^{1}H NMR Studies of T4 Gene 32 Protein: Effects of Zinc Removal and Reconstitution†

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Received March 20, 1989; Revised Manuscript Received July 5, 1989

ABSTRACT: Gene 32 protein (g32P), the single-stranded DNA binding protein from bacteriophage T4, contains 1 mol of Zn(II)/mol bound in a tetrahedral ligand field. ^{113}Cd NMR studies of Cd-substituted wild-type and mutant ($Cys^{166} \rightarrow Ser^{166}$) g32Ps show Cys^{77}, Cys^{87}, and Cys^{90} to provide three sulfur donor atoms as ligands to the metal ion [Giedroc, D. P., Johnson, B. A., Armitage, I. M., & Coleman, J. E. (1989) *Biochemistry 28*, 2410]. Proton NMR signals from the His and Trp side chains of the protein have been followed as a function of pH and metal ion removal by biosynthesizing the protein with amino acids carrying protons at specific positions in a background of perdeuteriated aromatic amino acids. Only one of the two pairs of His resonances (from His^{64} and His^{81}) titrates over the pH range 8.0–5.9. The nontitrating His side chain is most likely ligated to the metal ion. Upon Zn(II) removal, ^{1}H NMR spectra of the fully protonated g32P-(A+B) exhibit substantial signal broadening in several regions of the spectrum, while the His 2,4-^{1}H resonances are broadened beyond detection. The ^{1}H NMR spectral characteristics of the original protein are restored by reconstitution with stoichiometric Zn(II). The broadening of the ^{1}H NMR signals is not due to oligomerization of the protein, since small-angle X-ray scattering experiments show that the average radius of gyration of the apo-g32P-(A+B) is 25.0 Å and that of the reconstituted Zn(II)-g32P-(A+B) is 31.2 Å. These values suggest that apo-g32P-(A+B) is a monomer, while Zn(II)-g32P-(A+B) is a dimer. We propose that apo-g32P-(A+B) exhibits conformational flux at intermediate rates on the ^{1}H NMR time scale which accounts for the broadening of the NMR signal of the His protons. The conformational fluctuations that occur upon removal of Zn(II) appear to be localized to the immediate vicinity of the Zn(II) binding domain as evidenced by ^{1}H NMR spectra of selectively deuteriated Trp-g32P-(A+B). ^{1}H NMR signals from two of the five Trp side chains, proposed to be Trp^{72} and Trp^{116}, are significantly broadened upon Zn(II) removal, while the remaining signals of the other three Trp residues (Trp^{31}, Trp^{144}, and Trp^{168}) are unchanged.

Gene 32 protein (g32P)[1] is coded for by the genome of bacteriophage T4. The protein binds cooperatively to single-stranded regions of phage DNA formed during phage DNA replication, recombination, and repair. The presence of g32P is required for all these processes [for a review, see Chase and Williams (1986)]. Native g32P is a single polypeptide chain of 301 amino acids which forms oligomers in solution (Prigodich et al., 1984), a process that may be a reflection of its protein–protein interactions which occur when bound cooperatively to ssDNA (Prigodich et al., 1984; Pan et al., 1989). Limited proteolysis of g32P by trypsin removes an N-terminal 21 amino acids (the B domain) and a C-terminal 48 amino acids (the A domain) to generate g32P-(A+B) or "core" g32P (residues 22–253). The latter species shows less oligomerization and no cooperativity upon binding to ssDNA (Spicer et al., 1979). The g32P-(A+B) does, however, exhibit affinity essentially identical with that of the native protein for a single site on a ssDNA lattice (Spicer et al., 1979).

g32P contains 1 mol of Zn(II) per mole associated with the core domain and bound in a closed tetrahedral coordination complex which includes the S^- groups of Cys^{77}, Cys^{87}, and Cys^{90} as ligands (Giedroc et al., 1986, 1989). The Zn(II) ion can be removed by reaction of the protein with *p*-mercuribenzenesulfonate followed by dialysis against EDTA and thiols to reverse the reaction with the mercurial (Giedroc et al., 1986). Readdition of stoichiometric Zn(II) to apo-g32P regenerates a molecule with structural and functional properties indistinguishable from those of the native g32P (Giedroc et al., 1987, 1989; Keating et al., 1988). The spectroscopic data on g32P and its Cd(II)- and Co(II)-substituted derivatives suggest that the metal binding domain of the protein is formed by Cys^{77}-X_3-His^{81}-X_5-Cys^{87}-X_2-Cys^{90} constituting a S_3N donor set. As yet there is no direct evidence for the His ligation.

^{1}H NMR studies of the isolated proton resonances of the two His residues in the molecule as well as the ^{1}H signals from residues in the immediate vicinity of the Zn(II) binding domain in the Zn(II) and apo-g32P should provide information on the participation of His^{81} as a ligand as well as detect Zn(II)-induced conformational changes that occur in the region of the polypeptide forming the Zn(II) domain. We have carried out the NMR studies of specific sets of protons in g32P by biosynthesizing the protein with amino acids containing ^{2}H at most positions with only the specific sites of interest containing ^{1}H. The present paper presents the results of the NMR of the C2H and C4H protons of the His residues and the C2H and C5H protons of the Trp residues. The NMR studies are combined with small-angle X-ray scattering experiments to

† This work was supported by NIH Grants DK09070 and GM21919. The 500-MHz NMR spectrometer was supported by NIH Grant RR03475, NSF Grant DMB-8610557, and ACS Grant RD259.
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[1] Abbreviations: g32P, gene 32 protein; g32-(A+B), g32P core protein; g32P*, g32P residues 22–253; NMR, nuclear magnetic resonance; TSP, sodium (trimethylsilyl)tetradeuteriopropionate.

0006-2960/89/0428-8828$01.50/0

determine the quaternary structure of the protein during metal ion removal and restoration, information essential for the interpretation of the NMR data.

Materials and Methods

Preparation of Selectively Deuteriated g32Ps. Perdeuteriated Phe, Tyr, and Trp were obtained by proton/deuterium exchange in sulfuric acid (Griffith et al., 1976; Matthews et al., 1977) and were used without further purification. A host auxotrophic for aromatic amino acids was constructed by P1 transduction of the aroA::Tn10 locus from strain LCB273 into *Escherichia coli* CJ109 (cI^+, his^-) (CJ109::AroA). CJ109::AroA transformed with plasmid pYS6 containing g32P gene under transcriptional control of the phage λ P_L promoter (Shamoo et al., 1986) was grown in 1.2× M9 salts supplemented with 10 g/L glucose, 1.7 g/L citrate, 50–100 mg/L free nucleotide bases, 100 mg/L thiamin, 50 mg/L ampicillin, 100 mg/L appropriate protonated amino acid, and 30 mg/L deuteriated amino acids. g32P production was induced by adding nalidixic acid to 50 μg/mL at an OD_{600} = 1.0–1.5, and cells were harvested after 6–7 h. g32P was purified as previously described through the ssDNA–cellulose column step (Bittner et al., 1979). Limited tryptic proteolysis and purification of g32P-(A+B) was carried out according to the method of Giedroc et al. (1989).

Apo-g32P-(A+B) was obtained via chemical modification with *p*-mercuribenzenesulfonate (Sigma) in the presence of EDTA followed by reversal of the mercurial reaction with DTT followed by exhaustive dialysis (Giedroc et al., 1986). Residual Zn(II) content of apo-g32P-(A+B) was typically 0.05–0.10 mol of Zn(II)/mol.

^{1}H NMR Experiments. ^{1}H NMR spectra were recorded at 30 °C on a Bruker AM-500 spectrometer. Protein samples [0.2–0.25 mM in g32P-(A+B)] were prepared for NMR by D_2O-buffer exchange on a Sephadex G-25 spun column. For the pH titration of the His protons in g32P-(A+B), the protein was initially buffered with 50 mM sodium phosphate and 30 mM NaCl, pD* 8 (pD* refers to direct pH meter readings), with the pH adjusted by subsequent introduction of small amounts of DCl added directly to the protein sample to generate the indicated pD*. For the apo-g32P-(A+B) salt titration, the protein was buffered at 50 mM sodium phosphate, pH 7.8, with increasing amounts of chelexed 4 M NaCl added to give the indicated NaCl concentration. Chemical shifts are reported relative to TSP as an internal standard.

Small-Angle X-ray Scattering Experiments. Data were collected on a small-angle X-ray scattering station at Yale by using the line source from an Elliott GX6 rotating anode generator, typically operated at 45 kV and 45 mA. Typical data collection time was 1 h. Data to Q = 0.14 Å^{-1} were analyzed by using the Guinier approximation.

Results and Discussion

^{1}H NMR Spectra of the Histidyl Protons in g32P-(A+B). The complete spectrum of fully protonated g32P-(A+B) has been published previously (Prigodich et al., 1984). Figure 1A shows the spectrum of the aromatic region of the perdeuteriated, selectively protonated His g32P-(A+B). The proton resonances from the phenylalanines, tyrosines, and tryptophans have been essentially removed by the incorporation of perdeuteriated residues. There are only two histidines in g32P, His^{64} and His^{81}. The expected two sets of His proton resonances divide themselves into pairs A and B (7.60 and 7.34 ppm) and C and D (6.79 and 6.56 ppm) on the basis of their line width (Figure 1A) and pH titration behavior (Figure 2). The A–B pair has line widths of ~14 Hz, while the C–D pair

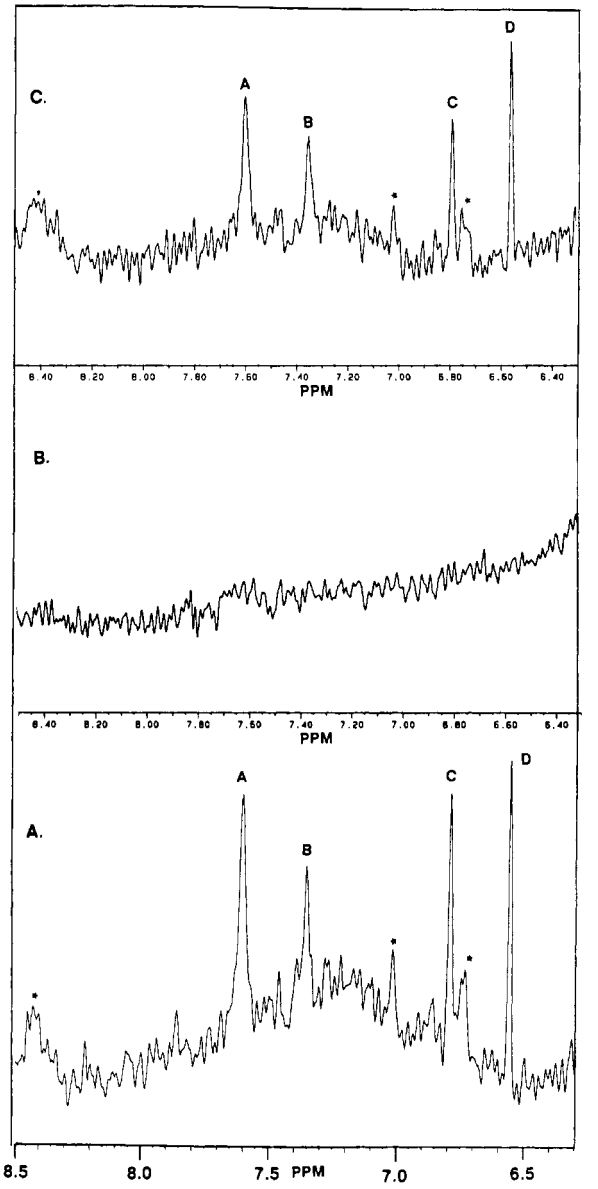

FIGURE 1: ^{1}H NMR spectra of the aromatic regions of (A) selectively deuteriated His g32P-(A+B) (0.2 mM, pH 8.0), (B) apo form of the same His g32P-(A+B) sample, and (C) the same His g32P-(A+B) sample as in (B) following reconstitution with stoichiometric Zn(II). Signals marked with an asterisk are from residual 3,5-^{1}H of tyrosines or nonexchangeable amide protons (compare with Figure 3A). All spectra contain 1024 transients.

has line widths of ~9 Hz, suggesting that the upfield pair of resonances belongs to a single His residue whose side chain is considerably more mobile than the His residue giving rise to the downfield signals. The latter His side chain may have a τ_r close to that calculated for the overall tumbling of the protein (see the following).

Upon removal of the Zn(II) ion both sets of His ^{1}H resonances become undetectable (Figure 1B). Readdition of the Zn(II) ion to the same sample of apo-g32P-(A+B) restores the His proton signals to the same amplitude and chemical shift as observed in the original Zn(II) core protein (Figure 1C). Therefore, addition of Zn(II) to apo-g32P-(A+B) completely restores a protein conformation indistinguishable from that of untreated g32P-(A+B), consistent with previous structural studies (Giedroc et al., 1987, 1989; Keating et al., 1988).

pH Titration of His g32P-(A+B). The primary structure of g32P contains the sequence Cys^{77}-X_3-His^{81}-X_5-Cys^{87}-X_2-Cys^{90}, which we have proposed constitutes the NS_3 ligand

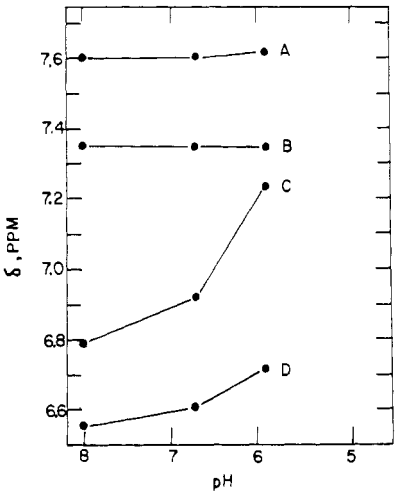

FIGURE 2: Chemical shifts of the His g32P-(A+B) protons at pH 8.0, 6.7, and 5.9.

donor set to the intrinsic Zn(II) ion (Giedroc et al., 1986). ^{113}Cd NMR and site-directed mutagenesis have provided strong evidence that Cys^{77}, Cys^{87}, and Cys^{90} contribute the S^- ligands to the Zn(II) (Giedroc et al., 1989); however, direct evidence for the involvement of a histidine side chain in zinc coordination is lacking. The pH nontitratability of histidine side chains that serve as metal ion ligands in metalloproteins has been well documented (Campbell et al., 1974). Figure 2 shows a plot of the chemical shifts of the His g32P-(A+B) protons at pH 8.0, 6.7, and 5.9. No change in chemical shift is observed for the resonance pair A–B within this pH range. In contrast, the resonance pair C–D shifts downfield 0.45 and 0.16 ppm, respectively, as the pH is lowered from 8.0 to 5.9. At the concentrations required for ^{1}H NMR, g32P-(A+B) precipitates below pH ~5.5 (D. Giedroc and J. Coleman, unpublished observations) such that titration behavior at lower pH values cannot be explored. The investigated pH range (8.0–5.9) should be sufficient to observe chemical shift changes of any nonliganded, solvent-accessible histidine residues. Obviously, the fact that all resonances broaden beyond detection in the His apo-g32P-(A+B) spectrum (Figure 1B) prevents determination of pK_a values in the metal-free protein. The most likely explanation for the failure of the A–B pair of signals to change with pH in the metalloprotein is that this particular His side chain is a ligand to the metal ion.

Signals of the second pair of His protons, C–D, exhibit an unusually large upfield shift in Zn(II)-g32P-(A+B), 0.7–1.2 ppm upfield of the chemical shift of "average" His resonances in proteins. This may be due to stacking of this histidine side chain with one or more other aromatic residues (Pan et al., 1989). The pK_a of this histidine can be estimated to be 6.0–6.5. Both line width, ~9 Hz, and titration behavior suggest that this histidine is largely exposed to the solvent.

Are Conformational Changes Responsible for the Broadening of His Proton Signals in Apo-g32P-(A+B)? Previous studies of g32P by limited proteolysis (Giedroc et al., 1987) and circular dichroism (Giedroc et al., 1989) reveal small conformational changes in both g32P and the proteolytic fragment, g32P-(A+B), upon Zn(II) removal. When ^{1}H NMR spectra of g32P-(A+B) are recorded under standard solution conditions of low ionic strength (50 mM NaCl), substantial signal broadening occurs throughout the spectrum of g32P-(A+B) as a result of metal ion removal (compare panels A and D in Figure 3). The line widths are sensitive to increasing ionic strength as some of them are noticeably narrowed when the NaCl concentration is raised to 1 M

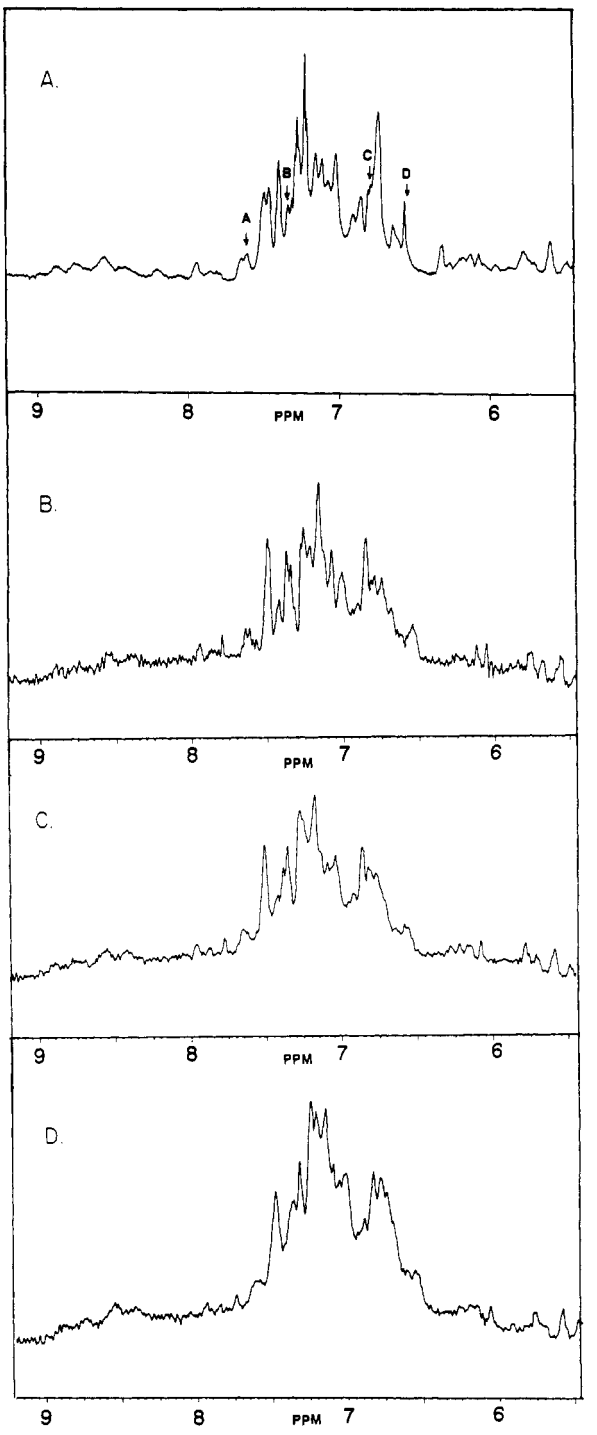

FIGURE 3: ^{1}H NMR spectra of fully protonated apo-g32P-(A+B) at pH 7.8 and at various NaCl concentrations. The spectrum of the Zn(II) form at 50 mM NaCl is shown in panel A for comparison to the apo-g32P-(A+B). (B) 1000 mM NaCl; (C) 500 mM NaCl; (D) 50 mM NaCl. The His protons (A–D) are marked in part A; note that they also disappear from this fully protonated spectrum when the Zn(II) ion is removed.

(panels C and B of Figure 3).

A general decrease in spectral resolution of the ^{1}H NMR spectrum of a protein could result from a significant change in the degree of oligomerization of the protein or from an alteration in the rate of an intramolecular chemical exchange process. NMR data alone cannot distinguish between these two possibilities. Measurement of the radius of gyration (R_G) of the protein molecule with X-ray scattering techniques, if performed under solution conditions that mimic the NMR experiment, can be used as a means to estimate the average

Table I: Radius of Gyration (R_G) of Apo- and Zn(II)-g32P-(A+B)

protein[a]	R_G (Å)
apo, 50 mM NaCl	25.0 ± 2.4
apo, 1000 mM NaCl	23.4 ± 4.2
Zn,[b] 50 mM NaCl	31.2 ± 1.5

[a] Protein concentration of 0.20 mM. [b] Reconstituted. Zn(II) is present in a slight molar excess.

mass of a molecular complex and thus the degree of oligomerization, provided one makes simple assumptions about shape. As shown in Table I, R_G values determined for the apo and Zn(II) forms of g32P-(A+B) at low ionic strength are 25.0 and 31.2 Å, respectively (Table I). Thus, apo-g32P-(A+B) is **not** characterized by a higher extent of oligomerization relative to the Zn(II) protein. Quite the contrary, the Zn-(II)-g32P-(A+B) appears to be a dimer, while apo-g32P-(A+B) is a monomer (see the following). Consistent with this, the radii of gyration for apo-g32P-(A+B) at both low and high ionic strength are indistinguishable (25.0 ± 2.4 vs 23.4 ± 4.2 Å) (Table I), indicating unchanged shape and degree of oligomerization as a result of increasing ionic strength.

The X-ray scattering data suggest that disruption of protein–protein dimer formation in Zn(II)-g32P-(A+B) under the NMR conditions of relatively high protein concentration occurs as a result of metal ion removal. Thus, the experimentally observed R_G^2 ratio of Zn(II) and apo-g32P-(A+B) is consistent with Zn(II)-g32P-(A+B) being present as a dimer in low ionic strength solution at a concentration of 0.2 mM. Furthermore, the Guinier plot shows increasing negative slope at smaller angles indicative of oligomerization (e.g., dimerization) of the Zn(II)-g32P-(A+B) molecule (data not shown).

For globular proteins, the radius of gyration (R_G) has the relationship to the radius, r, of a spherical protein monomer as follows:

$$[R_G^2]_{monomer} = {}^3/_5 r^2; \text{ hence } r_{apo} = 32.3 \text{ Å} \quad (1)$$

$$[R_G^2]_{dimer} = {}^9/_{10} r^2$$

$$[R_G^2]_{dimer}/[R_G^2]_{monomer} = {}^3/_2 = 1.5$$

$$\text{experimental: } [R_G^2]_{Zn(II)}/[R_G^2]_{apo} = 1.56$$

These calculations for the apo-g32P-(A+B) suggest that the apomonomer is likely to resemble an ellipsoid. If we assume average parameters for globular proteins (i.e., partial specific volume = 0.73 cm^3/g; extent of hydration = 0.3 g of H_2O/g), an independent value for the spherical radius of apo-g32P-(A+B) can be calculated to be about 22 Å. This value is significantly smaller than the radius calculated from the measured value of R_G, i.e., 32.3 Å (eq 1), a result that could be explained by an axial ratio considerably different from 1.

Since the small-angle X-ray scattering data collected under the same conditions as the NMR appear to remove oligomerization of the apo-g32P-(A+B) as an explanation for the broadening of the His proton resonances, a chemical exchange process within the apoprotein proceeding at a rate corresponding to intermediate exchange on the ^{1}H NMR time scale (10–100 s^{-1}) seems the more likely explanation for the broadening. The exchange rate between two or more conformational states of the apoprotein can be sensitive to ionic strength, which may explain the change in the narrowing of the proton signals from the apo-g32P-(A+B) at high salt concentrations (Figure 3). Attempts to slow the postulated exchange by lowering the temperature to 15 and then 5 °C have not been conclusive. While some narrowing is present at 15 °C, all signals begin to broaden at the lower temperature for other reasons.

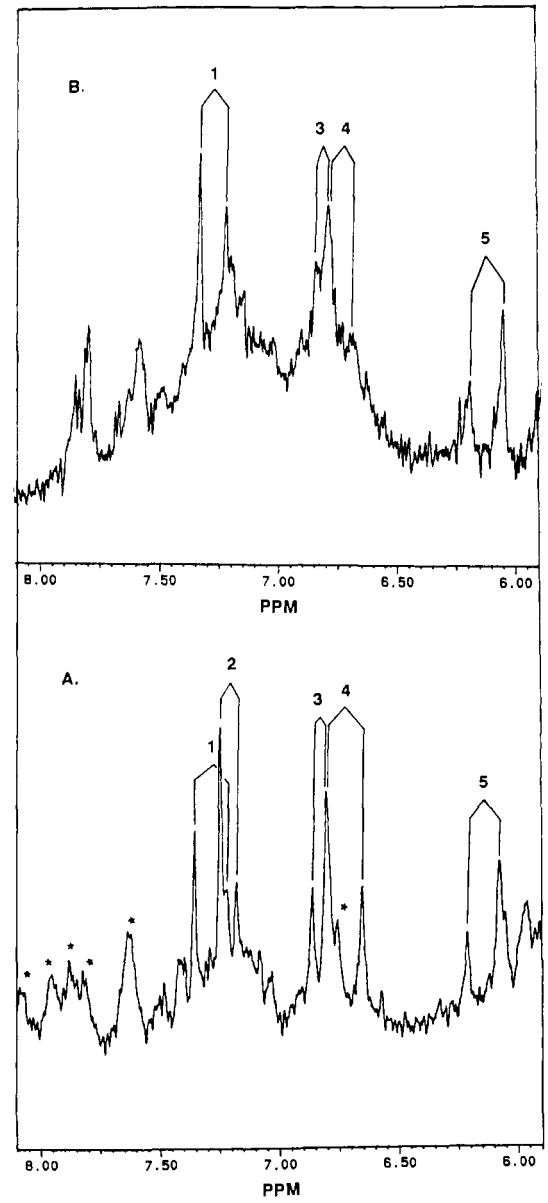

FIGURE 4: ^{1}H NMR spectra of selectively deuteriated Trp-g32P-(A+B). (A) Zn(II) form; (B) apo form. Both spectra were acquired at pH 7.8 in the standard buffer at 50 mM NaCl. Signals marked with an asterisk are from residual 3,5-^{1}H of tyrosines or nonexchangeable amide protons. The selectively deuteriated Trp is 50% protonated at the 2-position and 25% protonated at the 5-position of the indole ring.

^{1}H NMR Spectra of Selectively Protonated Trp g32P-(A+B). Additional support for a localized conformational exchange process in the apo-g32P-(A+B) can be obtained from the ^{1}H NMR spectra of protons at the 2- and 5-positions of the tryptophanyl side chains. Previous near-UV CD experiments showed that conformational perturbation of the side chains of several tryptophan and tyrosine residues occurred as a result of metal ion removal and reconstitution (Giedroc et al., 1989). As tryptophan residues are well distributed throughout the primary structure of the protein (positions 31, 72, 116, 144, and 168), we can ask whether the observed changes in conformational equilibria that occur upon metal ion removal are uniformly distributed throughout the molecule or localized in the immediate vicinity of the metal ion binding domain.

The tryptophan proton signals (H2 and H5 in a per-deuteriated background) from all five Trp residues (1–5) are well resolved (Figure 4A). In the apo-g32P-(A+B), only Trp^2

and Trp^4 exhibit substantial broadening in the apo form relative to the Zn(II) form and thus appear to be located in the immediate vicinity of the Zn(II) binding domain (Figure 4B). Trp^2 has been shown to be near the ssDNA binding site (Pan et al., 1989), making Trp^{116} a good candidate for Trp^2, since its immediate neighbor, Tyr^{115}, has already been shown to be involved in ssDNA binding (Prigodich et al., 1986). On the other hand, site-directed mutagenesis reveals that Tyr^{73} plays some role in maintenance of the folding of g32P around the Zn(II), since a Tyr^{73}-Ser^{73} mutant fails to incorporate Zn(II) and is susceptible to proteolysis in vivo and in vitro (Shamoo et al., 1989). It is therefore not unreasonable to suggest that Trp^{72} could be assigned to Trp^4 as it would be expected to be capable of sensing any structural changes in the immediately adjacent metal ion binding domain. In addition, this tryptophan does not appear to be directly a part of the nucleotide binding site (Pan et al., 1989).

In contrast, the proton resonances from Trp^1 and Trp^5 are essentially unchanged in apo-Trp-g32P(A+B). The latter signals also do not shift upon ssDNA binding (Pan et al., 1989). Finally, Trp^3 signals appear to undergo intermediate broadening in the apoprotein, and they broaden substantially upon ssDNA binding [see Pan et al. (1989)].

Conclusions. Broadening of specific ^1H NMR signals of fully protonated and selectively deuteriated g32P-(A+B) molecules can be used to probe the structural and dynamic changes that occur in g32P-(A+B) as a result of metal ion removal and reconstitution. We conclude that the region of the polypeptide chain affected by metal ion removal is localized and extends N-terminally as far as His^{64}, since the proton signals from this residue are broadened beyond detection in the spectrum of the apoprotein (Figure 1B), and C-terminally to Trp^{116}, since protons from this residue are also broadened by Zn(II) removal (Figure 4B). The X-ray scattering results suggest for the first time that Zn(II) g32P-(A+B) is a dimer in solution and that removal of the metal ion results in its dissociation to a monomer. It is tempting to speculate that the observed destabilization of monomer–monomer contacts in the apo-g32P-(A+B) fragment are responsible for the pronounced defect exhibited by apo-g32P in cooperative binding to ssDNA (Giedroc et al., 1987), a binding mode dependent on protein–protein interactions. Thus, both the Zn(II) binding domain and the N-terminal B domain can be considered to make separate but perhaps interactive contributions to the cooperativity of binding of g32P to ssDNA. The Zn(II) chelate can thus indirectly influence the multiple surface equilibria between protein–protein and protein–DNA required for the cooperative, high-affinity binding mode of g32P to "infinite"-length ssDNA lattices.

ACKNOWLEDGMENTS

We greatly appreciate the assistance of Michio Kataoka in carrying out the X-ray scattering experiments.

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