Zn(II) Coordination Domain Mutants of T4 Gene 32 Protein†

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ABSTRACT: Gene 32 protein (g32P), the replication accessory single-stranded nucleic acid binding protein from bacteriophage T4, contains 1 mol of Zn(II)/mol of protein. Zinc coordination provides structural stability to the DNA-binding core domain of the molecule, termed g32P–(A+B) (residues 22–253). Optical absorption studies with the Co(II)-substituted protein and ^{113}Cd NMR spectroscopy of ^{113}Cd(II)-substituted g32P–(A+B) show that the metal coordination sphere in g32P is characterized by approximately tetrahedral ligand symmetry and ligation by the Cys–S$^-$ atoms of Cys^{77}, Cys^{87}, and Cys^{90}. These studies predicted the involvement of a fourth protein-derived non-thiol ligand to complete the tetrahedral complex, postulated to be His^{81} on the basis of primary structure prediction and modeling [Giedroc, D. P., Johnson, B. A., Armitage, I. M., & Coleman, J. E. (1989) *Biochemistry 28*, 2410–2418]. To test this model, we have employed site-directed mutagenesis to substitute each of the two histidine residues in g32P (His^{64} and His^{81}), accompanied by purification and structural characterization of these single-site mutant proteins. We show that g32P's containing any of three substitutions at residue 64 (H64Q, H64N, and H64L) are isolated from *Escherichia coli* in a Zn(II)-free form [≤0.03 g·atom Zn(II)]. All derivatives show extremely weak affinity for the ssDNA homopolymer poly(dT). All are characterized by a far-UV–CD spectrum reduced in negative intensity relative to the wild-type protein. These structural features parallel those found for the known metal ligand mutant Cys^{87} → Ser^{87} (C87S) g32P. In contrast, g32P–(A+B) containing a substitution of His^{81} with glutamine (H81Q), alanine (H81A) or cysteine (H81C), contains stoichiometric Zn(II) as isolated and binds to polynucleotides with an affinity comparable to the wild-type g32P–(A+B). Spin-echo ^{1}H NMR spectra recorded for wild-type and H81Q g32P–(A+B) as a function of pH allow the assignment of His^{81} ring protons to δ = 6.81 and 6.57 ppm, respectively, at pH 7.8, corresponding to the C and D histidyl protons of ^{1}H-His–g32P–(A+B) [Pan, T., Giedroc, D. P., & Coleman, J. E. (1989) *Biochemistry 28*, 8828–8832]. These resonances shift downfield as the pH is reduced from 7.8 to 6.6 without metal dissociation, a result incompatible with His^{81} donating a ligand to the Zn(II) in wild-type g32P. Likewise, Cys^{81} in Zn(II) H81C g32P is readily reactive with 5,5′-dithiobis(2-nitrobenzoic acid), unlike metal ligands Cys^{77}, Cys^{87}, and Cys^{90}. Upon substitution of H81Q, H81A, and H81C g32P–(A+B) with Co(II), the d–d ligand field transition envelope is not detectably different from that in the wild-type protein. The natural CD spectra of all three mutant Co(II) complexes show the same minor alterations in the S$^-$ → Co(II) ligand-to-metal charge transfer region. ^{113}Cd substitution of the Gln, Ala, and Cys mutants gives a ^{113}Cd NMR signal at δ = 633 ± 1 ppm, compared to δ = 639 ppm for the wild-type protein. We conclude that it is unlikely that His^{81} forms a coordination bond to Zn(II) in g32P, as the original model predicted. Metal coordination by His^{64} in g32P remains a possibility in light of the inability of His^{64} mutant proteins to coordinate Zn(II).

Bacteriophage T4 gene 32 protein (g32P) binds preferentially and cooperatively to single-stranded nucleic acids relatively independent of sequence. This type of binding allows g32P to function in T4 replication, repair, and recombination by binding to ssDNA transiently formed during any one of these processes [for a review, see Karpel (1990) and Morrical and Alberts (1990)]. Limited proteolysis of g32P[1] provides evidence for two terminal structural domains positioned at the amino ("B" domain, residues 1–21) and carboxy termini ("A" domain, residues 254–301) of the DNA-binding core domain (residues 22–253) (Moise & Hosoda, 1976; Williams & Konigsberg, 1978). Genetic or proteolytic removal of the B domain to form g32P–B (Giedroc et al., 1990) or concomitant removal of the C-terminal A domain to form g32P–(A+B) (Spicer et al., 1979) results in high binding affinity by g32P for single-stranded nucleic acids but eliminates the cooperativity of binding as well as g32P monomer self-association (Giedroc et al., 1990). Thus, g32P's lacking the B domain bind noncooperatively (ω = 1) to polynucleotides. Recent ^{1}H NMR studies on complexes formed by g32P and its deletion

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[1] Abbreviations: bp, base pair; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); g32P, gene 32 protein, 301 amino acids; the N-terminal "B" domain (resdues 1–21); the C-terminal "A" domain (residues 254–301); g32P–(A+B), gene 32 protein lacking both the A and B domains, also referred to as g32P DNA-binding core fragment and g32P *III; HPLC, high-pressure liquid chromatography; LMCT, ligand-to-metal charge transfer; Na_3EDTA, trisodium ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; nt, nucleotide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ss, single-stranded; Tris, tris(hydroxymethyl)aminomethane.

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derivatives on oligo- and polynucleotides suggest that the protein–nucleic acid interface must be very similar for both the noncooperative and cooperative complexes (Pan et al., 1989b).

The ssDNA-binding core fragment contains the intrinsic Zn(II) ion (Giedroc et al., 1986). The metal coordination chelate undergoes only very small changes in structure upon single-stranded nucleic acid binding, detectable by difference visible–CD measurements (D. Giedroc, unpublished results). However, removal of the Zn(II) ion has an appreciable effect on the structure and thermodynamic stability of the g32P monomer and may well influence monomer self-association (Keating et al., 1988; Pan et al., 1989a). Zn(II) binding or substitution with another metal ion [e.g., Co(II) or Cd(II)] confers stability on the core domain as indicated by protection against proteolysis and enhanced thermal stability relative to the apoprotein (Giedroc et al., 1987; Keating et al., 1988). Zn(II) removal is accompanied by small but significant changes in backbone structure and aromatic side chain microenvironment as detected by CD spectroscopy of the peptide chromophores (Giedroc et al., 1989).

The structural changes which accompany Zn(II) removal, though small, have a significant effect on the nucleic acid binding properties of g32P in vitro. The magnitude of the cooperativity parameter appears to be primarily affected upon metal depletion, with a comparably smaller change in the magnitude of the noncooperative binding affinity (Giedroc et al., 1987; Nadler et al., 1990).[2] Fluorescence resonance energy transfer spectroscopy has been used to elucidate a series of distances between the fixed metal ion [Co(II)] energy acceptor and a variably positioned nucleotide base energy donor (Giedroc et al., 1991). The metal–nucleotide base distances range from 16 to 21 Å, relatively close in the context of the globular dimensions of the g32P–A[1] monomer (25 × 25 × 65 Å) (Grant et al., 1991). The Zn(II) complex appears to organize a complementary surface(s) required for high-affinity cooperative binding to nucleic acids, with the molecular details as yet undefined.

Optical spectroscopy of the Co(II)-substituted protein indicates tetrahedral symmetry of ligands about the metal ion (Giedroc et al., 1986). ^{113}Cd(II) substitution and NMR give a chemical shift, $\delta = 638 \pm 1$ ppm, consistent with coordination by at least three Cys side chains. These have been assigned to residues 77, 87, and 90 by site-directed mutagenesis and elimination (Giedroc et al., 1989). The fourth non-thiol ligand was proposed to be His^{81} (Giedroc et al., 1986). This same region of the molecule, Cys^{77}–X_3–His^{81}–X_5–Cys^{87}–X_2–Cys^{90}, was also predicted to form a Zn(II)-binding domain from a computer search of known protein sequences for potential Zn(II)-binding sites loosely based on the ligand pattern of transcription factor IIIA (Berg, 1986). *Direct* identification of the fourth metal ligand in g32P has been lacking. In this communication, we assess the structural and functional consequences of substitution of each of the two His in g32P: His^{64}, on the N-terminal side of the metal domain, and His^{81}, the proposed Zn(II) ligand. We show that both conservative and nonconservative substitution of His^{64} is deleterious to g32P structure. These g32P molecules do not incorporate Zn(II) in vivo. All show extremely weak affinity for ssDNA. On the other hand, replacement of the proposed metal ligand His^{81} with glutamine (H81Q), alanine (H81A), or cysteine (H81C) gives rise to g32P molecules characterized by an essentially wild-type globular structure containing stoichiometric Zn(II). ^{1}H NMR studies and metal ion substitution with Co(II) and ^{113}Cd(II) have permitted structural characterization of the metal–ligand complex in these mutant proteins. By all criteria, the coordination complex in each of the His^{81} mutant proteins is structurally similar to the wild-type protein complex. The implications of these findings on the proposed involvement of His^{81} in Zn(II) ligation in wild-type g32P are discussed.

Materials and Methods

Materials

All buffers were prepared with doubly distilled and deionized Milli-Q water. ssDNA-cellulose was prepared according to Alberts et al. (1968) as described (Giedroc et al., 1990). DE-52 was obtained from Whatman while phenyl-Sepharose CL-4B was purchased from Sigma. Sodium *p*-hydroxymercuriphenylsulfonate, DTNB, IPTG, trypsin, and chromatographically purified DNase were obtained from Sigma. Poly(dT) was purchased from the Midland Certified Reagent Co. (Midland, TX). Molecular biologicals were obtained from either New England Biolabs (Boston, MA), Boehringer-Mannheim (Indianapolis, IN), or Promega (Fisher Scientific).

Methods

Plasmid Constructions. All standard molecular biological methods were carried out according to Maniatis et al. (1982). The phagemid p32NB.25Nde (Giedroc et al., 1990) was the parent construction used for introduction or elimination of the translationally silent restriction endonuclease recognition sequences. p32NB.25Nde has a 1.1 kb insert, flanked by unique 5′ *Nhe*I and 3′ *Bam*HI restriction sites, and contains the T4 *gene 32* coding sequences and a consensus ribosome-binding site. This plasmid lacks the 5′ noncoding autogenous translational repression signals (Shamoo et al., 1986). This insert also contains a unique *Nde*I site at nucleotides +58–63 (+1 = ATG) which changes the codons for Asn^{20}–Lys^{21} to His^{20}–Met^{21} (Giedroc et al., 1990). The Met codon ultimately serves as the initiation codon for g32P's lacking the N-terminal B domain, i.e., g32P–B (Giedroc et al., 1990) and recombinant g32P–(A+B), under the inducible expression by T7 RNA polymerase (Studier et al., 1990). These plasmids are designated pT7g32–B.wt (Giedroc et al., 1990) and pT7core9.wt, respectively. In the latter plasmid, a translational stop codon (TAG) is positioned immediately following the codon for Ala^{252}; this results in expression of a g32P fragment containing residues 22–252 or recombinant g32P–(A+B). Intact g32P was expressed from derepression of the inducible λP_L promoter by temperature jump, from a plasmid designated $pP_L\phi$p32.wt. This plasmid is analogous to pP_Lg32.wt already described (Giedroc et al., 1989), except that T7 *gene 10* ribosome-binding site provides translational control. The detailed mutagenesis and subcloning strategies used for all wild-type and single-site mutant phagemids and overexpression plasmids are described in the Supplementary Material.

Purification of g32P's. g32P and derivatives were inducibly expressed on Luria Broth using methods outlined previously (Giedroc et al., 1989, 1990) for the induction of the λP_L and T7 promoters, respectively. All g32P's were purified from the low-speed supernatant lysis fraction using a combination of three chromatographic steps (DE-52, ssDNA-cellulose, and phenyl-Sepharose chromatographies). SDS–PAGE and UV-absorbance analysis of the g32P derivatives used in this study indicates that all were greater than 90% homogeneous and

[2] Recent experiments suggest that chemical removal of Zn(II) reduces the magnitudes of both K_{obs} for an isolated lattice site and ω, the latter by about 5-fold, regardless of whether finite-length oligonucleotides or model infinite-length homopolynucleotides are used (D. Giedroc and B. Bruner, unpublished results).

exhibit 280/260 ratios exceeding 1.75, indicating trace or no contamination by endogenous nucleic acid. Only H64N g32P–(A+B) showed any degree of degradation, evidenced by a clear protein doublet which cochromatograph on ssDNA-cellulose. Where indicated, core fragment g32P–(A+B) preparations were obtained from the appropriate intact g32P's by preparative limited proteolysis and purified to >99% homogeneity exactly as described previously (Giedroc et al., 1989). Amino acid analysis confirmed the presence of a single His in His^{64} and His^{81} mutants. Metal-free apo-g32P was prepared with sodium *p*-hydroxymercuriphenylsulfonate (PMPS) as described (Giedroc et al., 1986) and is designated apo(PMPS)-g32P.

^{113}Cd substitution and Co(II) substitution of the native Zn(II) was carried out essentially as described (Giedroc et al., 1989), with the Zn(II), Cd(II), and Co(II) content quantitated by flame atomic absorption on a Perkin-Elmer 2380 atomic absorption spectrophotometer. In the case of ^{113}Cd exchange, >75% substitution of the Zn(II) sites was achieved in all cases, whereas with Co(II) substitution >85% was obtained. The recorded extinction coefficients of the Co(II)-substituted mutant proteins were calculated from the concentration of bound Co(II) determined by atomic absorption. Apo(PMPS) contained 0.05 g·atom Zn(II) by atomic absorption.

NMR Methods. ^{113}Cd NMR spectra were recorded either at 30 °C on a Bruker AMX-500 spectrometer (110.9 MHz for ^{113}Cd) at Rice University or at 21 °C on a Varian XL-400 spectrometer (88.7 MHz for ^{113}Cd) at Texas A&M University, both equipped with a broad-band tunable probe that accommodates 10-mm (o.d.) NMR tubes. Chemical shifts are reported relative to 0.1 M $Cd(ClO_4)_2$. The protein samples were 0.3–0.5 mM ^{113}Cd(II) in 2.0 mL of 50 mM sodium phosphate and 30 mM NaCl, pH 8, containing 10–30% D_2O-exchanged buffer as a field lock. The actual concentration of bound ^{113}Cd(II) in each sample for NMR is indicated in the figure legends, as are other acquisition parameters. For the chloride titration experiments, the ^{113}Cd g32P–(A+B) derivative was dialyzed exhaustively against 50 mM sodium phosphate, pH 7.8, and returned to the NMR tube, and its spectrum was recorded at 0 M NaCl. Small aliquots of a Chelexed stock solution of 4 M NaCl were then incrementally added to the NMR tube to achieve the indicated Cl^- concentration.

Zn(II) g32P–(A+B) samples were prepared for ^1H NMR by exhaustive dialysis against 50 mM sodium phosphate and 30 mM NaCl, pH 7.8, followed by D_2O exchange through a 5-mL Sephadex G25C spun column equilibrated with D_2O-exchanged buffer. To reduce the pH, small aliquots of 1 M DCl were added directly to the pH 7.8 protein samples to achieve the indicated pH (direct pH reading of protein samples). Spin-echo ^1H NMR spectra (Campbell et al., 1975) were recorded at 30 °C, following presaturation of the residual HOD resonance, with a delay time τ = 15 ms and an acquisition time of 1.4 s. The number of transients is indicated in the figure legends. Chemical shifts are reported relative to internal TSP [3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt] (Aldrich).

Optical Spectroscopy. Visible spectra of Co(II)-substituted g32P's were recorded at ambient temperature on a single-beam Hewlett-Packard 8420A UV–vis diode array spectrophotometer. A reference solution of final TNGa [10 mM Tris-HCl, 0.1 M NaCl, 5% (v/v) glycerol, pH 8] dialyzate was used to electronically blank the instrument prior to scanning the sample. Co(II) concentration ranged from 60 to 250 μM in TNGa buffer in a 10-mm path length masked quartz cell. Natural circular dichroism spectra of Co(II)-substituted g32P's [50–240 μM Co(II) in TNGa] were recorded from 700 to 300 nm with a Jasco C-600 spectropolarimeter in a 2- or 10-mm path length rectangular cuvette at 25.0 ± 0.1 °C. One to four scans were recorded at 10–20 nm/min with a time constant of 2–4 s, spectral bandwidth of 1 nm, and step size of 0.2 nm. Actual acquisition parameters are indicated in the figure legends. Recorded ellipticity values were converted to Δε units, expressed as M $Co(II)^{-1}\cdot cm^{-1}$.

Ultraviolet Circular Dichroism. Far-UV–CD spectra were recorded at a scan rate of 20 nm/min in a 2-mm path length rectangular cell at 25.0 ± 0.1 °C with protein concentrations ranging from 2 to 5 μM in TNGa buffer. Typical acquisition parameters were 1–2-s time constant, 1-nm bandwidth, 0.2-nm step size, and a sensitivity of 20-50 mdeg. In all cases, 2–4 scans were signal averaged. Ellipticity was converted to Δε ($M_{peptidebond}^{-1}\cdot cm^{-1}$), with the protein concentration determined on an aliquot of the sample subjected to CD by UV absorption and quantitation with $\epsilon_{282} = 4.13 \times 10^4$ $M^{-1}\cdot cm^{-1}$ (Spicer et al., 1979). The near UV–CD spectra were collected in the same cell and buffer at a concentration of ≈200 μM. The scan rate, time constant, and bandwidth were as above for collection of the far-UV–CD spectra, with a sensitivity setting of 10 or 20 mdeg.

Fluorescence Quenching Experiments. The intrinsic fluorescence of wild-type and mutant g32P's was monitored with an SLM 8000C spectrofluorometer upon excitation at 292 nm (1-nm band-pass) and emission at 347 nm (4-nm band-pass). The temperature was maintained in a thermostated sample compartment at 20 ± 0.1 °C. Protein samples, prepared at 0.3–0.7 μM in 2.0 mL of T buffer (10 mM Tris-HCl, 0.1 mM Na_3EDTA) with 0.1 M NaCl, pH 8.1 (20 °C), were continuously gently stirred throughout the course of a titration with small (2–20-μL) aliquots of a concentrated stock solution of poly(dT), prepared and quantitated as described (Giedroc et al., 1990). The measured fluorescence values (F_i) were converted to corrected fluorescence values ($F_{corr,i}$) after accounting for dilution, inner filter correction [according to Birdsall et al. (1983)], and photobleaching (Overman et al., 1988). The extent of photobleaching, obtained by monitoring the protein fluorescence of companion nontitrated cuvette subjected to the same excitation protocol as the titrated cuvette, never exceeded 5% under the excitation conditions used. Q_{obs}, the observed fluorescence quenching after the *i*th addition, is given by $Q_{obs} = (F_{corr,max} - F_{corr,i})/F_{corr,max}$, where $F_{corr,max}$ is the maximum or initial protein fluorescence prior to titration with nucleic acid. The data obtained were subjected to a two-parameter (Q_{max} and K_{obs}) best-eye fit accorded by the large ligand noncooperative overlap binding model (McGhee & von Hippel, 1974) with the site size *n*, set to 7.0, exactly as described (Giedroc et al., 1990).

Other Methods. The free sulfhydryl content was determined for the indicated g32P's (6–10 μM) under denaturing conditions in 7.9 M urea, 10 mM Tris-HCl, 0.1 M NaCl, 5% (v/v) glycerol, and 0.1 mM Na_3EDTA upon addition of excess DTNB[1] to a final concentration of 140 μM. The absorbance change at 412 nm was recorded in a 10-mm path length cell and converted to moles of sulfhydryl groups using $\epsilon_{412} = 1.36 \times 10^4$ $M^{-1}\cdot cm^{-1}$. C87S g32P was found to contain 2.8 titratable thiols under fully reduced, denaturing conditions; other data are given in the text.

Results

Mutant g32P's and the Model for Zn(II) Coordination. The structure of the Zn(II) complex in g32P is not detectably altered upon proteolytic or genetic removal of either or both of the amino-terminal B and carboxy-terminal A domains

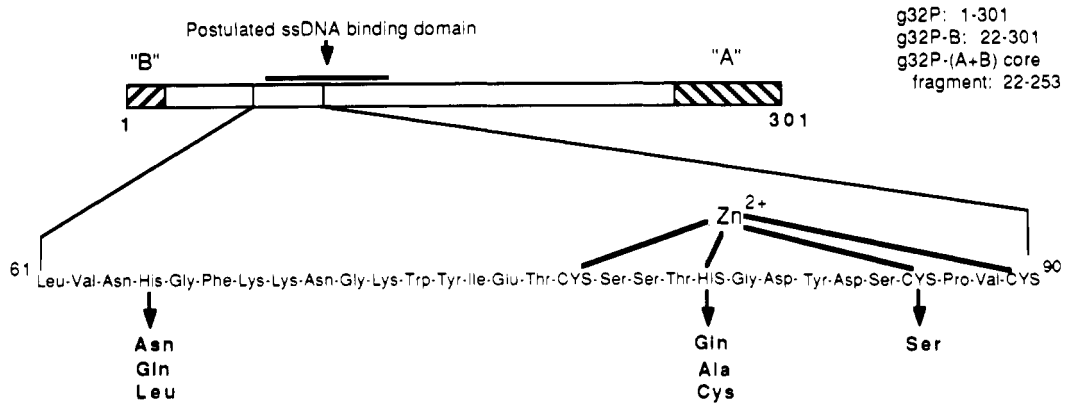

FIGURE 1: Previous model for the zinc coordination domain in g32P and single-site substitution mutants characterized in this study.

(Giedroc et al., 1987). Since we were originally interested in examining the effect of any one amino acid substitution in a variety of different contexts, mutations were expressed in either the context of the intact g32P molecule or as part of two g32P deletion mutants, one of which lacks the N-terminal B domain (g32P–B), and the other, both the A and B domains [g32P–(A+B) core fragment].[1] The construction of the recombinant plasmids designed to inducibly express recombinant g32P, g32P–B (Giedroc et al., 1990), and g32P–(A+B) (residues 22–252) are outlined under Materials and Methods and in the supplementary material.

The model for Zn(II) coordination by g32P and the mutants made to test this model is shown in Figure 1. All structural studies reported to date are consistent with the three cysteinyl ligands (residues 77, 87, and 90) and a fourth protein-derived non-thiol ligand, proposed to be His^{81}, to complete the tetrahedron (Giedroc et al., 1989). To explicitly test this, we have produced and characterized three single amino acid substitutions at His^{64}, H64Q g32P–B, H64L g32P–B and H64N g32P–(A+B); three at His^{81}, H81Q g32P–(A+B), H81A g32P, and H81C g32P; and the single Cys^{87} mutant, C87S g32P.

Binding Affinity of g32P Mutants for ssDNA-Cellulose Correlates with Zn(II) Incorporation by Escherichia coli. All g32P derivatives are expressed in soluble form to high intracellular levels on rich media. None of the mutant proteins showed any evidence for marked instability relative to the appropriate wild-type molecule during the course of standard purification protocol [cf. Giedroc et al. (1990)]. Single-stranded DNA-cellulose chromatography provided an initial indication of the affinity of g32P's for ssDNA. The higher the binding affinity, the higher the concentration of NaCl required to elute the protein, since the binding free energy has a large electrostatic component (Kowalczykowski et al., 1981). In cases where the binding to ssDNA-cellulose was relatively weak, phenyl-Sepharose chromatography was sufficient to give g32P preparations greater than 90% pure by SDS–PAGE[1] analysis. This level of purity was sufficient to estimate Zn(II) content by atomic absorption and global conformation by CD spectroscopy.

The data compiled in Table I indicate that all three His^{64} mutants, including the conservative Asn and Gln substitutions, are purified from *E. coli* in the Zn(II)-free form and bind to ssDNA-cellulose very weakly. These gross properties also characterize the known metal ligand mutant, C87S g32P. It proved impossible to reconstitute a nondialyzable metal complex of any kind with, for example, the H64L and H64Q g32P–B derivatives. In striking contrast, all three His^{81} substitution mutants as a group retain high-affinity binding to ssDNA-cellulose and contain stoichiometric Zn(II) by atomic absorption spectroscopy as isolated.

Table I: Gross Properties of Wild-Type and Mutant g32P's[a]

protein	ssDNA-cellulose binding[b] g32P	g32P–(A+B)	g32P–B	Zn(II)[c]	metal reconstitution/substitution[d]
wild-type	****	**	*	+	+
H64N		±		–	–
H64Q			–	–	–
H64L			–	–	–
H81Q		**		+	+
H81A	****	**		+	+
H81C	****	**		+	+
C87S	●			–	–

[a] Mutant proteins were expressed and initially characterized in the intact g32P, g32P–(A+B), and/or g32P–B forms as indicated. All properties are reported relative to the wild-type derivative in each case. [b] Elution from ssDNA-cellulose was with 2 M NaCl (****), 0.5 M NaCl (**), between 0.3 and 0.5 M NaCl (*) (Giedroc et al., 1990), 0.15 NaCl (±), and no binding (–). [c] Stoichiometric (1.0 ± 0.1) (+) or less than 0.05 g-atom (–) Zn(II) by atomic absorption on purified proteins. [d] Metal-free g32P's were anaerobically incubated with 10 mM DTT and excess Zn(II) or ^{113}Cd(II) and subjected to dialysis against TNGa buffer to remove loosely bound metals. Nondialyzable (+) or dialyzable (–) metal by AAS.

Far- and Near-Ultraviolet CD[1] Spectroscopy. CD spectroscopy in the far ultraviolet can be used to estimate the extent to which each single amino acid substitution alters the secondary structure content of the appropriate wild-type molecule. Although the far-UV spectra shown (Figure 2) do not extend to high enough energy to confidently estimate the fractional composition of each secondary structure in any case (Johnson, 1990), two conclusions can be drawn concerning the relative spectral features which characterize each mutant protein. All three Zn(II)-containing His^{81} mutants appear to possess secondary structure contents nearly indistinguishable from the appropriate wild-type species (Figure 2A,B). In contrast, H64N g32P–(A+B), representative of the His^{64} mutants, as well as C87S g32P, are characterized by $\Delta\epsilon$ values significantly less negative than that of the wild-type protein, suggestive of some loss in structure (Figure 2A,B). These spectral features are similar to those previously found for the metal-free apo-g32P, prepared via chemical expulsion of the Zn(II) from the native protein with sulfhydryl-modifying reagents [apo-(PMPS)] (Figure 2B) (Giedroc et al., 1989). A difference far-UV–CD spectrum (C87S–WT) shows the presence of two minima at ≈223 and 208 nm (Figure 2B, inset). Grossly similar secondary structural changes characterize the apo-(PMPS)-g32P (Figure 2B) [see also Giedroc et al. (1989)].

Optical activity in the near-UV reflects the asymmetric environment of one or more of the aromatic side chains in a

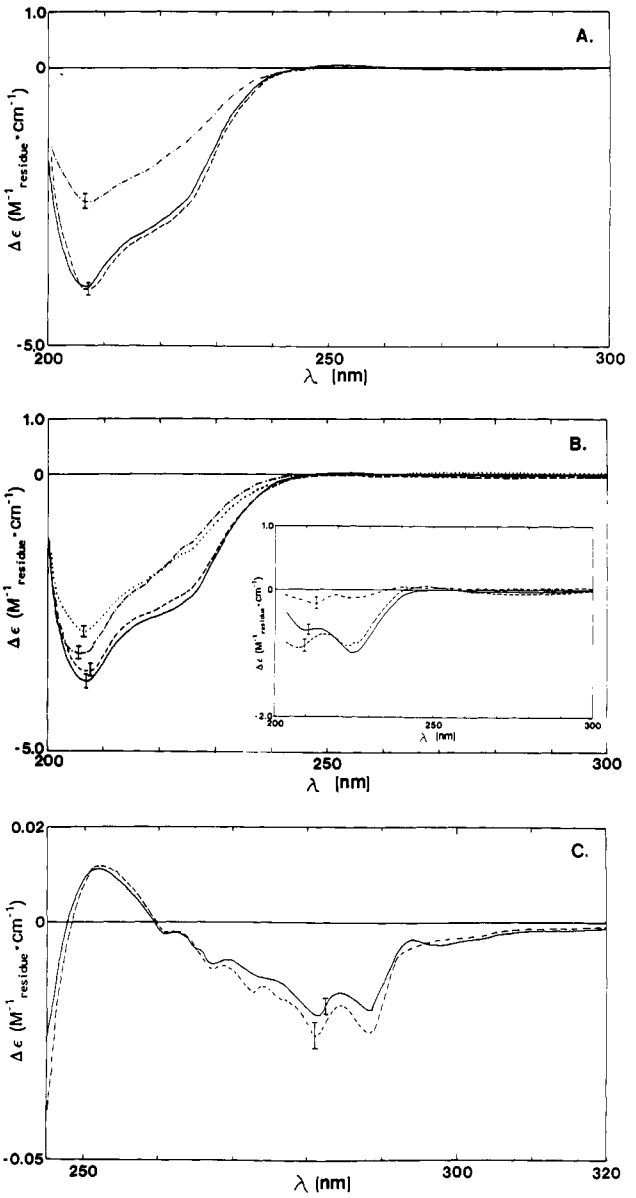

FIGURE 2: Far- and near-ultraviolet circular dichroism spectra of wild-type and mutant g32Ps in 10 mM Tris-HCl, 0.1 M NaCl, and 5% (v/v) glycerol, pH 8.0. (A) Far-UV-CD spectra of WT g32P-(A+B) (solid curve), H81A and H81Q g32P-(A+B)'s (dashed curve), and H64N g32P-(A+B) (dot-dashed curve). (B) Far-UV-CD spectra of WT g32P (solid curve), H81A g32P (dashed curve), C87S g32P (dot-dashed curve), and apo(PMPS)-g32P (dotted curve). (Inset) Difference far-UV-CD: WT-apo(PMPS)-g32P (dashed curve); WT-C87S g32P (solid curve); WT-H81A g32P (dot-dashed curve). (C) Near-UV-CD spectra of H81A g32P (dashed curve) and C87S g32P (solid curve). Acquisition parameters are given under Materials and Methods.

protein, dominated by electronic transitions of both Trp and Tyr. Spectral changes or perturbations here are usually attributed to electronic interactions which become manifest as a result of tertiary structural interactions. In wild-type g32P, two negative Cotton effects at ≈289 and 282 nm are attributed to one or more Trp, while the positive peak at ≈245 nm probably arises from a phenolic ring transition(s) (Anderson & Coleman, 1975; Giedroc et al., 1989). The two spectra shown (Figure 2C) are representative of wild-type and all mutant g32P's thus far isolated and indicate that most of the general features of the near-UV-CD spectra of the wild-type molecule persist in *all* mutant proteins, independent of whether Zn(II) is bound or not. Thus, although the reduction in negative ellipticity is easily measured in the far-UV-CD

Table II: Equilibrium Binding Parameters Obtained from Reverse Fluorescence Titrations of Wild-Type and Mutant g32P Derivatives with Poly(dT)[a]

protein	K_{obs} (M^{-1})	Q_{max}
wild-type g32P-(A+B)	8.0×10^6	0.415
H81A g32P-(A+B)	7.0×10^6	0.408
H81Q g32P-(A+B)	2.2×10^7	0.425
H64N g32P-(A+B)	1.5×10^3	0.425[b]
C87S g32P	3×10^2	0.425[b]

[a] Conditions: 10 mM Tris-HCl, 0.1 mM Na_3EDTA, pH 8.1, and 0.05 M NaCl at 20 °C. Protein concentrations varied from 0.3 to 1.0 μM. Parameters were obtained from best-eye-fit theoretical isotherms drawn through the experimental data points predicted by the McGhee-von Hippel large ligand noncooperative overlap binding model with fixed $n = 7$ and $\omega = 1$ [cf. Giedroc et al. (1990)]. The uncertainty in K_{obs} is ≈20%. [b] Q_{max} values estimated from other derivatives, since this binding affinity was too weak to obtain saturated binding isotherms.

spectra in the His[64] and Cys[87] mutants, they are inconsistent with a complete loss of ordered secondary (and globular) structure in these molecules. These spectral data are consistent with limited proteolysis experiments, which provide evidence that the C-terminal region of the core domain of the molecule (amino acids 112–253) does not require Zn(II) binding for its folding (H. Qiu and D. Giedroc, unpublished observations).

Quantitative Binding Studies of Mutant g32P's to the Homopolynucleotide Poly(dT). In vitro equilibrium ssDNA binding affinity has been estimated for each of the mutant g32P's, generally as the g32P-(A+B) derivatives. The effect of these mutations on the cooperative binding properties of g32P are beyond the scope of this paper and will be reported elsewhere. The wild-type core fragment binds noncooperatively and nonstoichiometrically to poly(dT) near neutral pH and 20 °C (Giedroc et al., 1990). The binding of wild-type and mutant g32P's to poly(dT) was monitored at pH 8.1, 0.1 M NaCl, and 20 °C by measuring the quenching of the intrinsic Trp fluorescence upon association with nucleic acid in a reverse titration of ligand (g32P) with lattice [poly(dT)]. Best-fit theoretical isotherms superimposed on the experimental data described by the noncooperative large ligand overlap model of McGhee and von Hippel (1974) (see Materials and Methods) give the parameters (K_{obs}, Q_{max}, and site size n) compiled in Table II.

H81Q and H81A g32P-(A+B) bind with a K_{obs} similar to the wild-type recombinant protein (Table II). On the other hand, the recombinant H64N g32P-(A+B) shows an affinity some three to four orders of magnitude lower, while the C87S protein is basically inactive as an ssDNA-binding protein in this assay ($K_{obs} \approx 300\ M^{-1}$). Other experiments which monitored the fluorescence of the nucleic acid molecule also revealed little complex association with C87S g32P (data not shown). These quantitative results are consistent with those described in Table I.

Free Sulfhydryl Reactivity of Wild-Type and His[81] Mutant Proteins. The reactivity of cysteine residues to DTNB can provide an indication of the accessibility and, by inference, the metal coordination state of the Cys side chains in g32P. Figure 3 shows a time course of the sulfhydryl reactivity of the wild-type, H81A, H81C, and C166S g32P's under native (closed symbols) or denatured metal-chelated (open symbols) conditions. Under fully reduced and denaturing conditions, H81C, WT, H81A, and C166S g32P's give five, four, four, and three free thiols, respectively, as expected from their primary structures. Under native metallated conditions, wild-type g32P is characterized by a fast phase of reactivity in which one thiol reacts, followed by a much slower phase, which if allowed to continue results in quantitative reactivity

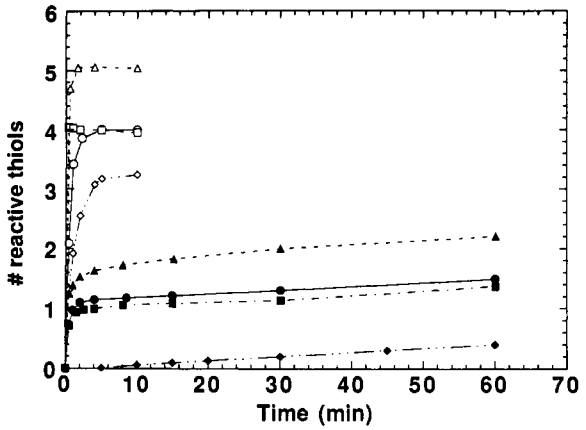

FIGURE 3: Time course of reaction of DTNB with wild-type and single-site mutant g32P's under native (filled symbols) and denaturing metal-chelated (open symbols) conditions. The protein concentration ranged from 6 to 10 μM with DTNB present in excess (140 μM) in either TNGa, 0.1 mM Na_3EDTA (filled symbols), or TNGa, 0.1 mM Na_3EDTA, and 7 M urea (open symbols). Symbols: (○, ●) wild-type g32P, (□, ■) H81A g32P, (△, ▲) H81C g32P, and (◇, ◆) C166S g32P. The corrected absorbance change at 412 nm was converted to the number of reactive thiols using $\epsilon_{412} = 1.36 \times 10^4$ $M^{-1}\cdot cm^{-1}$.

of all four thiols and complete loss of bound Zn(II).

Upon comparison of the reactivities of H81A and WT proteins with that of C166S g32P, it is clear that Cys^{166} is the fast-reacting thiol since C166S g32P is characterized by only the slow phase of DTNB reactivity. This slow phase must represent reactivity of metal ligand cysteines at residues 77, 87, and 90. Significantly, H81C g32P is characterized by a fast reaction of approximately *two* thiols, followed by the same slow rate of reactivity of the metal ligand cysteines found in the other g32P derivatives. Since Cys^{81} is not found in H81A and WT g32P's, the simplest interpretation of these data is that Cys^{81} is accessible to modification by DTNB in the Zn(II)-coordinated form of the protein, a result unexpected in the event of a Cys^{81}–S^-–Zn(II) coordination bond.

Spin-Echo ^1H NMR Spectra of Wild-Type and H81Q g32P–(A+B). Due to the complexity of the aromatic region of the NMR spectrum of g32P–(A+B), Pan et al. (1989a) used selective deuteriation techniques to identify two pairs of histidyl resonances in the ^1H NMR spectrum of ^1H-His–g32P–(A+B). One pair was positioned at 6.81 and 6.57 ppm at pH 8.0, designated the C and D protons, respectively. They were characterized by the same narrow line width and were shown to shift downfield as the pH was reduced from 8.0 to 5.9. g32P–(A+B) begins to precipitate at pH values below 5.5, so that complete titrations could not be obtained; however, the pK_a was estimated to be 6.0–6.5. (Pan et al., 1989a). These characteristics are consistent with this His being largely exposed to solvent (Pan et al., 1989a). The second proton pair, designated A (δ = 7.61 ppm) and B (δ = 7.35 ppm), was found to have broader resonances, and their shifts were insensitive to pH changes over the same range. The A–B pair is thus buried from solvent and/or ligated to the Zn(II) ion.

One-dimensional ^1H NMR spectra of fully protonated wild-type and H81Q g32P–(A+B) reveal that both spectra are extremely similar at pH 7.8, indicative of at most minor conformational changes which occur upon substitution of His^{81} with Gln (spectra not shown). However, the H81Q spectrum is of generally slightly lower quality due to uniform broadening throughout the spectrum, suggestive of enhanced self-association of the mutant. Spin-echo pulse sequences have long been used to selectively enhance the detection of protons with relatively long relaxation times in proteins. His ring signals are most readily detected with this technique (Campbell et al., 1975). Recording a series of such spectra at a variety of pH values allows definitive identification of resonances corresponding to imidazole ring protons exposed to solvent. Since g32P–(A+B) contains just two His, recording of spin-echo spectra of wild-type and H81Q g32P–(A+B) over the same pH range will allow the assignment of the His^{81} and His^{64} ring proton resonances to the A, B, C, or D resonances of Pan et al. (1989a).

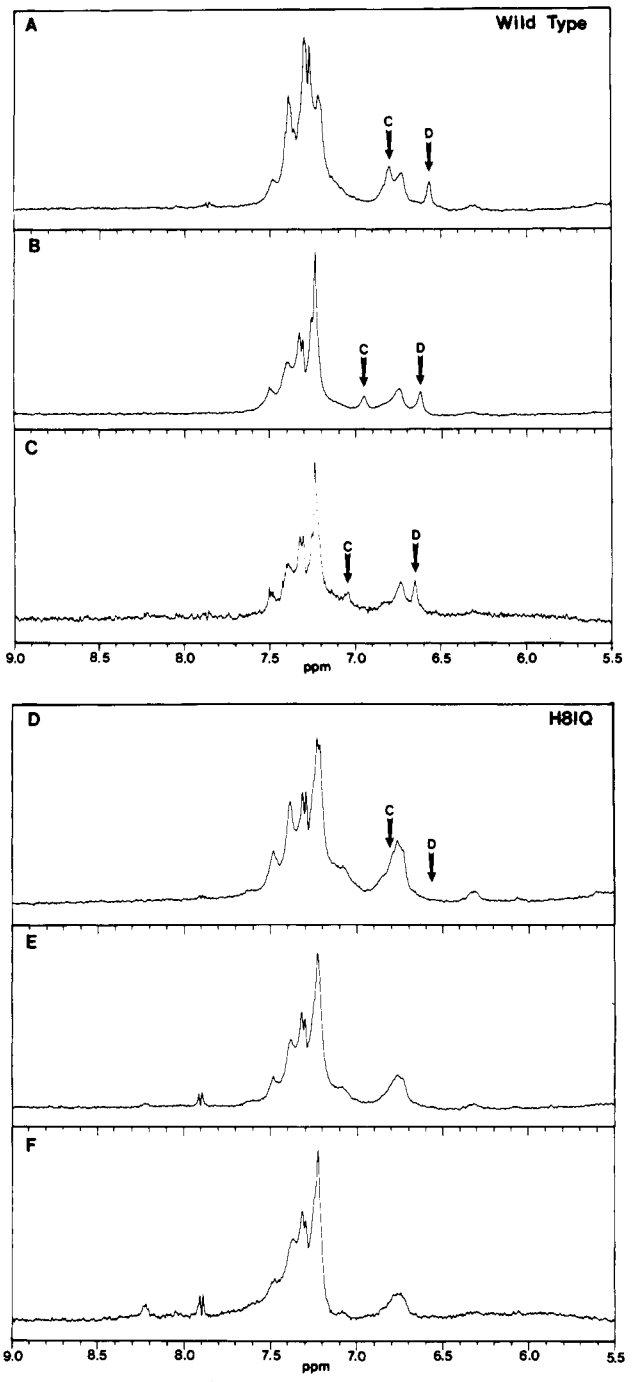

FIGURE 4: Spin-echo ^1H NMR spectra of the aromatic region of wild-type (0.98 mM) and H81Q (0.72 mM) g32P–(A+B) as a function of pH in 50 mM sodium phosphate and 30 mM NaCl, at 30 °C. (A) WT, pH 7.80, 1098 transients; (B) WT, pH 7.05, 958 transients; (C) WT, pH 6.64, 957 transients; (D) H81Q, pH 7.80, 882 transients; (E) H81Q, pH 7.00, 1794 transients; (F) H81Q, pH 6.64, 1046 transients. All other acquisition parameters are given under Materials and Methods.

Figure 4 shows spin-echo spectra of wild-type (panels A–C) and mutant H81Q (panels D–F) g32P–(A+B) recorded at pH 7.8, 7.0, and 6.6, respectively. Histidyl protons C and D of

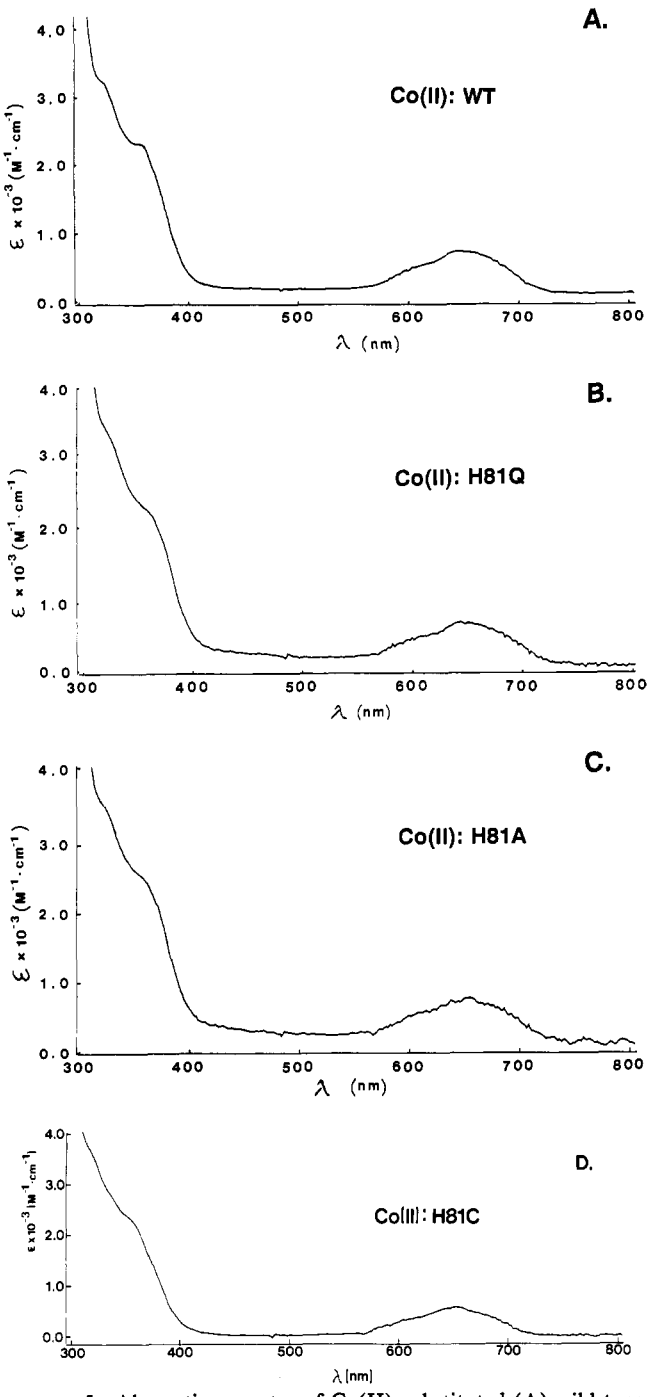

FIGURE 5: Absorption spectra of Co(II)-substituted (A) wild-type g32P, 167 μM Co(II); (B) H81Q g32P, 62 μM Co(II); (C) H81A g32P, 272 μM Co(II); and (D) H81C g32P, 131 μM Co(II). See Materials and Methods for experimental details.

Pan et al. (1989a) are easily identified in the wild-type spectra from their known chemical shifts and titration behavior (panels A–C). These resonance lines are clearly absent in the corresponding H81Q g32P–(A+B) spectra (panels D–F), with the remainder of the spectrum relatively unperturbed. Particularly, at pH 7.0 and 6.6, the wild-type and mutant spectra appear indistinguishable, outside of the C–D proton pair present only in the wild-type protein. This firmly assigns the C–D pair as deriving from His^{81}. The A–B proton pair is therefore assigned by elimination to His^{64}. His^{64} protons could not be readily identified in the spin-echo spectra of either molecule at any pH due to spectral crowding and/or unfavorable relaxation effects, the latter consistent with its broader line width (Pan et al., 1989a).[3] An extremely acidic pK_a or

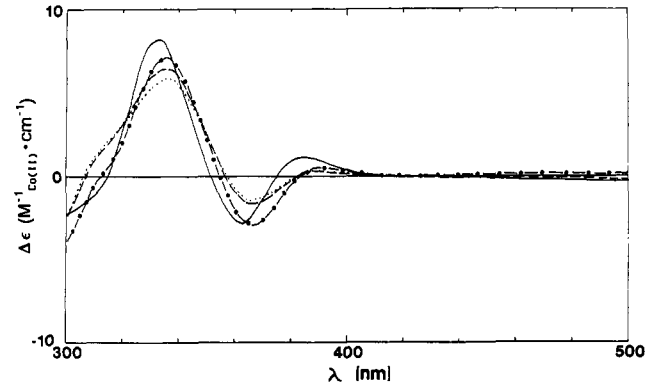

FIGURE 6: Natural circular dichroism of Co(II)-substituted wild-type (solid curve), H81Q (dotted curve), H81A (dashed curve), and H81C (dot-dashed curve) g32P's. Conditions: TNGa buffer, 25 °C, WT, 56 μM Co(II), two scans, 4-s time constant, 10 nm/min scan rate; H81Q, 62 μM Co(II), one scan, 2-s time constant, 20 nm/min; H81A, 272 μM Co(II), two scans, 2-s time constant, 20 nm/min; H81C, 131 μM Co(II), four scans, 4 s time constant, 10 nm/min.

nontitratability would characterize a histidine coordinated to a metal ion through one of its two nitrogen atoms, since protonation of a hystidyl ligand requires dissociation of the imidazole–metal bond (Ulrich & Markley, 1978). Protonation of His^{81} has little effect on coordination of the metal ion, since the ^{113}Cd chemical shift of ^{113}Cd-substituted wild-type g32P–(A+B) varies by just 1 ppm over the same pH range (δ = 638.5, 638.0, and 637.5 ppm at pH 7.8, 7.0, and 6.6, respectively). These data are consistent with His^{81} being relatively exposed to the solvent and nonliganding in wild-type g32P–(A+B).

Metal Ion Substitution and Spectroscopy of His^{81} Mutant Proteins. Although the His^{64} and Cys^{87} mutant g32P's proved refractory to metal ion binding in vivo and in vitro (Table I), the His^{81} substitution mutants, by virtue of their tight Zn(II) binding, are excellent targets for substitution of the Zn(II) with spectroscopically useful metal ions, e.g., Co(II) and ^{113}Cd(II). In previous studies with the wild-type protein, both Co(II) and Cd(II) have been shown to be excellent substitutes for Zn(II) since they maintain the structural integrity and high-affinity cooperative binding of the molecule [for a review, see Coleman and Giedroc (1989)]. In this way, subtle features of the coordination geometry which result specifically from replacement of the imidazole side chain with a polar but nonliganding side chain Gln (H81Q), the nonliganding methyl group of Ala (H81A), and the potential liganding cysteinyl side chain (H81C) can be probed.

The optical absorption spectra of all three His^{81} mutant Co(II) derivatives (panels B–D) compared to wild-type g32P–(A+B) (panel A) are shown in Figure 5. It is striking that regardless of substitution, the energies and intensities of the ligand field transitions and the $S^- \rightarrow$ Co(II) ligand-to-metal charge transfer bands are extremely similar to those in the wild-type protein. There is no spectral evidence for additional cysteinyl ligation to Co(II) in the H81C mutant (Figure 5D), consistent with reactivity of Cys^{81} to DTNB in the metallated state (Figure 3). The natural CD of the $S^- \rightarrow$ Co(II) LMCT region (Figure 6) shows detectable but quite small alterations in the mutant proteins relative to the wild-type protein. These appear independent of the nature of the substitution at residue 81.

[3] Spin-echo spectra collected at shorter delay times (τ = 10 and 5 ms) reveal resonances potentially attributable to His^{64} protons. However, as τ decreases, the technique loses selectivity, making it impossible to obtain a firm assignment.

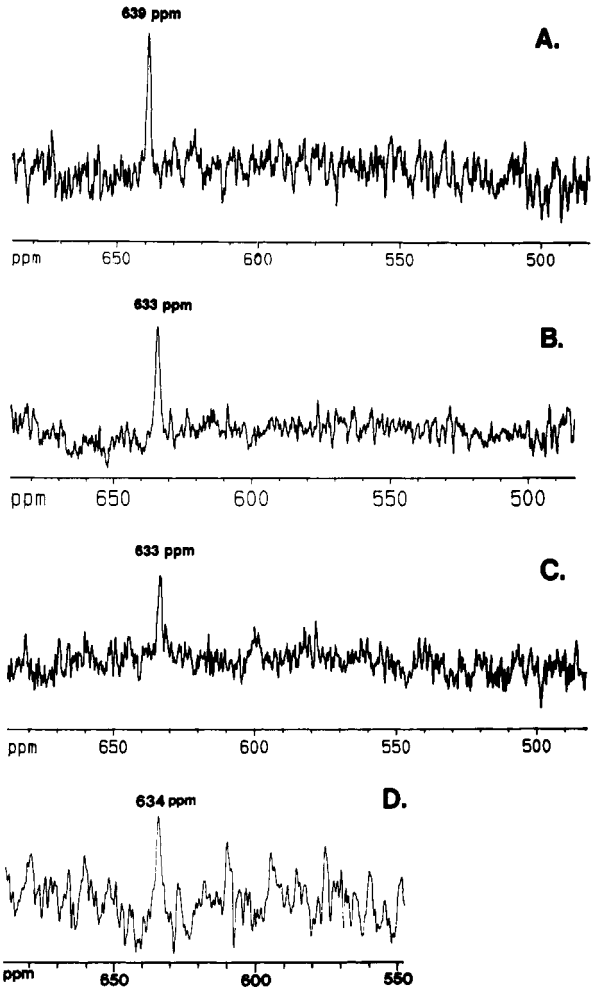

FIGURE 7: ^{113}Cd NMR spectra of ^{113}Cd-substituted wild-type, H81Q, H81A, and H81C g32P-(A+B) core fragments in 50 mM sodium phosphate and 30 mM NaCl, pH 7.8. Conditions: (A) WT (111 MHz), 0.38 mM ^{113}Cd(II), 3200 transients, 1-s recycle delay, 0.36-s acquisition time, and 45° pulse angle, with 55-Hz digital broadening; (B) H81Q (111 MHz), 0.52 mM ^{113}Cd(II), 4500 transients, and all other parameters as in panel A. At 89 MHz, δ = 633.1, 633.3, and 633.5 ppm at 0, 50, and 250 mM NaCl in 50 mM sodium phosphate, pH 7.8; (C) H81A (111 MHz), 0.28 mM ^{113}Cd(II), 2800 transients, 50-Hz line broadening, and all other parameters as in panel A. At 89 MHz, δ = 633.6 and 633.3 ppm at 30 mM and 0.61 M NaCl, respectively, in 50 mM sodium phosphate, pH 7.8; (D) H81C (89 MHz), 0.35 mM ^{113}Cd(II), 15600 transients, 2.5-s recycle delay, 0.3-s acquisition time, and 68° pulse angle, with 80-Hz digital broadening. In panels A–C, the complete spectral window that was scanned is shown. In panel D, this and multiple spectra were collected to 775 and 805 ppm to lower field. No additional resonances were detected. The region shown was selected to facilitate comparison of all spectra.

^{113}Cd(II) substitution and ^{113}Cd NMR will be more sensitive to the nature of the metal–ligand bonds (bond length and angle) (Lacelle et al., 1984) and to dynamic changes in structure (Armitage & Otvos, 1982) than will Co(II) substitution of the intrinsic Zn(II). H81Q, H81A, and H81C mutants give essentially identical ^{113}Cd NMR chemical shifts, 633–634 ppm (Figure 7B-D), slightly upfield of the wild-type protein (δ = 639 ppm) (Figure 7A) recorded under the same solution conditions. These ^{113}Cd NMR spectra obtained for the His^{81} mutants are consistent with the Co(II) substitution data, in that δ appears relatively insensitive to the nature of the substitution at residue 81 (see Discussion). The ^{113}Cd chemical shifts of both the H81Q and H81A g32P-(A+B) are insensitive to exogenous Cl^- (see the legend to Figure 7), evidence that a new open or solvent(anion)-accessible inner coordination site has not been created as a result of His^{81} mutagenesis (Giedroc et al., 1989).

Discussion

Since the original demonstration of Zn(II) binding by T4 gene 32 protein (Giedroc et al., 1986), there has been considerable effort directed toward understanding the role of the Zn(II) domain in replication-mode ssDNA binding (Giedroc et al., 1987; Nadler et al., 1990), in addition to its mRNA-binding activity in autogenous translational repression of g32P biosynthesis (Shamoo et al., 1991). Such a molecular understanding begins with direct identification of the ligand donors in the primary structure of the molecule. In this report, we use site-directed mutagenesis to introduce single amino acid substitutions in the metal domain region of g32P and examine the structural and functional consequences of these perturbations.

Substitution of the proposed Zn(II) ligand His^{81} with two nonliganding amino acids (Gln and Ala) appears to affect the globular structure and ssDNA binding of the molecule very little. Both mutant proteins bind tightly to ssDNA (Table II). It is of interest that a previously mapped His^{81} → Tyr mutation (ts53; Gauss et al., 1987) bestows on T4 a temperature-sensitive phenotype, rather than a nonfunctional or nonviable one. This might suggest that substitution of His^{81} does not greatly disrupt tertiary structural folding or function of the molecule at permissive temperature, but rather destabilizes the structure of the protein or the protein–nucleic acid complex at restrictive temperatures. If our substitutions with Ala, Gln, or Cys behave comparably to the ts53 mutant, we anticipate that the complex is destablized, since our preliminary microcalorimetry experiments show that the T_m of the H81A and wild-type g32P's are identical (D. Giedroc and H. Qiu, unpublished observations). A preliminary thermodynamic investigation of the H81A mutant–ssDNA interactions reveals that, at 20 °C, the cooperativity parameter is reduced by a factor of 5 ($\omega \approx$ 120) relative to that of the wild-type protein ($\omega \approx$ 600), suggestive of a subtly altered protein–DNA complex (H. Qiu and D. Giedroc, unpublished data).

Previous studies which investigated the consequences of mutagenesis of known histidine ligands to Zn(II) in two "zinc finger" proteins have revealed deleterious structural and functional effects. When either of the two His ligands in one zinc finger of yeast ADR1 are converted to Ala, the resulting protein is nonfunctional (Thukral et al., 1991). This contrasts with Ala replacement of a nonliganding His situated between the second and third metal ligands, which has more subtle effects on function. In substitution of the His in Cys_3–His metal-binding retroviral-like zinc finger from Rauscher murine leukemia virus with Gln, the optical spectrum of the Co(II) derivative is markedly altered in both the visible and near-UV regions, consistent with a change in first Co(II) coordination shell (Green & Berg, 1990).

In contrast, metal coordination by H81A and H81Q g32P's appears relatively unaffected. His^{81} mutants are characterized by structural properties expected from substitution of a nonliganding and solvent-exposed His. Spin-echo ^{1}H NMR spectra of the wild-type protein are consistent with the mutagenesis studies and reveal that the His^{81} imidazole ring must be largely exposed to solvent and nonliganding (Figure 4). The optical spectra of the Co(II) derivatives and the ^{113}Cd NMR spectra of the ^{113}Cd-substituted mutant derivatives are identical to one another (Figures 5–7). This is strong evidence that metal coordination of g32P is relatively insensitive to the nature of the substitution at residue 81. The small changes in the coordination environment that we do observe relative to the wild-type protein likely arise from a subtle change(s) in S^-–

metal bond distance and/or angle required to accommodate substitution of an amino acid side chain positioned between two metal ligands in the primary structure, Cys^{77} and Cys^{87}. This change in structure would be expected to detectably alter the optical activity in LMCT region of the Co(II) chromophore in the near-UV (Figure 6) [cf. Johnson and Schachman (1983)]. A slight lengthening of one or more Cd–S^- bonds would likewise tend to reduce the deshielding influence of one or more coordinated Cys, moving δ of ^{113}Cd to slightly higher field (639 to 633 ppm; Figure 7) (Lacelle et al., 1984). For comparison, ^{113}Cd substitution and NMR of the two Cys_3His Zn(II) sites of HIV-1 nucleocapsid protein gave resonance lines positioned at 640 and 659 ppm, a difference of 19 ppm (Fitzgerald & Coleman, 1991), far larger than the 6 ppm difference we observe in wild-type and H81Q (H81A) g32P's. The ^{113}Cd chemical shifts of both mutant molecules are insensitive to changes in exogenous Cl^- concentration, suggesting that anionic ligands from solution do not have access to the inner coordination sphere of the metal ion, analogous to the wild-type protein (Giedroc et al., 1989). Thus, four protein-derived ligands likely complete the tetrahedron. All data strongly suggest that the wild-type, H81Q, and H81A g32P's are characterized by the same set of metal–ligand coordination bonds.

Substitution of His^{81} with cysteine does not result in the formation of a tetrathiolate metal coordination complex. This would be expected if one of the two imidazole side chain nitrogen atoms of His^{81} was within coordination distance of the metal ion (Vallee & Auld, 1990; Krizek et al., 1991). The spectroscopic properties of this mutant are comparable in every case to the Ala and Gln mutants. Cys^{81} in metallated H81C g32P is readily reactive with DTNB (Figure 3), unlike known metal–ligand cysteines. There is no indication from the Co(II) absorption spectrum of H81C g32P that a new S^-–Co(II) bond is formed in the complex. The intensity and shape of the absorption and CD spectra of the LMCT region is similar to that of the Ala and Gln mutants, while the "d–d" transition region does not move to even marginally lower energy or increase in intensity. Either or both would be expected from replacement of an existing non-thiol–Co(II) coordination bond with a Cys^{81}–S^-–Co(II) bond in a tetrahedral or distorted tetrahedral complex (Giedroc & Coleman, 1986; Corwin et al., 1988; Krizek et al., 1991). In addition, the intensity observed for the ligand field region of Co(II)-substituted H81C g32P effectively rules out expansion of the Co(II) coordination shell from four to five coordinate, since this would be expected to occur with a considerable loss of intensity (Corwin et al., 1987).

The ^{113}Cd NMR spectra obtained for freshly prepared ^{113}Cd-substituted H81C protein gives the same slightly upfield-shifted resonance (634 ppm) as the Ala and Gln substitutions. However, the signal-to-noise ratio in the NMR spectrum of the fully relaxed H81C species deteriorates over time, obscuring its detection under typical solution conditions and ^{113}Cd concentrations used for the other derivatives (Figure 7D). This process appears appreciably faster at 30 °C than at 21 °C. After a period of days at 30 °C over which no signal is observed, a weak downfield-shifted resonance is ultimately detected at δ = 680 ppm. Although this new resonance is $\approx$20 ppm to lower field than any NS_3 liganding site reported to date (Fitzgerald & Coleman, 1991), it is 70 ppm upfield from the ^{113}Cd resonance of ^{113}Cd-substituted mononuclear tetrathiolate site of liver alcohol dehydrogenase (δ = 750 ppm) (Bobsein & Meyers, 1982). Over time, Cys^{81} might replace the true fourth ligand in g32P–(A+B) or expand the coordination shell from four to five coordinate, both with a frequency intermediate to slow on the ^{113}Cd chemical shift time scale. These structural changes might ultimately arise from a slow inter- or intramolecular oxidation of Cys^{81} (H. Qiu and D. Giedroc, unpublished results).

In contrast to the His^{81} mutants, substitution of His^{64} is consistently deleterious to the structure and function of g32P. The structural properties of these mutants are not readily distinguished from the known metal ligand donor mutant, C87S g32P (Figure 2 and Table II). The significance of these results is difficult to assess in the absence of a tertiary structure model of the protein. At this level, these substitution mutants must formally be considered folding mutants, not unlike the $Tyr^{73} \rightarrow$ Ser mutant characterized by Shamoo et al. (1989). All molecules are easily isolated but show very little affinity for ssDNA-cellulose, fail to incorporate Zn(II), and show a moderately reduced negative ellipticity in the far-UV CD spectrum, relative to the wild-type protein. Therefore, it is not sufficient evidence that the lack of Zn(II) coordination in a mutant protein necessarily requires that the replaced side chain donate a ligand in the wild-type protein, although obviously it remains a formal possibility. Thus far, other substitutions of His^{64} with, for example, a potentially liganding Cys side chain have proven difficult to express, perhaps suggestive of a far more deleterious alteration in structure than that introduced by Asn, Gln, or Leu replacement. As such, it is not currently possible to test the proposal that His^{64} or Cys^{64} is indeed a ligand using mutagenesis techniques.[4]

Upon elimination of His^{81} as candidate ligand, His^{64} would be required to form a Cys_3His coordination donor set in g32P, since this is the remaining His in the molecule. Previous ^1H NMR studies clearly indicate that conformational changes which occur in g32P–(A+B) upon metal depletion extend to the protons of His^{64} (Pan et al., 1989a). Such a donor set would represent a novel disposition of metal coordination ligands within the primary structure of a zinc-containing nucleic acid binding protein (see Figure 1). However, His^{64} is truely among a significant number of other possibilities in the absence of direct evidence for its involvement. Our preliminary multiple-quantum heteronuclear $^1H(^{113}Cd)$ and ^{15}N NMR experiments suggest that such spectroscopic evidence may be difficult to obtain.[4] We emphasize that there is no compelling justification a priori to select His^{81} over His^{64} as a metal ligand in the absence of physical data. G32P is not a member of a family of evolutionarily structurally homologous proteins [outside of other T-even phage g32P's which are $\approx$98% identical (McPheeters et al., 1988)], like, for example, the retroviral *gag* gene-derived zinc finger nucleocapsid protein family (Berg, 1986; Summers, 1991), with which g32P is often compared on the basis of arguments of anticipated functional similarities (Coleman & Giedroc, 1989; Shamoo et al., 1991). In the absence of such evolutionary guidance, the prediction

[4] ^1H and ^{15}N NMR techniques are currently being applied to the wild-type and His^{81} mutant in an effort to directly detect an imidazole nitrogen–metal coordination bond. Thusfar, application of a one-dimensional heteronuclear $^1H(^{113}Cd)$ multiple-quantum correlation pulse sequence to ^{113}Cd g32P–(A+B) [cf. Live et al. (1981)] reveals that, under conditions where metal ligand Cys β-protons are easily detected, ^{113}Cd coupling to either or both imidazole C2 and C4 protons cannot be observed (D. Giedroc and G. King, unpublished results). Likewise, ^{113}Cd substitution and NMR of uniformly ^{15}N-labeled wild-type and H81Q g32P–(A+B), fail to provide evidence in either case for one-bond ^{15}N–^{113}Cd coupling, which has been observed in other systems (Evelhoch et al., 1981) (D. Giedroc, H. Qiu, and G. King, unpublished results). The lack of coupling in either case does not rule out histidyl coordination in the event that His proton signals are broad [cf. Summers (1988)] and/or $^3J_{H-Cd}$ or $^1J_{N-Cd}$ coupling constants are vanishingly small.

of structural significance exclusively from amino acid sequence data (Berg, 1986; Giedroc et al., 1986; Shamoo et al., 1991) becomes an uncertain proposition.

Supplementary Material Available

A detailed description of all plasmid constructions and site-directed mutagenesis procedures (4 pages). Ordering information is given on any current masthead page.

Registry No. Zn, 7440-66-6; His, 71-00-1.

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