ^{113}Cd-substituted metal site in a metalloprotein that specifies a NS_3 ligand donor set. Distance geometry modeling shows that such a donor set can be easily accommodated by the side chains of Cys^{77}, His^{81}, Cys^{87}, and Cys^{90} of gene 32 protein.

Registry No. $d(pA)_6$, 24512-53-6; Cys, 52-90-4; His, 71-00-1; Cd, 7440-43-9; Zn, 7440-66-6.

References

Anderson, R. A., & Coleman, J. E. (1975) *Biochemistry 14*, 5485.

Armitage, I. M., & Otvos, J. D. (1982) *Biol. Mag. Reson. 4*, 79.

Bailey, D. B., Ellis, P. D., & Fee, J. A. (1980) *Biochemistry 19*, 591.

Bittner, M., Burke, R. L., & Alberts, B. M. (1979) *J. Biol. Chem. 254*, 9565.

Bobsein, B. R., & Myers, R. J. (1980) *J. Am. Chem. Soc. 102*, 2454.

Chang, C. T., Wu, C.-S. C., & Wang, J. T. (1978) *Anal. Biochem. 91*, 13.

Chase, J. W., & Williams, K. R. (1986) *Annu. Rev. Biochem. 55*, 103.

Coleman, J. E., & Giedroc, D. P. (1989) *Met. Ions Biol. Syst. 25*.

Corwin, D. T., Jr., Gruff, E. S., & Koch, S. A. (1987) *J. Chem. Soc., Chem. Commun.*, 966.

Engeseth, H. R., McMillin, D. R., & Otvos, J. D. (1984) *J. Biol. Chem. 259*, 4822.

Ernst, R. R., Bodenhausen, G., & Wokaun, A. (1987) in *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, p 203, Oxford University Press, Oxford, U.K.

Farrar, T. C., & Becker, E. D. (1971) in *Pulse and Fourier Transform NMR*, p 59, Academic Press, New York.

Gettins, P., & Coleman, J. E. (1984) *J. Biol. Chem. 259*, 11036.

Gettins, P., & Cunningham, L. W. (1986) *Biochemistry 25*, 5004.

Giedroc, D. P., Ling, N., & Puett, D. (1983) *Biochemistry 22*, 5584.

Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., & Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452.

Giedroc, D. P., Keating, K. M., Williams, K. R., & Coleman, J. E. (1987) *Biochemistry 26*, 5251.

Irion, M., Weldon, N., & Weiss, A. (1978) *J. Magn. Reson. 30*, 457.

Johnson, B. A. (1987) *J. Mol. Graphics 5*, 167.

Kagi, J. H. R., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2435.

Keating, K. M., Giedroc, D. P., Harris, L. D., Ghosaini, L. R., Williams, K. R., Sturtevant, J. M., & Coleman, J. E. (1987) *UCLA Symp. Mol. Cell. Biol., New Ser. 69*, 35.

Keating, K. M., Ghosaini, L. R., Giedroc, D. P., Williams, K. R., Coleman, J. E., & Sturtevant, J. M. (1988) *Biochemistry 27*, 5240.
Kowalczykowski, S. C., Lonberg, N. C., Newport, J. W., Paul, L. S., & von Hippel, P. H. (1980) *Biophys. J. 32*, 403.
Lacelle, S., Stevens, W. C., Kurtz, D. M., Jr., Richardson, J. W., Jr., & Jacobson, R. A. (1984) *Inorg. Chem. 23*, 930.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Maret, W., Anderson, I., Dietrich, H., Schneider-Bernlohr, H., Einersson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501.
McFarland, T. M., & Coleman, J. E. (1972) *Eur. J. Biochem. 29*, 521.
Moise, H., & Hosoda, J. (1976) *Nature (London) 259*, 455.
Otvos, J. D., & Armitage, I. M. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094.
Otvos, J. D., & Armitage, I. M. (1980b) *Biochemistry 19*, 4031.
Prigodich, R. V., Casas-Finet, J., Williams, K. R., Konigsberg, W. H., & Coleman, J. E. (1984) *Biochemistry 23*, 522.
Prigodich, R. V., Shamoo, Y., Williams, K. R., Chase, J. W., Konigsberg, W. H., & Coleman, J. E. (1986) *Biochemistry 25*, 3666.
Schoot-Uiterkamp, A. J. M., Armitage, I. M., & Coleman, J. E. (1980) *J. Biol. Chem. 255*, 3911.
Shamoo, Y., Adari, H., Konigsberg, W. H., Williams, K. R., & Chase, J. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8844.
Shamoo, Y., Roberts, W. J., Coleman, J. E., Williams, K. R., & Konigsberg, W. H. (1987) *UCLA Symp. Mol. Cell. Biol., New Ser. 69*, 385.
Spicer, E. K., Williams, K. R., & Konigsberg, W. H. (1979) *J. Biol. Chem. 254*, 6433.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764.
Vasak, M., Overnell, J., & Good, M. (1987) *Experientia, Suppl. 52*, 179.
Ward, R. L. (1969) *Biochemistry 8*, 1879.
Williams, K. R., & Konigsberg, W. H. (1978) *J. Biol. Chem. 253*, 2463.
Williams, K. R., LoPresti, M. B., & Setoguchi, M. (1981) *J. Biol. Chem. 256*, 1754.

Characteristics in Tyrosine Coordinations of Four Hemoglobins M Probed by Resonance Raman Spectroscopy†

Masako Nagai,‡ Yoshimasa Yoneyama,§ and Teizo Kitagawa*,‖

Biological Laboratory, Kanazawa University School of Allied Medical Professions, Kanazawa, Ishikawa 920, Japan, Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920, Japan, and Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki 444, Japan

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ABSTRACT: Resonance Raman spectra of four hemoglobins (Hbs) M with tyrosinate ligand, that is, Hb M Saskatoon (β distal His → Tyr), Hb M Hyde Park (β proximal His → Tyr), Hb M Boston (α distal His → Tyr), and Hb M Iwate (α proximal His → Tyr), were investigated in order to elucidate structural origins for distinctly facile reducibility of the abnormal subunit of Hb M Saskatoon in comparison with other Hbs M. All of the Hbs M exhibited the fingerprint bands for the Fe–tyrosinate proteins around 1600, 1500, and 1270 cm^{-1}. However, Hb M Saskatoon had the lowest Fe–tyrosinate stretching frequency and was the only one to display the Raman spectral pattern of a six-coordinate heme for the abnormal β subunit; the others displayed the patterns of a five-coordinate heme. The absorption intensity of Hb M Saskatoon at 600 nm indicated a transition with a midpoint pH at 5.2, whereas that of Hb M Boston was independent of pH from 7.2 to 4.8. The fingerprint bands for the tyrosinate coordination as well as the Fe–tyrosinate stretching band disappeared for Hb M Saskatoon at pH 5.0, and the resultant Raman spectrum resembled that of metHb A, while those bands were clearly observed for Hb M Boston at pH 5.0 and for two Hbs M at pH 10.0. These observations suggest that the unusual characteristics of the heme in the abnormal β chain of Hb M Saskatoon result from the weak Fe–tyrosinate bond, which allows weak coordination of the proximal histidine, giving rise to the six-coordinate high-spin state at pH 7. At pH 5, the coordination geometry is modified due to protonation of the tyrosinate.

Hemoglobin (Hb)[1] M is a group of mutant Hbs in which the heme iron in the abnormal chain is retained in the ferric state under physiological conditions. The amino acid substitutions of Hbs M are classified into two categories; one includes replacement of the proximal or distal histidine (His) to tyrosine (Tyr), and the other includes replacement of a residue near the distal His of the β chains to glutamate or aspartate (Gerald & Efron, 1961; Heller et al., 1966; Steadman et al., 1970; Cohen-Solal et al., 1973). Hb M Milwaukee (β67Val → Glu) is a representative of the latter category while the former one is further assorted depending on whether the

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* To whom correspondence should be addressed.
‡ Biological Laboratory.
§ Department of Biochemistry.
‖ Institute for Molecular Science.

[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; RR, resonance Raman; His, histidine; Tyr, tyrosine; ENDOR, electron nuclear double resonance; Val, valine; Glu, glutamic acid.

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