Effects of Substitution of Proposed Zn(II) Ligand His^{81} or His^{64} in Phage T4 Gene 32 Protein: Spectroscopic Evidence for a Novel Zinc Coordination Complex†

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ABSTRACT: T4 gene 32 protein (gp32), the prototype helix-destabilizing or single-stranded (ss) DNA binding protein, contains one tightly coordinated Zn^{2+} ion bound tetrahedrally by three cysteines (residues 77, 87, and 90) and a fourth non-thiol donor. In previous work, it was shown that the proposed non-thiol ligand His^{81} could be readily substituted with nonliganding glutamine and alanine residues without deleterious effects on gp32 structure and simple assays of ssDNA binding. In this paper we show that exchange broadening of bulk $^{35}Cl^-$ anion by protein-bound Zn(II) is not observed in the His^{81} → Ala (H81A) mutant, unless the coordination site is disrupted with an organomercurial, *p*-mercuriphenylsulfonate. This suggests that, in the mutant protein, anions, and by implication solvent molecules, do not gain access to a newly formed inner shell Zn(II) coordination site as a result of mutagenesis. H81A gp32 is characterized by nearly wild-type helix-destabilizing activity on poly(d[A-T]) and highly cooperative binding to the polynucleotide poly(A) at pH 7.7 over the temperature range from 20 to 42 °C at 0.35 M NaCl, exhibiting only a ~2.5–4-fold decrease in poly(A) affinity. Limited proteolysis experiments show that an additional tryptic cleavage site maps to the Arg^{111}-Lys^{112} bond within the protease-resistant core domain of the H81A gp32 following long incubation times and results in the accumulation of a 16-kDa subcore fragment. This new cleavage site is within the internal LAST motif, which has been proposed to be directly involved in cooperative ssDNA binding [Casas-Finet, J. R., & Karpel, R. L. (1993) *Biochemistry 32,* 9735–9744]. Thus substitution of His^{81} with Ala subtly alters the conformation or dynamics of the backbone around the LAST motif, which may be manifest as a moderately lower cooperative binding affinity of H81A gp32 for polynucleotides. H81A gp32, however, is fully functional in stimulating *in vitro* homologous pairing catalyzed by the T4 recombinase uvsX protein. Since substitution of His^{81} with a nonliganding Ala is nearly silent, we propose an alternative mode of Zn(II) coordination in T4 gene 32 protein, involving His^{64} rather than His^{81} as the fourth non-thiol ligand. That replacement of His^{64}, and not His^{81}, with Cys results in marked changes in the first coordination sphere of ligands as evidenced by the optical spectrum of Co(II)-substituted H64C gp32 is consistent with the noninvolvement of His^{81} and implicates a novel His^{64}-X_{12}-Cys^{77}-X_9-Cys^{87}-X_2-Cys^{90} coordination motif, unique among zinc-containing nucleic acid binding proteins.

Bacteriophage T4 gene 32 protein (gp32,[1] 301 amino acids) is the most extensively studied member of a large class of helix-destabilizing proteins which bind with high cooperativity to single-stranded (ss) nucleic acids and play accessory roles in DNA replication and recombination (Karpel, 1990). The cooperative binding of gp32 may impart a particular conformation on the ss nucleic acid which is readily utilized by the DNA polymerase or recombinase. On the basis of limited proteolysis experiments three functional domains have long been proposed for gp32 (Spicer et al., 1979). The N-terminal "B" domain (residues 1–21) has been shown to be required for cooperative binding (Giedroc et al., 1990), while the C-terminal "A" domain (residues 254–301) plays a functional role in heterologous protein–protein interactions which direct the formation of multiprotein complexes which carry out replication (Hurley et al., 1993) and recombination (Jiang et al., 1993). The core domain (residues 22–253), designated as gp32-(A+B), appears to contain the major determinants for ssDNA binding (Prigodich et al., 1986).

It has been established that gp32 contains an intrinsic Zn^{2+} ion, tetrahedrally coordinated in a site contained within the proposed single-stranded DNA binding core domain (Giedroc et al., 1986). The function of zinc coordination has been extensively studied. Among the roles proposed for Zn(II) ligation are structural integrity of the core fragment (Giedroc et al., 1986, 1987), gp32 monomer association and therefore the ssDNA binding cooperativity (Nadler et al., 1990), and autoregulation of its own synthesis at the transitional level (Shamoo et al., 1991). Recently we have shown that Zn(II) complex formation in gp32 is directly coupled to its biological function as a recombination accessory protein (Qiu et al., 1994). In the absence of high-resolution structural data, however, the mechanistic details of the role of Zn(II) coordination are still not well understood. Indeed, recent work suggests that a number of distinct metal-free apo-gp32s can be prepared which appear to differ significantly in their functional activity (Qiu et al., 1994).

Even the identification of the metal coordination ligands in gp32 requires further definition. There are four cysteines and two histidines in the core domain of gp32 (residues 22–253). ^{113}Cd NMR studies show that the metal binding site

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[1] Abbreviations: gp32, T4 gene 32 protein; ss, single-stranded; ds, double-stranded; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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is formed by three cysteines and a fourth non-thiol-protein-derived ligand (Giedroc et al., 1989). Physical and biochemical studies have shown that Cys^{166} is not a metal ligand and is not required for tight and functional ssDNA binding (Giedroc et al., 1989; Qiu et al., 1994). By elimination, Zn(II) coordination by gp32 uses the side-chains S^- atoms of Cys^{77}, Cys^{87}, and Cys^{90} and a fourth non-thiol ligand. On the basis of sequence comparisons with "zinc finger" Zn(II) sites in a number of retroviral single-stranded nucleic acid binding proteins, the fourth ligand was proposed to be His^{81} (Berg, 1986; Giedroc et al., 1986). This would result in an S_3N zinc binding motif formed by 14 contiguous amino acids. The only other histidine residue, His^{64}, lies just N-terminal to this region of the proposed metal binding site, and ^1H NMR studies have shown that the protons of both His^{81} and His^{64} are modulated as a result of Zn(II) removal (Pan et al., 1989).

Spin-echo ^1H NMR measurements of the wild-type gp32 and characterization of several His^{81} mutants reveal that His^{81} exhibits spectroscopic properties not readily compatible with a His^{81} imidazole N–metal bond. His^{64} mutants, on the other hand, do not coordinate Zn(II), leaving His^{64} as a possible metal ligand (Giedroc et al., 1992). In this paper, we report a detailed characterization of the H81A mutant protein. We have carried out uvsX-catalyzed homologous pairing experiments, which can be strongly stimulated by gp32, as a functional assay for its activity as a recombination accessory protein. Here, gp32 has been proposed to function in the assembly of the presynaptic filament and to directly influence postsynaptic phases of recombination (Kodadek, 1990; Jiang et al., 1993). Our binding and biochemical data show clearly that the substitution of His^{81} with Ala perturbs gp32 structure and function to only a modest extent and is incompatible with His^{81} donating a metal ligand in the wild-type protein. In striking contrast, substitution of His^{64} with Cys results in the formation of a non-native first coordination sphere of ligands about the metal ion. The implications of these results for the mode of Zn(II) coordination are discussed.

MATERIALS AND METHODS

Materials

All buffers were prepared with doubly distilled and deionized water. Buffer salts were obtained from Sigma. Some commonly used buffers are as follows: T buffer, 10 mM Tris-HCl, pH 7.8, and 0.1 mM EDTA; TNGa buffer, 10 mM Tris-HCl, pH 7.8, 0.1 M NaCl, and 5% glycerol; H buffer, 10 mM Hepes, pH 7.7, and 0.1 mM EDTA. The stock solutions of 2 M Tris-HCl and 4 M NaCl were passed through a Chelex-100 column (Bio-Rad) prior to buffer preparation. For use in anaerobic experiments, buffer solutions were degassed with several cycles of evacuation and argon purging on a Schlenck line for about 1 h prior to being brought into an argon-atmosphere anaerobic glove box. Protein samples and metal stock solutions were degassed in the same way for 20 min.

Poly(A) and poly[d(A-T)] were purchased from the Midland Certified Reagent Company (Midland, TX). Poly(A) was fractionated on Sepharose-CL-4B and dialyzed exhaustively against T/0.1 M NaCl. The concentration of poly(A) nucleotides was determined in this buffer using ϵ = 10 300 M (nucleotide)$^{-1}$ cm^{-1} at 260 nm (Kowalczykowski et al., 1981). The concentration of poly[d(A-T)] was determined using ϵ = 6700 M (nucleotide)$^{-1}$ cm^{-1} at 260 nm. TPCK-treated trypsin was purchased from Sigma . A 10 mg/mL stock solution of trypsin was made in 1 mM HCl, stored at −20 °C, and diluted into appropriate reaction buffer as required.

Methods

Proteins. Wild-type and mutant gp32s were prepared from an overproducing strain of *Escherichia coli* TB1 transformed with $pP_L\phi$gp32.wt, $pP_L\phi$gp32.H81A, and $pP_L\phi$gp32.H64C by the heat induction of the phage λP_L promoter as previously described (Giedroc et al., 1992). All gp32s were purified from the cell lysate using a combination of column chromatographic techniques (DE-52, ssDNA–cellulose, and phenol-Sepharose columns) as previously described (Giedroc et al., 1992). The purity of the proteins was determined on SDS–PAGE to be greater than 95% homogeneous. Zinc content of gp32s was determined by flame atomic absorption on a Perkin-Elmer 2380 spectrophotometer. Metal-free apo-*S*-methylated gp32 was prepared by incubation with methyl methanethiosulfonate (MMTS) as previously described (Qiu et al., 1994) and designated as apo-*S*-methylated gp32. The metal-free apo-gp32 prepared with sodium *p*-mecuriphenylsulfonate (PMPS) (Giedroc et al., 1986) is designated apo-(PMPS)-gp32. T4 uvsX protein was kindly provided by F. Salinas in Dr. T. Kodadek's laboratory (University of Texas at Austin).

Poly[d(A-T)] Melting Experiments. Ultraviolet melting profiles were determined at 260 nm on a Cary 1 scanning spectrophotometer equipped with a temperature controller essentially as previously described (Giedroc et al., 1987; Villemain & Giedroc, 1993). Stock solutions of gp32s in TNGa buffer and poly[d(A-T)] in T/0.1 M NaCl were diluted in T/0.15 M NaCl to a volume of 400 μL and loaded into cuvettes preequilibrated at 5 °C. The solutions were allowed to equilibrate for 5–10 min, after which time a 0.5 °C/min temperature increase was initiated. The association constant (K_a) can be estimated from thermal melting profiles from the equation (Crothers, 1971)

$$K_a = (e^{\Delta H \Delta T_m / B_c R T_m T_m'} - 1)/a$$

where ΔH, the heat of formation of double-stranded poly[d(A-T)], is −8000 cal/(mole of base pairs); T_m' and T_m refer to the melting temperature of poly[d(A-T)] in the presence and absence of gp32, respectively; $\Delta T_m = T_m' - T_m$; R, the gas constant, is 1.987 cal deg^{-1} mol^{-1}; a is the free gp32 concentration at T_m'; and B_c is the density of the protein binding site on the dsDNA. Assuming a site size of 7.5 for the monomer of gp32, $B_c = 1/(7.5/2) = 0.267$ (Giedroc et al., 1987), while a = [gp32] − 0.5[poly[d(A-T)] binding sites]. This calculation assumes identical enthalpies of complex formation for the wild-type and H81A gp32s, or a difference otherwise small compared to the heat of formation of a duplex of 8–10 nucleotides. Estimates of the van't Hoff enthalpies from the data presented here (Table 1) are −12.8 and −15.8 kcal/mol for the H81A and wild-type proteins, respectively, compared to −80 kcal/mol for formation of a 10-bp $d(A-T)_5$ duplex, which suggests that this approximation is valid.

Circular Dichroism Binding Experiments. The diminution in the positive Cotton band of poly(A) at 264 nm was monitored upon titration with small aliquots of gp32 protein on a Jasco C-600 spectropolarimeter. About 15–25 μM poly(A) in 1700 μL of H buffer with various concentrations of NaCl was incubated at the appropriate temperature for 5 min before data acquisition. Typical acquisition parameters were a 1–2-s time constant, a 1-nm bandwidth, a 0.2-nm step size, and a sensitivity of 20 mdeg. Four scans in a window of 262–266

Table 1: Equilibrium Binding Parameters Obtained for Wild-Type and H81A gp32s on Poly(A) at pH 7.7, 0.35 M NaCl, and the Indicated Temperature[a]

T (°C)	parameter[b]	wild-type gp32	H81A gp32	wild-type/ H81A
20	K_{int} (M^{-1})	860 (150)	590 (80)	1.5
	ω	1700 (300)	600 (80)	2.9
	K_{app} ($K_{int}\omega$) (M^{-1})	1.5×10^6	3.5×10^5	4.2
30	K_{int} (M^{-1})	480 (50)	400 (50)	1.2
	ω	1500 (150)	670 (80)	2.2
	K_{app} ($K_{int}\omega$) (M^{-1})	7.2×10^5	2.7×10^5	2.7
37	K_{int} (M^{-1})	310 (20)	320 (40)	1.0
	ω	1400 (150)	550 (70)	2.5
	K_{app} ($K_{int}\omega$) (M^{-1})	4.3×10^5	1.8×10^5	2.4
42	K_{int} (M^{-1})	300 (40)	430 (40)	0.7
	ω	740 (90)	200 (50)	3.7
	K_{app} ($K_{int}\omega$) (M^{-1})	2.2×10^5	8.6×10^4	2.6

[a] Determined from a nonlinear least squares parameter optimization of K_{int} and ω of circular dichroism binding isotherms like those shown in Figure 2. The 67% confidence intervals are shown in parentheses. [b] The binding site size, n, was fixed at $n = 11$ nucleotides, and only those data points $\Theta \leq 0.4$ were used in the analysis. See Materials and Methods and the text for details.

nm were averaged to increase the signal-to-noise ratio. After each addition of gp32 aliquots, the cuvette was stirred for 1 min. The stirring was then ceased, and the CD spectrum was recorded as indicated. The small negative contribution of the aromatic CD of gp32 was subtracted from the UV-CD spectra of gp32–poly(A) complex, and the dilution was then corrected; the resulting CD intensity at 264 nm was used for the calculation of fractional saturation. Fractional saturation (Θ) recorded at the ith addition of gp32 to poly(A) was calculated from $\Theta = (\text{mdeg}_i - \text{mdeg}_{init})/(\text{mdeg}_{final} - \text{mdeg}_{init})$, which assumes that the change in the CD intensity at 264 nm is linear and directly proportional to the protein binding density. The titration data was then subjected to a nonlinear least squares minimization routine (JANA) created for NONLIN by Dr. M. L. Johnson (Johnson & Faunt, 1992; Villemain & Giedroc, 1993). This algorithm was used to extract values of K_{int} and ω with n fixed at 11 (see Results) from the McGhee–von Hippel closed-form expression for the cooperative large-ligand overlap binding model (McGhee & von Hippel, 1974) as amended by Bujalowski et al. (1989):

$$\nu/L_F = K_{int}(1 - n\nu)\{[2\omega(1 - n\nu)]/[(2\omega - 1)(1 - n\nu) + \nu + R]\}^{n-1}\{[1 - (n + 1)\nu + R]/[2(1 - n\nu)]\}^2$$

where $R = \{[1 - (n + 1)\nu]^2 + 4\omega\nu(1 - n\nu)\}^{1/2}$; ν (binding density) $= \Theta/n$; and $L_F = L_T - L_B$, where L_F is free ligand ([gp32]) and L_B is the bound gp32, $L_B = \nu[\text{poly(A)}_T]$. Once Θ is known, ν is calculated from $= \Theta/n$.

Limited Proteolysis by Trypsin. To 500 μL of gp32 (100 μM) in TNGa buffer preequilibrated at 16 °C was added trypsin prepared as a 1 mg/mL solution in 1 mM HCl to a final concentration of 0.5 or 2.5 mg of trypsin/(μmol of gp32) as indicated. Ten-microliter aliquots were withdrawn at the indicated times following trypsin addition and were mixed with 50 μL of 1× SDS–PAGE gel-loading buffer, heated for 15 min at 75 °C, and stored on ice until all samples were ready for electrophoresis through a 15% SDS–PAGE denaturing gel.

^{35}Cl NMR Spectroscopy. ^{35}Cl NMR spectra were recorded at ambient temperature on a Varian XL-400 spectrometer operating at 39.1 MHz for ^{35}Cl equipped with a broad-band tunable probe that accommodates 10-mm (o.d.) NMR tubes. The protein samples were 0.2 mM gp32 in 2.0 mL of 50 mM sodium phosphate and 0.2 M NaCl, pH 7.7, containing 20–50% D_2O exchanged buffer as a field lock. A sweep width of 2000 Hz was used for data acquisition. Other acquisition parameters are listed in the caption to Figure 1.

Protein Chemistry. The proteolytic fragment generated from extensive tryptic cleavage of H81A gp32 was excised and electroeluted from an SDS–PAGE gel. The sample was dialyzed into 50 mM ammonium bicarbonate and taken to dryness in a speed vacuum. This dried sample was submitted to the Biotechnology Support Laboratory at Texas A&M University for amino acid analysis and N-terminal amino acid sequencing.

Metal Reconstitution of gp32s and Optical Absorption Spectroscopy. Co(II)-substituted wild-type gp32 was prepared as previously described by metal exchange (Giedroc et al., 1986). H64C gp32 was isolated in a Zn(II)-free form with a large fraction of oxidized thiols (≈1.5 reactive thiols by DTNB titration). This protein was dialyzed extensively into metal-free TNGa buffer and concentrated to 1 mM using a 10-mL Amicon concentrator cell. A freshly made metal-free DTT stock solution (1.0 M) was then added to the protein to a final concentration of 100 mM and incubated overnight at 4 °C. The reduced protein was then introduced into the glovebox and dialyzed exhaustively against several changes of 0.5 L of TNGa buffer over the course of 24 h. This protein following dialysis contained 5.3 reactive thiols under denaturing conditions (5 expected) with no residual thiol remaining in the dialyzate. About 1.1 molar equiv of a stock solution of Zn(II), Co(II), and Cd(II) was then added to three aliquots of the reduced and metal-free protein. The green Co(II)-substituted protein was loaded into an anaerobic cuvette in the glovebox, and the visible absorption spectrum was collected within 30 min on a Hewlett-Packard 7452 spectrophotometer at ambient temperature. The absorption spectrum showed no changes over the course of days provided the sample remained in an anaerobic environment. The Zn(II) protein was also scanned in a similar way. The Zn(II)- and Co(II)-substituted wild-type proteins were scanned as described previously (Giedroc et al., 1992). Tabulated spectral data were then imported into Kaleidagraph (Synergy Software, Reading, PA) where spectral subtraction of the Zn(II) protein was performed. The absorbance obtained for H64C gp32 was converted to extinction, assuming complete metal site occupancy in this derivative.

RESULTS

^{35}Cl NMR Spectroscopy of gp32s. Previous experiments have shown that both ^{113}Cd(II)-substituted H81A and H81Q gp32 give a ^{113}Cd chemical shift only 5 ppm upfield of wild-type gp32, a very small change (Giedroc et al., 1992). Like that of the wild-type protein, the chemical shift is insensitive to the addition of exogenous anions (e.g., Cl^-) from solution. This suggests that Cl^- does not undergo rapid exchange with a solvent water molecule at either the wild-type or the H81A mutant ^{113}Cd coordination site. ^{35}Cl NMR is a much more sensitive probe of solvent access by Cl^- to the native Zn(II) site in gp32 (Gettins & Coleman, 1984). ^{35}Cl is a quadrupolar nucleus with a nuclear spin $I = 3/2$ and gives sharp resonances in solution. Complexation of Cl^- with metal ions greatly increases the unsymmetrical environment around the nucleus and will therefore significantly broaden the resonance line of bulk solution $Na^{35}Cl$. A set of ^{35}Cl NMR spectra obtained for 0.2 mM H81A gp32 in 0.2 M NaCl in sodium phosphate buffer are shown in Figure 1. Metal-free H81A gp32 was obtained by chemical modification of the protein with *p*-mercuriphenylsulfonate (PMPS) followed by extensive

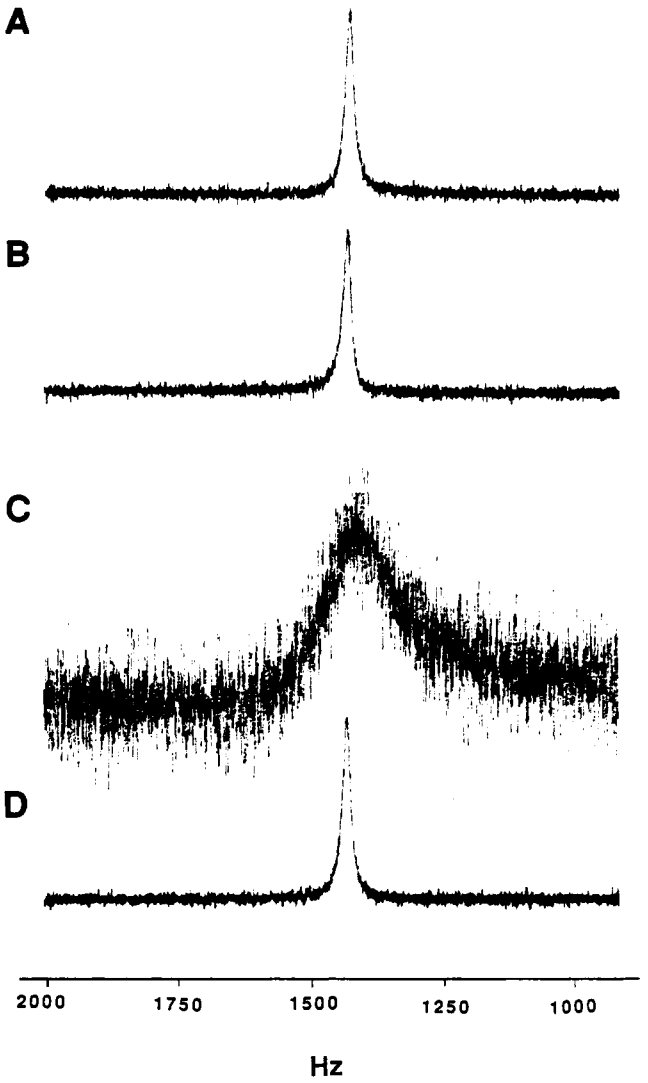

FIGURE 1: ^{35}Cl NMR spectra of apo(PMPS) H81A gp32 (panel A), Zn(II) H81A gp32 (panel B), Zn(II) H81A gp32 + 2 mM PMPS (panel C), and Zn(II) H81A + 2 mM PMPS + 2.5 mM DTT (panel D) in 10 mM sodium phosphate, pH 7.8, and 0.2 M NaCl. The addition of PMPS and dithiothreitol was carried out *in situ* in the NMR tube (panels B–D were recorded sequentially on the same sample). Twenty-four transients were collected in all experiments and subjected to Fourier transformation without exponential line broadening. A sweep width of only 1000 Hz is shown.

dialysis into metal-free buffer as described in Materials and Methods (Giedroc et al., 1986). This gp32 protein serves as a control in which no metal ion is present to complex the Cl^- ion. The height at half-width (designated as $\upsilon_{1/2}$) of the ^{35}Cl resonance in the presence of 0.2 mM apo(PMPS) H81A gp32 is 19 Hz (Figure 1, panel A). This is just slightly greater than the $\upsilon_{1/2}$ of the same buffer solution in the absence of protein (12 Hz; spectra not shown), the difference presumably resulting from the nonspecific interaction of Cl^- ions with amino acid side chains. The spectrum of Zn(II) H81A gp32 (panel B) gives exactly the same $\upsilon_{1/2}$ as apo-H81A protein. Severe broadening of the ^{35}Cl line is effected only by the addition of *p*-mercuriphenylsulfonate *in situ*, which reacts with liganding cysteines (Giedroc et al., 1986) and exposes solvent-accessible Zn(II) coordination sites ($\upsilon_{1/2}$ increased to 122 Hz as shown in Figure 1, panel C). Reversal of the reaction by addition of excess dithiothreitol in the NMR tube results in the restoration of the sharp resonance (panel D, $\upsilon_{1/2}$ = 19 Hz), consistent with the proposal that Zn(II) complex is reformed. These same findings characterize the Zn(II)-containing wild-type protein as well (Giedroc et al., 1989). A rapidly-exchanging Cl^- anion does not have access to the first Zn(II) coordination sphere of H81A gp32, suggesting that a new open or solvent-accessible coordination site has *not* been created on gp32 as a result of $His^{81} \rightarrow$ Ala mutagenesis.

Equilibrium Binding of H81A gp32 to the Model Homopolynucleotide Poly(A). Zn(II) coordination has been proposed to be very important in maintaining highly cooperative binding (Giedroc et al., 1987; Keating et al., 1988). More recent data (Nadler et al., 1990; Villemain & Giedroc, 1993) suggest that apo-gp32 is characterized by a relatively modest ~5–10-fold decrease in cooperative binding affinity at 0.25 M NaCl, pH 8.1, 20 °C. In contrast, substitution of the known metal ligand Cys^{87} with nonliganding Ser in gp32 results in a much larger decrease in its overall binding affinity to ss nucleic acids, exhibiting barely detectable binding to poly(dT) under the same solution conditions (Giedroc et al., 1992). The noncooperative binding affinity of H81A gp32-(A+B) core fragment for poly(dT) is essentially indistinguishable from that of the wild-type protein under these same conditions (Giedroc et al., 1992).

Since the functional conformation of gp32 is one that binds cooperatively to single-stranded nucleic acids, we wished to further characterize the effect of substitution of His^{81} on the cooperative binding mode. Here, perturbations in the magnitude of the cooperativity parameter ω and/or the intrinsic binding constant, K_{int}, of an H81A gp32 monomer for an isolated lattice site, where $K_{app} = K_{int}\omega$, could potentially be resolved. To do this, we carried out a series of binding experiments with wild-type and H81A gp32s binding to poly(A) over a wide range of NaCl concentration and temperature and analyzed these data to obtain a two-parameter (K_{int} and ω) fit to the McGee–von Hippel large-ligand linear lattice binding model (McGhee & von Hippel, 1974). The RNA homopolymer poly(A) was chosen for these experiments since it binds gp32 with a moderate affinity at moderate NaCl concentrations and has been used extensively in previous quantitative analyses of gp32 binding equilibria (Newport et al., 1981; Villemain & Giedroc, 1993).

Complex formation between gp32s and poly(A) was monitored by measuring the diminution of the intense positive Cotton band in the UV circular dichroism spectrum of poly(A) at 264 nm (Jensen et al., 1976; Khan & Giedroc, 1992). All binding experiments were carried out in the "forward" mode, in which a fixed input concentration of poly(A) lattice is titrated with ligand gp32 (Villemain & Giedroc, 1993). All measurements were carried out at pH 7.7 in 10 mM Hepes/0.1 mM EDTA (H buffer) with the indicated NaCl concentration at the indicated temperature.

The binding site size *n* (the number of nucleotides occluded by one gp32 monomer) was independently determined to be 11 ± 1 nucleotides by collecting binding isotherms under low salt concentration (H/50 mM NaCl, 20 °C; data not shown).[2] Representative titrations performed under nonstoichiometric conditions for wild-type and H81A gp32s are shown in Figure 2 and plotted as Θ, or the fractional saturation of 25 μM poly(A) nucleotide, vs input gp32 concentration. The solid lines specify nonlinear least squares fits of the data to the large ligand cooperative overlap binding model at fixed *n* with the parameters K_{int} and ω optimized. For the reasons discussed

[2] The apparent occluded site size for both wild-type and H81A gp32 on poly(A) under identical solution conditions is 7.5–8 nucleotides when measured with a "reverse" titration (Villemain & Giedroc, 1993) vs 11 ± 1 nucleotides in the forward circular dichroism titration. The molecular basis for this discrepancy is unknown.

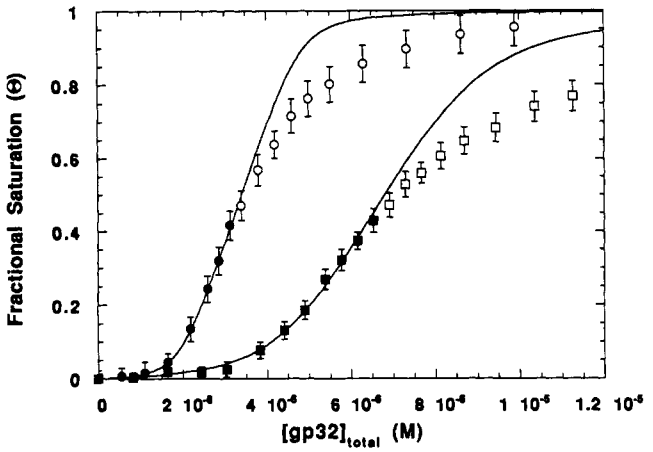

FIGURE 2: Representative forward CD binding titrations presented as fractional saturation (Θ) vs total gp32 concentration. Conditions: H/0.35 M NaCl, pH 7.7, 37 °C. The experimental data points obtained for wild-type (●, ○) and H81A gp32s (■, □), respectively, are indicated with the uncertainties in the measurements. The solid curves represent the nonlinear least squares fits of data points confined to low fractional saturation, i.e., $\Theta \leq 0.4$ (filled symbols) and described by the parameters presented in Table 1.

previously (Villemain & Giedroc, 1993), the data points included in the analysis were limited to those obtained at low lattice saturation ($\Theta \leq 0.4$) under which the infinite lattice approximation in the model is most valid. Table 1 summarizes the equilibrium binding parameters obtained between 20 and 42 °C at H/0.35 M NaCl, pH 7.7. As can be seen, H81A gp32 binds to poly(A) with high affinity and cooperativity, both characteristics of the wild-type protein. The overall binding affinity of H81A gp32 to poly(A) given by $K_{int}\omega$ only falls 2.5–4-fold, depending on the solution conditions. This modest decrease is mainly reflected in a decrease in the magnitude of the cooperativity parameter ω, with the intrinsic binding constant K_{int} apparently less affected. The same ~2–3-fold decrease in binding affinity upon Ala^{81} substitution was also noted over a [NaCl] range extending from 0.27 to 0.39 M at 20 °C in H buffer, pH 7.7 (data not shown).

Helix-Destabilizing Activity of gp32s on the Alternating Copolymer Poly[d(A-T)]. We determined the helix-destabilizing activity of H81A gp32 on the model alternating DNA copolymer poly[d(A-T)] as an independent assay of binding affinity. Poly[d(A-T)] is partially double-stranded in solution and therefore undergoes a helix-to-coil transition upon thermal denaturation, as monitored by measuring the polynucleotide absorption as a function of temperature. The melting temperature (T_m) at which this transition occurs is a reflection of the stability of the helix under defined solution conditions. The difference between the T_m values obtained in the presence and absence of saturating gp32 is related by the apparent binding affinity, K_a, as well as the enthalpy of the formation of the complex. Under conditions where the enthalpies of protein–nucleic acid complex formation are comparable (Table 1), this assay will reflect the relative abilities of H81A and wild-type gp32s to destabilize the helical form of poly[d(A-T)].

Figure 3 shows the complete melting profiles obtained for the wild-type and H81A gp32s at T/0.15 M NaCl, pH 8. Under these conditions, the free poly[d(A-T)] melts with a T_m of 64.5 °C (Figure 3, No protein). In the presence of saturating wild-type and H81A gp32 proteins, the T_m is reduced to 24.3 and 25.1 °C, respectively (Figure 3, WT and H81A). Calculation of binding affinities of the two proteins from their T_m's gives $K_a = 1.0 \times 10^8$ M^{-1} for wild-type gp32

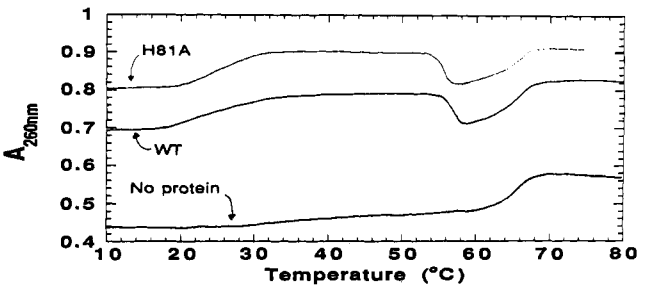

FIGURE 3: Thermal denaturation of poly[d(A-T)] in the presence (curve WT, wild-type gp32; H81A, H81A gp32) and absence (No protein) of gp32. The melting of poly[d(A-T)] was monitored at 260 nm as a function of temperature with a ramp rate of 0.5 °C/min.

and $K_a = 0.9 \times 10^8$ M^{-1} for H81A gp32. This relative difference between the two proteins in their affinities for poly[d(A-T)] is even smaller than that determined for poly(A) from CD titrations (Table 1). However, both experiments support the contention of a highly active H81A mutant. The negative transitions observed at about 56 °C in curves WT and H81A in Figure 3 correspond to thermal unfolding of the protein with the simultaneous renaturation of the copolymer, which then melts at the characteristic denaturation temperature (about 64.5 °C).

Limited Proteolysis of gp32s with Trypsin. Facile removal of the terminal A and B domains with the nearly complete protection of the core fragment from further proteolysis is a characteristic feature of wild-type gp32 (Williams & Konigsberg, 1978). This proteolysis pattern absolutely requires an intact metal domain, in that removal of the metal from the wild-type protein gives rise to extensive cleavage within the core domain of the molecule (Giedroc et al., 1986). We have used this assay as discussed below to detect what must be very slight conformational differences between the His^{81} mutant and wild-type proteins, and compare these proteolysis patterns with those obtained for two metal-free [C87S and apo(PMPS)] gp32 derivatives.

As representative of the results from the His^{81} mutants, Figure 4 shows the products of limited proteolysis by trypsin of intact H81A (panel B) and the wild-type protein (panel A). Proteolysis of the wild-type protein gives rise exclusively to 26-kDa gp32-(A+B) core fragment. After 25 and 33 h of incubation, however, a very small amount of a new fragment of ~16 kDa begins to appear, not previously observed. With H81A gp32, the core fragment is again the major proteolytic product formed at early time points (1.5–2 h); this can be easily isolated and purified. However, the 16-kDa fragment begins to appear at much earlier times than in the wild-type protein, such that, at the penultimate time point (22 h of incubation), the 16-kDa fragment becomes the major proteolytic product remaining. H81Q and H81C gp32 substitution mutants give qualitatively the same results (data not shown).

The 16-kDa subcore fragment has been mapped to the C-terminal region of the core fragment by amino acid analysis and N-terminal sequencing. N-terminal sequencing of the gel-purified fragment (see Materials and Methods) gives the following yield of amino acids at each cycle: Lys(301 pmol)-Thr(198)-Ser(100)-Ala(143)-Asn(134)-Ile(128)-Leu(106)-X-Val(158)-Lys(101)-Asp(122)-Pro(93)-Ala(99)-Ala(127)-Pro(60)-Glu(44). This sequence exactly matches the primary structure deduced from the published nucleotide sequence of gene *32* (Krisch & Allet, 1982), with Lys in cycle 1 corresponding to Lys^{112} and the sequence continuing to Glu^{127}, and X = Val^{119}, revealing tryptic cleavage following Arg^{111}.

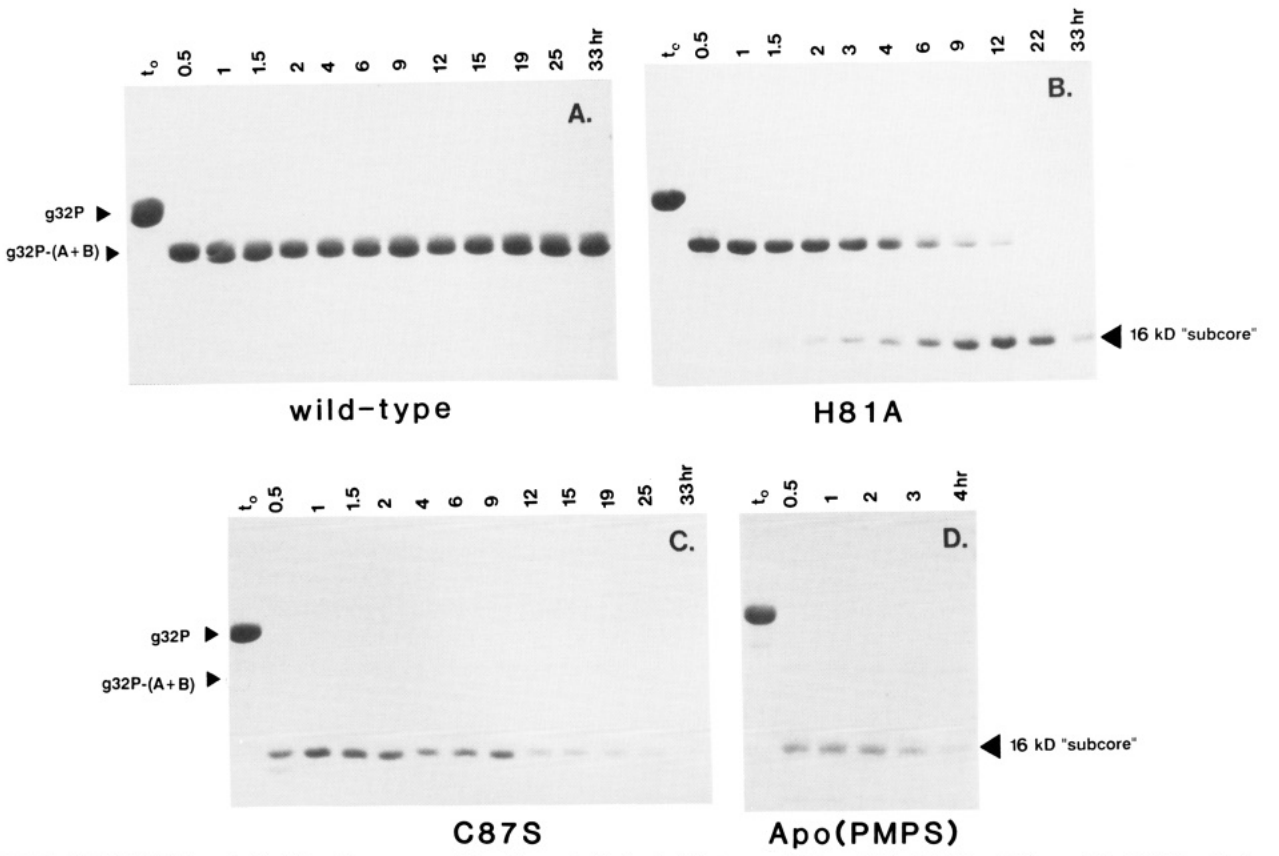

FIGURE 4: SDS–PAGE analysis of the time course of tryptic proteolysis of wild-type gp32 (panel A), H81A gp32 (panel B), C87S gp32 (panel C), and apo(PMPS)-gp32 (panel D). The electrophoretic migrations of intact gp32, gp32-(A+B), and the 16-kDa subcore fragment are indicated. Each 500-μL incubation mixture contained 100 μM gp32 and 0.5 mg of trypsin/(μmol of protein) (panels A–C) or 2.5 mg of trypsin/(μmol of protein) (panel D) in TNGa buffer at 16 °C. Ten-microliter aliquots were removed at the indicated times in hours (t_0 = before trypsin addition) and processed for electrophoresis as described in Materials and Methods.

The apparent molecular weight of the fragment on SDS–PAGE gel and amino acid analysis are consistent with this fragment extending to or near the C-terminus of the core domain, Lys^{253}, although the exact C-terminus was not determined. The amino-terminal one-third of the core domain (residues 22–111) is apparently degraded to smaller fragments before or concomitant with its liberation from the gp32-(A+B) core fragment upon cleavage following Arg^{111}. It is noteworthy that the amino acid sequence at the cleavage site, Lys^{110}-Arg^{111}-↓Lys^{112}-Thr^{113}-Ser^{114}, has been designated as the internal $(Lys/Arg)_3(Ser/Thr)_2$, or LAST motif, which has been proposed to be important in some aspect of cooperative binding by gp32 to ss nucleic acids (Casas-Finet et al., 1992; Casas-Finet & Karpel, 1993).

Panels C and D show the same limited proteolysis experiment carried out with two metal-free forms of gp32, recombinant C87S gp32 and apo(PMPS)-gp32, respectively. It is striking that in both cases the gp32-(A+B) core fragment is not a stable proteolytic intermediate, particularly in the C87S molecule (Figure 4C). Instead, the 16-kDa subcore is the major product at the short incubation times and proves to be relatively refractory to further proteolytic cleavage even though 15 additional potential tryptic cleavage sites are contained within this fragment. Quantitation of these gels reveals that the rate of degradation of the 16-kDa subcore may be very nearly the same in C87S and H81A gp32s (data not shown). This suggests that the subcore has considerable globular structure, corroborated by the significant far- and near-UV CD ellipticity exhibited by this fragment (data not shown). The subcore is devoid of Zn(II) [0.03 g-atom Zn(II) by atomic absorption] and shows no affinity for ssDNA–cellulose (data not shown).

uvsX-Catalyzed Homologous Pairing Is Enhanced by H81A gp32. T4 uvsX protein catalyzes the pairing reaction between homologous sequences of linear duplex and ss circular M13mp19 DNA in the presence of ATP. Although uvsX can perform this reaction alone, single-strand binding protein gp32 strongly stimulates this reaction by reducing the threshold concentration of uvsX protein required for homologous pairing to take place (Kodadek, 1990). The gp32-concentration dependence of the stimulation of uvsX activity has been used previously as an indicator of the relative recombination accessory activity of gp32s (Qiu et al., 1994).

Figure 5 shows a representative agarose gel electrophoresis assay of gp32-stimulated uvsX-catalyzed homologous pairing reactions. The conversion of a ^{32}P-labeled linear duplex into high molecular weight aggregates consisting of many single- and double-stranded species was monitored (Formosa & Alberts, 1986; Kodadek et al., 1988). The uvsX concentration was fixed at 1 μM, under which no homologous pairing occurs. Individual gp32s were then titrated into these reaction mixtures, and the gp32-concentration dependence of the conversion of the duplex to recombination products was determined. As is shown in Figure 5, H81A gp32 (panel A) and wild-type gp32 (panel B) possess indistinguishable accessory activities in this simple assay under these solution conditions, revealing that H81A gp32 appears as effective as wild-type gp32 in facilitating uvsX-mediated pairing *in vitro*. The maximum pairing occurs at about 1.5–2.0 μM gp32 in both cases. Apparent inhibition was seen at high gp32 concentration (>4 μM), possibly due to the competitive binding

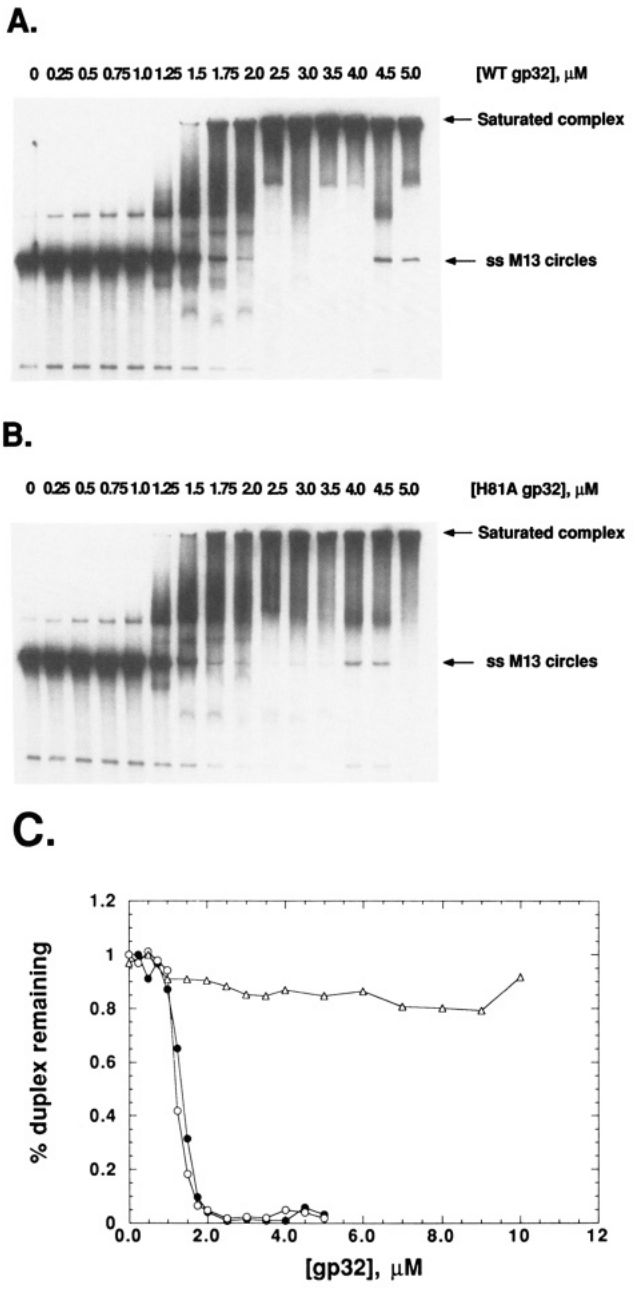

FIGURE 5: gp32 concentration dependence of the stimulation of the uvsX-catalyzed homologous pairing reaction by wild-type gp32 (panel A) and H81A gp32 (panel B). The starting ^{32}P-labeled linear duplex M13mp19 DNA and the high molecular weight aggregation products are indicated to the right of the gel. Panel C results from the quantitation of the disappearance of the linear duplex in the reaction with wild-type (●), H81A (○), and apo-*S*-methylated gp32 (△), the latter taken from quantitation of the data presented previously (Qiu et al., 1994).

of gp32 and uvsX to the ssDNA substrate. The disappearance of the labeled duplex was quantitated with the results shown in Figure 5C. These findings with H81A gp32 contrast sharply to those obtained for a metal-free derivative of gp32, apo-*S*-methylated gp32 (Qiu et al., 1994), where no apparent stimulation of homologous pairing was observed up to 10 μM protein.

Substitution of His^{64} with Cys Results in the Formation of a Non-Native Metal Ligand Donor Set. The data presented above and previously (Giedroc et al., 1992) suggest that substitution of His^{81} with Ala leads to a relatively small change in gp32 structure and function, inconsistent with a His^{81}–Zn(II) coordination bond. We have previously reported that

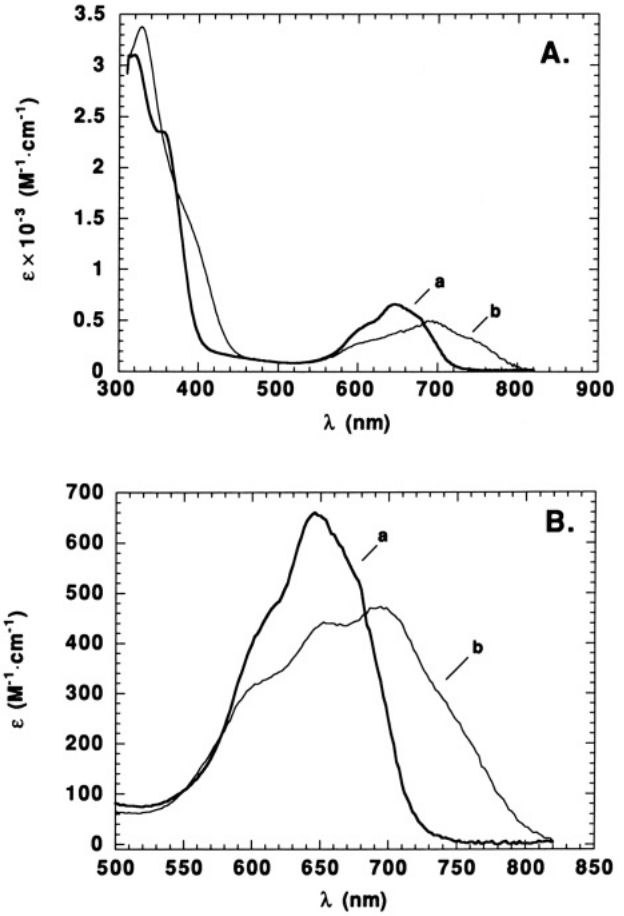

FIGURE 6: Corrected optical absorption spectra of Co(II)-substituted wild-type gp32 (curve a) and H64C gp32 (curve b) at ambient temperature and in TNGa buffer, pH 8.1. The spectral contribution from the same concentration of Zn(II) protein has been subtracted in both cases. (A) Complete spectra: 100 μM H64C gp32, 0.6 mM wild-type gp32. (B) Ligand field transition region: [wild-type gp32] = 0.6 mM; [H64C gp32] = 1.0 mM.

three substitution mutants of His^{64} (H64N, H64Q, and H64L gp32) are purified from *E. coli* as metal-free proteins (Giedroc et al., 1992). This prompted us to create and characterize His^{64}→Cys (H64C) gp32. This substitution should introduce significant alterations in the spectroscopic properties of gp32 related to metal coordination in the event that Cys^{64} would substitute for His^{64} in the native Zn(II) coordination set.

As the native Zn(II) complex is spectroscopically silent, we chose to substitute the Zn(II) with Co(II). The complete corrected optical absorption spectra for the wild-type (curve a) [cf. Giedroc et al. (1986)] and H64C (curve b) gp32s are shown in Figure 6A, while the ligand field transition region of each protein is expanded in Figure 6B. As is immediately apparent, the Co(II)-substituted protein is characterized by a significantly red-shifted "d-d" visible absorption envelope and contains additional alterations in the S$^-$→Co(II) ligand-to-metal charge-transfer region ($\lambda \leq 450$ nm) relative to the Co(II)-substituted wild-type protein. These pronounced spectral shifts are clearly indicative of a change in the first coordination shell of ligands about the Co^{2+} ion in H64C gp32. Further, they are consistent with the formation of a new tetrathiolate coordination complex formed by the side chains of Cys^{64}, Cys^{77}, Cys^{87}, and Cys^{90} (Corwin et al., 1988; Swenson et al., 1978; Garmer & Krauss, 1993; Krizek et al., 1993; Formicka-Kozlowska et al., 1988; Vásak et al., 1981), although more work must be done to substantiate this proposal.

DISCUSSION

Bacteriophage T4 gene 32 protein has been used as a model system for a large family of sequence nonspecific ssDNA binding proteins (Karpel, 1990). Ever since the original finding that gp32 is a Zn(II) metalloprotein (Giedroc et al., 1986), the structure and function of the Zn(II) coordination site have been under extensive investigation. A molecular understanding of Zn(II) binding begins with the identification of the metal-liganding residues. In this paper we report the structural and functional consequences of substitution of the proposed metal ligand His^{81}. The proposal that His^{81} donates a metal ligand in gp32 has been widely accepted [cf. Shamoo et al. (1991)], although no direct physical evidence for its involvement has ever been reported, having instead been based on modeling and amino acid sequence comparisons of gp32 with the S_3N retroviral-type zinc finger domain (South et al., 1991) found in the nucleocapsid protein (NCP) family (Giedroc et al., 1986, 1989; Berg, 1986). gp32 and NCPs are clearly not evolutionary homologous, but it was reasoned that they may well share some structural and functional similarities (Giedroc et al., 1986; Shamoo et al., 1991).

Our characterization of gp32 metal domain mutants (Giedroc et al., 1992; this work) does not support the predicted coordination domain model. All mutants with substitutions at His^{81} (H81A, H81Q, and H81C) have been purified as zinc metalloproteins with only very subtle changes in overall conformation and metal site geometry (Giedroc et al., 1992). There is no direct evidence for an altered coordination set, particularly in the H81C gp32 mutant (Giedroc et al., 1992). The ^{35}Cl NMR data on the H81A mutant (Figure 1) show that the inner coordination shell of Zn(II) in H81A gp32, like that in the wild-type protein, does not have access to a rapidly exchanging solvent ligand(s). These data coupled with previous ^{113}Cd NMR studies of wild-type and His^{81} mutants support the simplest interpretation that H81A gp32 is utilizing the same coordination ligands as the wild-type protein. In contrast to the metal ligand mutant C87S gp32, which binds to ssDNA very weakly (Giedroc et al., 1992), H81A gp32 exhibits highly cooperative ss nucleic acid binding activity (Table 1) and has essentially wild-type-like helix-destabilizing activity as measured by the depression of melting temperature of poly[d(A-T)] copolymer (Figure 3). Furthermore, H81A and wild-type gp32s possess indistinguishable biochemical activities in promoting the uvsX-catalyzed homologous pairing reaction (Figure 5). This is also in contrast to apo-C87S gp32 and apo-*S*-methylated gp32 (Qiu et al., 1994). Previous studies which investigated the consequences of mutagenesis of a known zinc-liganding histidine in retroviral nucleocapsid proteins and in yeast ADR1, a TFIIIA-type zinc finger protein, have revealed far more deleterious structural and functional effects (Thukral et al., 1991; Green & Berg, 1990). We therefore conclude that His^{81} in gp32 cannot be donating a metal coordination ligand to the Zn^{2+} ion, as proposed in the original model (Giedroc et al., 1989).

Structural Zn(II) sites in metalloproteins in general, and in zinc-containing nucleic acid binding proteins specifically, use exclusively histidines and cysteines as Zn(II) ligands. Acidic amino acid ligands, prevalent in catalytic zinc sites, are thus far lacking, perhaps due to the energetic cost required to bury a highly polarizable carboxylate functionality (Giedroc, 1994; Vallee & Auld, 1990). If we then limit the Zn(II) ligands to only histidines and cysteines in gp32 and further eliminate His^{81} from consideration, the only other possible liganding candidate is His^{64}. Figure 6 reveals that substitution of His^{64} with Cys results in striking changes in the optical spectrum of gp32 which can only result from a change in the first ligand shell (Garmer & Krauss, 1993). The movement of the ligand field transition envelope to lower energies and a significant alteration in the $S^- \rightarrow Co(II)$ ligand-to-metal change-transfer region in the near-UV are both consistent with the proposal that a new tetrathiolate ligand field complex has been formed (Garmer & Krauss, 1993; Krizek et al., 1993). These features of the visible absorption spectrum of the mutant protein, in particular a transition with an absorption maximum ≥ 750 nm, are found in published spectra for Co(II)-substituted aspartate transcarbamoylase (Garmer & Krauss, 1993), the structural zinc site of liver alcohol dehydrogenase (Formicka-Kozlowska et al., 1988) and mammalian metallothionein (Vasák et al., 1981), all of which are known to adopt distorted, tetrahedral, tetrathiolate Co(II) coordination complexes. It will be necessary, however, to characterize the Co(II) complexes of other amino acid substitution variants at position 64 to ensure that these spectral changes arise specifically from a coordination bond from Cys^{64}, rather than from addition of an exogenous solvent ligand or formation of some other non-native complex. Interestingly, Co(II), and we assume Zn(II), binds quite weakly to this mutant site, which readily undergoes rearrangement concomitant with cysteine thiol oxidation and metal release following a brief exposure to air.[3] Indeed, an anaerobic environment is required to form the complex with Zn(II), Co(II), or Cd(II), and this therefore explains our observations that H64C gp32 is purified in a metal-free form in air.[3] These characteristics of H64C gp32 relative to the wild-type protein are not unlike those previously described for His $\rightarrow$ Cys Zn(II) ligand substitution of human carbonic anhydrase II (Alexander et al., 1993).

Although a complete characterization of this mutant is beyond the scope of this paper, the relatively subtle structural and functional consequences of H81A substitution coupled with a perturbation of the first coordination ligand shell in the H64C mutant are compelling enough to lead us to propose an alternate Zn(II) coordination model for gp32 as shown in Figure 7. While this coordination scheme bears no sequence homology or similarity with other known zinc-containing DNA binding proteins, it is supported by all of the structural and biochemical evidence. In particular, Co(II)-substituted H81C gp32 has spectroscopic properties indistinguishable from those of the wild-type protein (Giedroc et al., 1992).

Zn(II) coordination has been shown to be important in maintaining the structural integrity of the gp32-(A+B) core domain, as evidenced by the resistance to further tryptic proteolysis (Giedroc et al., 1986, 1987). Our partial proteolysis results (Figure 5) reveal that the H81A substitution is not, however, completely transparent. The simplest interpretation of the proteolysis experiments is that the transient exposure of an additional tryptic site(s) immediately following Arg^{111} occurs with a higher frequency relative to the wild-type protein. Cleavage at this new site(s) appears to result in efficient degradation of the amino-terminal metal domain region of the core with concomitant accumulation of the carboxyl-terminal 16-kDa subcore fragment (residues 112–253) (Figure 5). The wild-type core fragment also is similarly cleaved, but the 16-kDa fragment is just detectable only after extensive incubation with trypsin. Under the same conditions, C87S gp32 and apo(PMPS)-gp32 are efficiently degraded directly to the 16-kDa fragment, with no (C87S) or little [apo(PMPS)] accumulation of the core fragment [see also Giedroc et al. (1986)]. Upon depletion of the core protein, degradation of

[3] H. Qiu and D. P. Giedroc, unpublished observations.

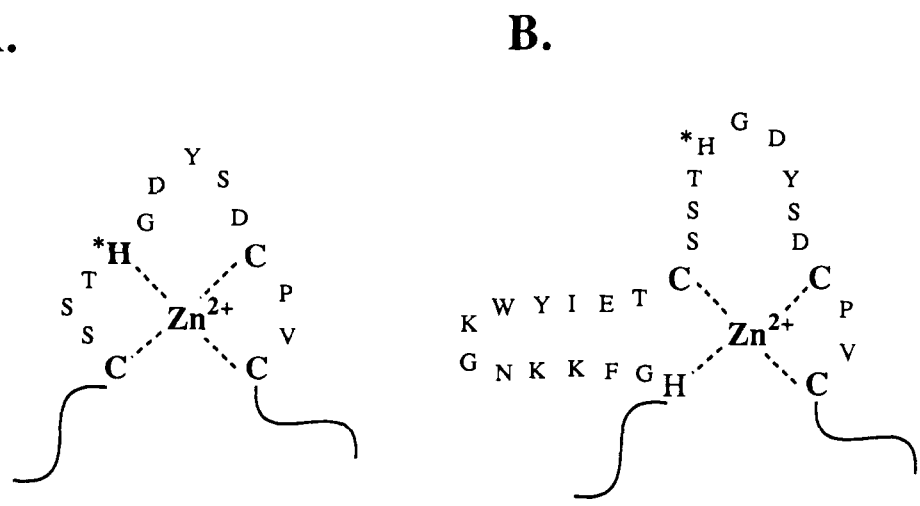

FIGURE 7: A new model for Zn(II) coordination in gp32. The previous model for Zn(II) coordination in gp32, involving His^{81} (*) as the non-thiol metal ligand, is shown in panel A. Panel B shows the new coordination model involving His^{64}, Cys^{77}, Cys^{87}, and Cys^{90} as metal ligands.

16-kDa subcore occurs at similar rates from the apo-C87S and H81A molecules. This suggests that, in both molecules, this region of the core fragment is folded similarly, independent of prior coordination of Zn(II) to the protein.

The additional tryptic cleavage site in H81A gp32 lies within the internal LAST motif (residues 110–114) (Casas-Finet et al., 1992). Interestingly, this LAST motif appears to be found in other nucleic acid binding proteins and is also repeated in the N-terminal B domain of gp32 (Lys^3-Arg^4-Lys^5-Ser^6-Thr^7). We have recently shown that substitution of Lys^3 and Arg^4 with a number of amino acids can produce a small or a very large perturbation of the cooperative binding of gp32 in a manner dependent on the nature of the substitution (Villemain & Giedroc, 1993). Casas-Finet et al. (1992, 1993) have proposed that an as yet unmapped peptide interactive site exists within the core domain of gp32 which interacts with the core LAST motif when gp32 is not bound to nucleic acids. In their model, it is this site which is destined to interact with the N-terminal LAST motif when gp32 binds cooperatively to ssDNA substrate, exposing the internal LAST motif for ssDNA binding. There is as yet no direct evidence that the internal LAST motif interacts with nucleic acid. However, our studies do reveal that substitution of His^{81} with Ala directly or indirectly alters the solvent accessibility of the Arg^{111}-Lys^{112} bond within the internal LAST motif which in turn modestly reduces the cooperative binding affinity of the protein for poly(A) (Table 1).

In summary, we have proposed that Zn(II) utilizes a novel HCCC coordination strategy in gp32 involving the His^{64}-X_{12}-Cys^{77}-X_9-Cys^{87}-X_2-Cys^{90} sequence. This disposition of ligands within the primary structure has not been found in any other zinc-containing single-stranded or duplex nucleic acid binding protein, although the C-terminal zinc site in the glucocorticoid hormone receptor formed by the sequence Cys-X_5-Cys-X_9-Cys-X_2-Cys bears some resemblance when one considers only the C-terminal three Cys ligands (Luisi et al., 1991). However, outside of the Cys-X_2-Cys sequence, amino acids within the X_9 sequence show no sequence homology between the two proteins. High-resolution structural studies of gp32 are required to determine whether there is a structural relationship between the two sites. The identification of a novel zinc coordination strategy at the level of the primary structural disposition of ligands which we now define for gp32 has also been noted in numerous gene regulatory proteins for which global folding topologies or high-resolution structures are available (Kosa et al., 1994; Everett et al., 1993; Qian et al., 1993; Omichinski et al., 1993). This general lack of sequence similarity among zinc binding sites derived from different protein classes is perhaps not surprising and merely reflects the unique requirements of a particular protein to create a site which is well suited to engage in a defined set of macromolecular interactions.

ADDED IN PROOF

X-ray crystallographic studies of gp32 also implicate coordination of the Zn^{2+} ion by His^{64}, Cys^{77}, Cys^{87}, and Cys^{90} (Y. Shamoo and T. Steitz, personal communication).

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