The Function of Zinc in Gene 32 Protein from T4†

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ABSTRACT: Gene 32 protein (g32P), the single-stranded DNA binding protein from bacteriophage T4, contains 1 mol of Zn(II) bound in a tetrahedral complex to $-S^-$ ligands, proposed on spectral evidence to include Cys-77, Cys-87, and Cys-90 [Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., & Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452]. The Zn(II) can be completely removed by treatment with the mercurial reagent *p*-(hydroxymercuri)benzenesulfonate and ethylenediaminetetraacetic acid. The resultant apo-g32P is rapidly digested by trypsin in contrast to the zinc protein which undergoes specific limited proteolysis to yield a resistant DNA-binding core. Rebinding of Zn(II) to the apoprotein restores the same limited susceptibility to proteolysis displayed by the native Zn(II) protein. In the presence of 150 mM NaCl, Zn(II) g32P reduces the melting temperature T_m of poly[d(A-T)] by 47 °C, while apo-g32P is unable to melt poly[d(A-T)] at this salt concentration, as the protein thermally unfolds before melting can take place. At 25 mM NaCl, however, apo-g32P lowers the T_m of poly[d(A-T)] by 36 °C, but the melting curve is broad compared to the steep cooperative melting induced by Zn(II) g32P. Association constants K_a calculated from the poly[d(A-T)] melting curves for Zn(II) and apo-g32P differ by 3 orders of magnitude, 4.8×10^{10} M^{-1} and 4.3×10^7 M^{-1}, respectively. K_{int} for the binding of Zn(II) g32P and apo-g32P to a single-site nucleotide lattice, $d(pT)_8$, are within an order of magnitude, 3.9×10^5 M^{-1} and 7.5×10^4 M^{-1}, respectively, as measured by fluorescence quenching. On a two-site nucleotide lattice, $d(pT)_{16}$, Zn(II) g32P exhibits a 100-fold increase in K_a (4.8×10^7 M^{-1}), due to the cooperativity of the binding, i.e., the cooperativity parameter, $\omega = 3800$. In contrast, the K_a of apo-g32P for $d(pT)_{16}$ increases only minimally, $K_a = 2.9 \times 10^5$ M^{-1}, and $\omega = 3.7$. Cd(II) and Co(II) provide adequate substitutes for the intrinsic Zn(II) ion in establishing highly cooperative binding to both poly[d(A-T)] and $d(pT)_{16}$. Thus Zn(II) organizes a subdomain within the core (residues 22–253) of g32P that is essential along with the amino-terminal domain (residues 1–21) in maintaining the protein–protein interactions necessary for the cooperative binding of g32P to a single-stranded DNA lattice.

Gene 32 protein (g32P) from bacteriophage T4 is a member of a class of proteins that binds preferentially and cooperatively to single-stranded nucleic acid lattices (Alberts & Frey, 1970; Alberts et al., 1980; Coleman & Oakley, 1980; Chase & Williams, 1986). Other members of this class include gene 5 protein from bacteriophage fd, *Escherichia coli* SSB, and the single-stranded DNA binding protein from adenovirus.

Probing of the structure of oligonucleotide complexes of these proteins by one- and two-dimensional ^1H NMR methods implicates the participation of aromatic amino acids in complex formation through partial intercalation of the aromatic rings with the nucleic acid bases (O'Connor & Coleman, 1983; Alma et al., 1981; Prigodich et al., 1984, 1986; King & Coleman, 1987). The nucleotide binding region of g32P appears to contain five Tyr and two Phe (Prigodich et al., 1984, 1986), while that of g5P appears to contain one Tyr and one Phe (King & Coleman, 1987). Gene 32 protein (M_r 33 488; 301 amino acids) is a three-domain protein in which the trypsin-resistant core, residues 22–253, termed g32P-(A+B) (Moise & Hosoda, 1976; Williams & Konigsberg, 1978), contains all the determinants required to interact with oligonucleotides, but it does so noncooperatively (Williams et al., 1979; Spicer et al., 1979). The amino-terminal domain (residues 1–21, B region) enables the protein to bind cooperatively to DNA and increases the intrinsic association constant by ~3 orders of magnitude (Spicer et al., 1979). The carboxy-terminal domain (residues 254–301, A region) provides a kinetic barrier to the melting of native dsDNAs, a role perhaps important in the in vivo function of the molecule (Alberts & Frey, 1970; Jensen et al., 1976). The carboxy-terminal domain also interacts with other proteins of the T4 replisome (Burke et al., 1980).

We have recently shown that intact g32P and the tryptic core contain a tightly bound Zn(II) ion (Giedroc et al., 1986). The intrinsic Zn(II) is tetrahedrally coordinated as shown by the visible absorption spectrum of the Co(II) derivative. The presence of S^- to Co(II) charge-transfer bands in the near-UV suggests coordination to Cys side chains. From a variety of data the best candidates for ligands are Cys-77, His-81, Cys-87, and Cys-90 (Giedroc et al., 1986). The structural integrity of the core as measured by its susceptibility to proteolysis is absolutely dependent on the presence of this Zn(II) ion. Removal of the metal, while not abolishing DNA binding, does appear to weaken it considerably. In this paper, we address whether the Zn(II) is absolutely essential for any phase of nucleic acid binding and how it might function in carrying out such a role.

Materials and Methods

Gene 32 Protein. Homogeneous g32P was prepared by standard procedures employing ssDNA–cellulose and phenyl-Sepharose chromatographies (Bittner et al., 1979). All g32P preparations were routinely dialyzed into TNG[1] buffer

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TNG, 10 mM Tris-HCl, 0.2 M NaCl, 5% v/v glycerol, pH 8; TAE, 40 mM Tris-acetate, 1 mM EDTA, pH 8; PMBS, *p*-(hydroxymercuri)benzenesulfonic acid; DTT, dithiothreitol; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; TPCK, L-(1-tosylamido)-2-phenylethyl chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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and checked for Zn(II) content and for the absence of proteolytic degradation upon storage. Cd(II) and Co(II) derivatives of g32P were prepared as previously described (Giedroc et al., 1986). These metallo-g32Ps contained 0.85–1.06 mol/mol of the indicated metal [Cd(II) or Co(II)] with the balance of sites filled with Zn(II). Apo-g32P was prepared by PMBS treatment with subsequent dialysis against EDTA and thiol (Giedroc et al., 1986). The residual Zn(II) content of apo-g32P ranged from 0.03 to 0.18 mol of Zn(II)/mol. Metal content of various metallo-g32Ps was determined on an Instrumentation Laboratories IL157 atomic absorption spectrometer (Giedroc et al., 1986). Protein concentrations were determined from UV absorption, $\epsilon_{280} = 4.1 \times 10^4$ M^{-1} cm^{-1}, for metal-substituted, metal-free, and native g32Ps (Williams et al., 1979). Amino acid analysis on selected samples indicated the concentrations to be within 10% of those calculated from UV absorption.

Nucleic Acid Ligands. Various nucleic acid lattices were used for binding studies without further treatment and were routinely dissolved in 10 mM Tris-HCl and 0.1 mM EDTA, pH 8. Their concentrations were determined by using the following extinction coefficients expressed per mole of P_i: polyriboethenoadenylic acid (Pharmacia P-L Biochemicals), $\epsilon_{280} = 5.1 \times 10^3$ M^{-1} cm^{-1}; $d(pT)_8$, $d(pT)_{16}$, and poly(dT) (Pharmacia P-L Biochemicals), $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1}; poly[d(A-T)] (Miles), $\epsilon_{260} = 6.7 \times 10^3$ M^{-1} cm^{-1}; fd DNA, $\epsilon_{260} = 8.69 \times 10^3$ M^{-1} cm^{-1} per mole of P_i and 5.57×10^7 cm^{-1} per mole of the 6408-base ssDNA circle. The fd DNA was prepared by repeated phenol extraction (3 times) of fd phage stocks, followed by two extractions, each with phenol/$CHCl_3$ (1:1) and ether, subsequent ethanol precipitation from sodium acetate, and dissolution into 10 mM Tris-HCl and 1 mM EDTA, pH 8. This DNA contained >90% intact circles with the remainder comprising once-nicked linear species and was used without further purification. All DNA binding studies were performed in acid-washed cuvettes with buffers prepared by dilution of Chelexed (Bio-Rad) stock solutions into metal-free double distilled, deionized water in the presence of 0.1–1 mM EDTA. The EDTA prevents the reconstitution of apo-g32P even if significant Zn(II) contamination occurs during sample manipulation.

Reconstitution Studies. Apo-g32P [0.05–0.18 mol of Zn(II)/mol] was incubated with exogenous $ZnCl_2$ or $^{65}ZnCl_2$, DTT, or EDTA as indicated. In analytical studies, the loosely bound metals were removed by passage through a spun column equilibrated in TNG/0.1 mM EDTA. Control studies showed that 0.1 mM EDTA in the buffer was sufficient to remove loosely bound Zn(II) ions added to the native protein. Limited trypsinization of native g32P, reconstituted g32P, and apo-g32P was performed as outlined previously (Williams & Konigsberg, 1978).

Polyriboethenoadenylic Acid Binding Studies. Polyriboethenoadenylic acid, 5×10^{-6} M (2.0 mL), in 10 mM Tris-HCl and 0.1 mM EDTA, pH 8, containing the indicated amount of NaCl was titrated with g32P and the fluorescence emission determined at 405 nm after excitation at 320 nm. The change in fluorescence (ΔF) upon protein addition was normalized to a scale of 0–100, where 0 represents the fluorescence in the absence of protein and 100 represents the maximum ΔF value. Binding constants (K_a) were determined under conditions where binding was not stoichiometric according to Kowalczykowski et al. (1986).

g32P Binding to $d(pT)_8$, $d(pT)_{16}$, and Poly(dT). g32P, 1×10^{-6} M, in 10 mM Tris-HCl and 0.1 mM EDTA, pH 8 (2 mL), was titrated with the indicated nucleic acid by addition of 5-20-μL aliquots from a concentrated stock solution prepared in the same buffer. The protein fluorescence at 347 nm was determined by exciting at 282 nm. The changes in g32P fluorescence upon nucleic acid addition (fluorescence quenching, % Q) were corrected for dilution, photobleaching, and absorption of the incident light by each lattice. Values of site size (n) and minimum estimates of apparent binding constants (K_a) were calculated for stoichiometric binding curves [i.e., $d(pT)_{16}$ and poly(dT) titrations] according to $K_a = \theta/(1-\theta)^2[g32P]$, where θ represents the fractional saturation of the protein at the stoichiometric point (Kelly et al., 1976). K_{int} values for the $d(pT)_8$ titrations were calculated from the slope of plots of $1/\Delta F$ vs. $1/[d(pT)_8]_{free}$ (Kelly et al., 1976). The cooperativity parameter, ω, was calculated for $d(pT)_{16}$ titrations from the equation $K_a = (S_2\omega)^{1/2}K_{int}$. S_2 is a statistical factor that takes into account the number of possible binding modes two g32P molecules can assume on one $d(pT)_{16}$ molecule, taken to be 4 (Kelly et al., 1976).

Thermal Denaturation of Poly[d(A-T)] Induced by g32Ps. Stock solutions of g32Ps in TNG buffer and poly[d(A-T)] in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1 M NaCl were diluted into ice-cold buffer without NaCl to the indicated protein concentrations. Additional NaCl was then added to adjust to the desired NaCl concentration in a volume of 250 μL. These solutions were loaded into cuvettes preequilibrated at 10–16 °C as indicated, and the temperature was maintained for 5 min, after which time a 1 °C/min temperature increase was initiated. Melting profiles were determined at 260 nm on a Gilford Model 251 spectrophotometer equipped with a Model 2527 thermoprogrammer connected to a Hewlett-Packard Model 7225B plotter. Approximate association constants were calculated according to 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)] $\cong$ −8000 cal/mol of base pairs (Crothers, 1971); T_m' and T_m refer to the melting temperature of poly[d(A-T)] in the presence and absence of g32P, respectively; $\Delta T_m = T_m' - T_m$; R is the gas constant = 1.987 cal deg^{-1} mol^{-1}; a is the free g32P concentration at T_m'; and B_c is the density of protein binding sites on the dsDNA. Assuming a site size of 7 for the monomer of g32P, $B_c = 1/(7/2) = 2/7 = 0.286$ [cf. Williams et al. (1983)], while $a = [g32P] - (1/2)[poly[d(A-T)]]$. A linear relationship, log [NaCl] vs. log K_a, permits an extrapolation of K_a values determined at various NaCl concentrations to other concentrations of NaCl (Jensen et al., 1976).

Endonuclease Digestion of fd DNA–g32P Complexes. A concentration of *Neurospora crassa* single-stranded endonuclease (Pharmacia P-L Biochemicals) that gave quantitative digestion of fd DNA in approximately 2 min at 37 °C was determined (5.5 units/mL) and used to probe the nuclease susceptibility of fd DNA complexed with either Zn(II) g32P or apo-g32P. Reaction mixtures contained 100 mM Tris-HCl, 10 mM $MgCl_2$, pH 8, and 5.25×10^{-6} M g32P sites (equivalent to 3.9×10^{-5} M fd DNA phosphate) with and without 38.5 milliunits of *N. crassa* nuclease. Each reaction contained 15.8×10^{-6} M g32P in a total volume of 70 μL. Following preincubation of the ssDNA–g32P complex for 5 min at 37 °C, endonuclease was added and 10-μL aliquots were withdrawn at each time point and mixed with 190 μL of 1 M NaCl and 25 mM EDTA on ice to stop the nuclease activity and dissociate the complex. These solutions were then rapidly mixed with an equal volume of phenol/$CHCl_3$ (1:1), the aqueous phase was extracted, and the phenol phase was back-extracted with 150 μL of 1 M NaCl and 25 mM EDTA,

pH 8. The aqueous phases were combined, 2 μg of tRNA was added and precipitated with ethanol at −70 °C, for 30 min, and the pellet was recovered and dissolved in 10 mM Tris-HCl and 1 mM EDTA, pH 8. The DNAs were electrophoresed through a 1.3% agarose gel in TAE[1] buffer, stained with ethidium bromide, and visualized by excitation under a UV lamp source. Control incubations of the ssDNA–protein complex at 37 °C for 30 min with no nuclease present were carried through the same manipulations and revealed quantitative recovery of the DNA.

Results

Preparation of Zn(II)-Reconstituted g32P from Metal-Free Apo-g32P. Since apo-g32P can be totally proteolyzed by trypsin (Giedroc et al., 1986), an assay for the successful reconstitution of the metalloprotein was devised, using gel electrophoresis to test for prevention of this proteolysis. Preliminary experiments had shown that the presence of a reducing agent (e.g., DTT) facilitated the reincorporation of Zn(II) into the apo-g32P, while EDTA present prior to Zn(II) addition efficiently prevented the reconstitution (data not shown). Therefore, apo-g32P containing ~0.05 mol of Zn(II)/mol was titrated with $ZnCl_2$ in a reducing environment. After 1.5 h at 22 °C, EDTA was added to 1 mM, the protein was desalted by passing over G-25 Sephadex in TNG/0.1 mM EDTA buffer, and the Zn(II) content and protease susceptibility of the protein were determined. As a control, native g32P was subject to the same manipulations. Time courses for the limited trypsin digestion of native g32P under conditions of no added zinc and in the presence of 5 mol equiv of excess Zn(II) are shown in Figure 1A, lanes 1–4 and 5–8, respectively. The gel patterns for apo-g32P treated with 0, 1.2, and 5 mol equiv of Zn(II) are presented in lanes 9–12, 13–16, and 17–20, respectively. Following the final gel filtration, all the reconstituted g32Ps contain approximately 1 mol of Zn(II)/mol, while the apo-g32P not treated with Zn(II) remains Zn(II) free. The presence of a single Zn(II) ion ensures the resistance of the core fragment (residues 22–253) to further proteolysis, while the apo-g32P is proteolyzed to small fragments (Figure 1A). Resistance refers to the absence of additional cleavage after the initial removal of the amino- and carboxy-terminal domains, residues 1–21 and 254–301.

That exogenous Zn(II) is populating previously unoccupied Zn(II) sites on apo-g32P is shown by adding 1.1 mol equiv of $^{65}ZnCl_2$ to apo-g32P containing 0.18 mol of Zn(II). The resulting protein following dialysis to remove loosely associated metal ions contained 0.99 mol/mol total Zn(II) by atomic absorption and 0.75 mol/mol ^{65}Zn(II). The protease susceptibility of this ^{65}Zn(II)-reconstituted g32P [^{65}Zn(II)-R g32P] is shown in Figure 1B, lanes 6–9, compared to native Zn(II) g32P (lanes 2–5) and indicates that rebinding of Zn(II) restored resistance to protease attack. This g32P sample was used to test for restoration of function as described below.

Binding of Apo- and Metallo-g32Ps to Polyriboethenoadenylic Acid. The binding of various g32Ps to RNA was probed by titration of the fluorescent RNA, polyriboethenoadenylic acid, with g32P. Complex formation was monitored by an increase in the fluorescence of the RNA due primarily to unstacking of the nucleic acid bases upon protein binding. Titrations were carried out at low (25 mM), intermediate (200 mM), and high (400 mM) NaCl concentrations. Representative titrations of Zn(II) and apo-g32P at low (open symbols) and high (closed symbols) salt are shown in panel A of Figure 2. The displacement of the high-salt curve for the apo-g32P significantly to the right shows that under these conditions the metal-free g32P binds less tightly to this ssRNA lattice than does native g32P. Panels B and C show low- and high-salt titrations with the Cd(II) and Co(II) g32Ps respectively. Stoichiometric binding points were derived from the low-salt titrations, while association constants were calculated from the curves generated at 400 mM NaCl and are compiled in Table I. The data indicate that at 400 mM NaCl $K_a \sim (1\text{–}4) \times 10^7\ M^{-1}$ for Co(II), Cd(II), and Zn(II) g32Ps, while $K_a \sim 1.4 \times 10^6\ M^{-1}$ for apo-g32P.

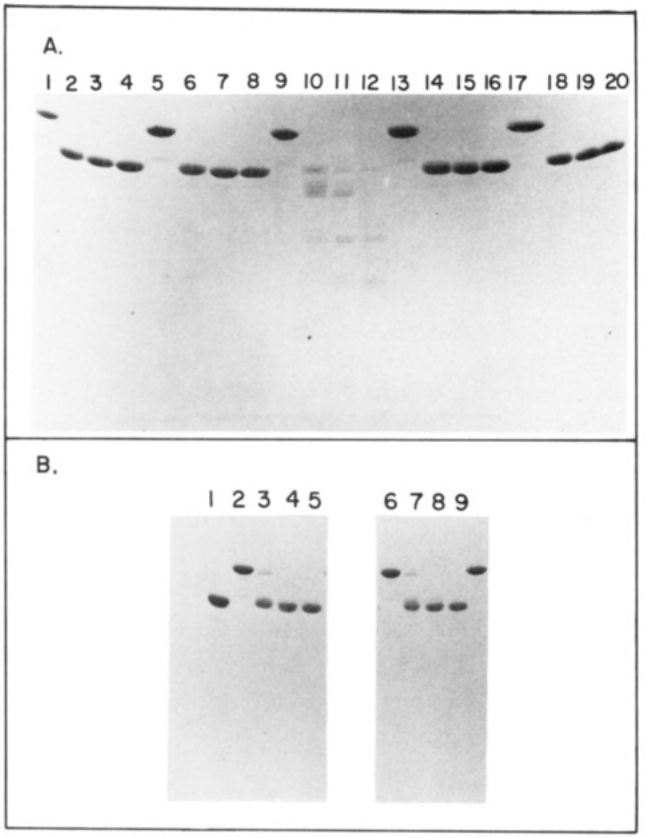

FIGURE 1: Reconstitution of apo-g32P with exogenous $ZnCl_2$. (A) A 174 μM concentration of native g32P or apo-g32P was incubated for 90 min, at 22 °C, with either 209 (1.2 mol equiv) or 870 μM (5 mol equiv) reagent-grade $ZnCl_2$ in the presence of 2.5 mM DTT in TNG buffer in a volume of 150 μL. EDTA (1 mM) was then added, and the $ZnCl_2$-treated samples were desalted on a 1-mL Sephadex G-25 spun column equilibrated with TNG/0.1 mM EDTA, collected, and measured for Zn(II) content. To assay for reconstitution, 7.16 μM g32P in TNG (100 μL) was treated with 9.6 μg/mL trypsin–TPCK (1:25 weight ratio trypsin:g32P) at 22 °C, and aliquots were removed at 0, 10, 30, and 60 min after addition of trypsin and subjected to SDS–PAGE on 16% Laemmli (1970) gels. Lanes 1–4, native g32P, 0.98 mol of Zn(II); lanes 5–8, native g32P + 5 mol equiv of $ZnCl_2$, 0.92 mol of Zn(II) found; lanes 9–12, apo-g32P, 0.05 mol of Zn(II) found; lanes 13–16, apo-g32P + 1.2 mol equiv of $ZnCl_2$, 0.90 mol of Zn(II) found; lanes 17–20, apo-g32P + 5 mol equiv of $ZnCl_2$, 0.82 mol of Zn(II) found. (B) Following dialysis against TNG/5 mM DTT, 120 μM apo-g32P [0.18 mol of Zn(II)] was treated with 132 μM $^{65}ZnCl_2$ in 1.0 mL, incubated 60 min, at 22 °C, and desalted by exhaustive dialysis against TNG/0.1 mM EDTA. The resulting g32P was subjected to Zn(II) analysis and, with native g32P, limiting proteolysis exactly as outlined in panel A. Lane 1, g32P-(A+B) standard; lanes 2–5, native g32P; lanes 6–9, ^{65}Zn(II)-reconstituted g32P [^{65}Zn(II)-R g32P], 0.99 mol of Zn(II) with 0.75 mol of ^{65}Zn(II).

Binding of Apo- and Metallo-g32Ps to $d(pT)_8$, $d(pT)_{16}$, and Poly(dT). As outlined under Materials and Methods, the apparent association constant, K_a, involves two terms: K_{int}, the intrinsic association constant for a single site, and ω, the cooperativity parameter (Kelly et al., 1976). For native g32P, determination of K_a values for $d(pT)_8$ and $d(pT)_{16}$ binding provides an approximate value of ω, since $d(pT)_8$ represents a single noncooperative binding site ($\omega = 1$), while $d(pT)_{16}$

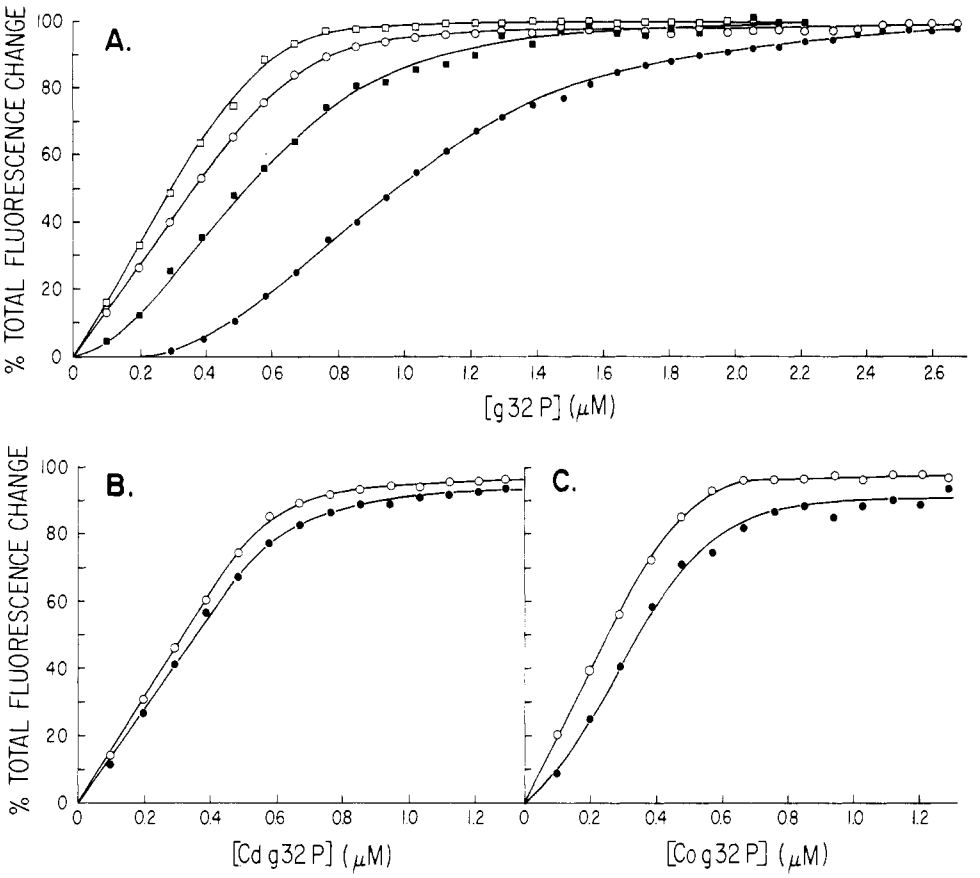

FIGURE 2: Titration of polyriboethenoadenylic acid with metallo-g32Ps. Polyriboethenoadenylic acid (5×10^{-6} M) was titrated with various g32Ps in 10 mM Tris-HCl and 0.1 mM EDTA, pH 8, with the indicated NaCl concentration as described under Materials and Methods, where the increase in the fluorescence (ΔF) of the RNA in each case was normalized to a scale of 0–100, where 100 represents the maximal fluorescence enhancement. The open symbols represent titrations performed at 25 mM NaCl, while the closed symbols represent those carried out at 400 mM NaCl. (A) Native g32P (□, ■); apo-g32P (○, ●). (B) Cd(II) g32P. (C) Co(II) g32P. The maximum fluorescence enhancements (ΔF_{max}) for the metallo- and apo-g32Ps at 400 mM NaCl are as follows: native, 92%; apo, 92%; Cd(II), 110%; and Co(II), 44%.

Table I: Association Constants[a] and Apparent Site Sizes[b] Derived from the Binding of Apo- and Metallo-g32Ps to Polyriboethenoadenylic Acid

g32P	K_a ($\times 10^{-7}$ M^{-1})	n^b	g32P	K_a ($\times 10^{-7}$ M^{-1})	n^b
Zn(II)	1.1 (±0.1)	8.1	Co(II)	4.2	9.5
Cd(II)	3.1	7.6	apo[c]	0.14 (±0.02)	6.7

[a] $K_a = K_{int}\omega$ and is derived from the 400 mM NaCl titrations presented in Figure 2, assuming a stoichiometry of 7.5 nucleotides per g32P. In those instances where multiple determinations were made, the average deviations are given in parentheses. [b] n, stoichiometry of binding (nucleotides/protein) derived from 25 mM NaCl titrations in Figure 2. [c] The apoprotein used in these experiments contained 0.03 mol of Zn(II)/mol.

Table II: Association Constants (M^{-1}) and ω Values Derived from the Binding of Apo- and Metallo-g32Ps to $d(pT)_8$, $d(pT)_{16}$, and Poly(dT)[a]

	K_a (M^{-1})				
g32P	$d(pT)_8$	$d(pT)_{16}$	ω	poly(dT)	n
Zn(II)	3.9×10^5	4.8×10^7	3800	2.0×10^8	6.9
Cd(II)	3.9×10^5	2.7×10^7	2000	$3.4 (\pm 2.2) \times 10^8$	6.4 (±0.6)
Co(II)	5.2×10^5	4.1×10^7	1500	$3.0 (\pm 0.2) \times 10^8$	6.3 (±0.1)
apo	$7.5 (\pm 1.9) \times 10^4$	2.9×10^5	3.7	$2.6 (\pm 1.5) \times 10^7$	4.9 (±0.5)

[a] Derived according to procedures outlined under Materials and Methods from the data presented in Figure 3. Association constants were calculated on the basis of the estimated site sizes (n) listed above that were determined from the poly(dT) titrations. K_a values for poly(dT) are minimum estimates; the actual K_a for poly(dT) could be considerably larger than the values listed above. The cooperativity parameter ω is based on a comparison of the K_a for $d(pT)_8$ and $d(pT)_{16}$ described under Materials and Methods.

provides two contiguous binding sites and reflects cooperative ($\omega > 1$) as well as stoichiometric binding (Kelly et al., 1976).

The $d(pT)_8$ binding isotherm for native g32P reflects true noncooperative binding with no evidence of a stoichiometric point as shown in Figure 3A (left panel). In contrast, g32P binding to $d(pT)_{16}$ gives rise to stoichiometric binding with $n \cong 7$ (Figure 3A, right panel). A similar curve is generated for poly(dT) binding (Figure 3A, right panel). K_a values calculated from these titrations are compiled in Table II and indicate that K_a increases by ~2 orders of magnitude in proceeding from $d(pT)_8$ to $d(pT)_{16}$, which yields an ω value of 3800 (Table II). Cd(II) (Figure 3B) and Co(II) (Figure 3C) g32Ps show very similar binding functions; i.e., stoichiometric and saturable binding is observed for the binding to $d(pT)_{16}$ and poly(dT) (right panels), but not to $d(pT)_8$ (left panels). As with Zn(II) g32P, there is a 10^2-fold increase in K_a due to cooperative binding ($\omega \cong 10^3$). K_a's for $d(pT)_8$ are approximately equal for the various metal-substituted g32Ps, indicating no significant alteration in the DNA binding surface upon metal ion substitution.

Figure 3D presents the binding characteristics of the apoprotein to $d(pT)_8$, $d(pT)_{16}$, and poly(dT). In appearance, the $d(pT)_{16}$ and $d(pT)_8$ curves look similar (Figure 3D, left panel). Neither reflects stoichiometric binding. In fact, stoichiometric binding occurs only with poly(dT) (Figure 3D, right panel). As outlined in Table II, K_a values for $d(pT)_8$ and $d(pT)_{16}$ differ

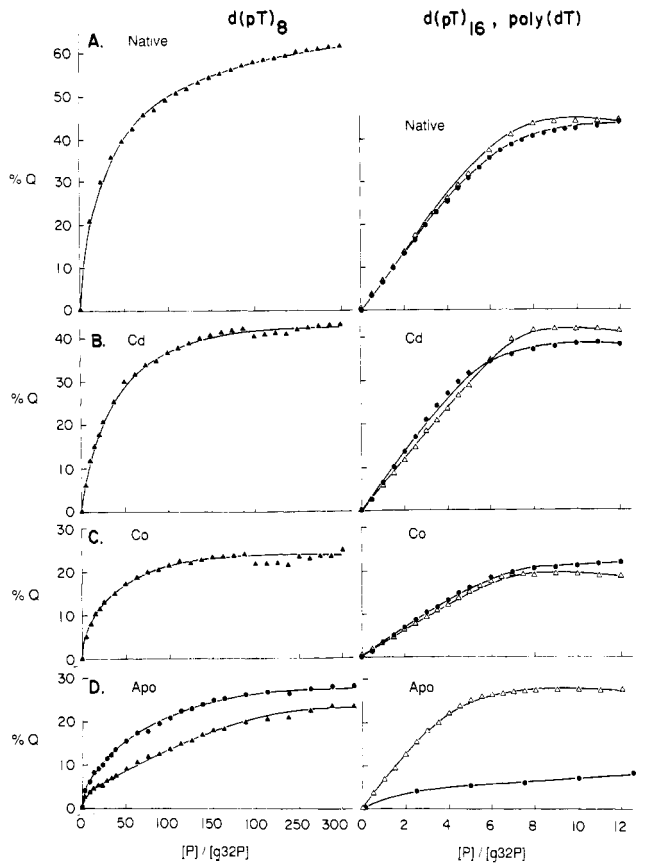

FIGURE 3: Titration of metallo-g32Ps with various nucleic acid lattices. The fluorescence emission spectra of all g32Ps at 1×10^{-6} M exhibit a broad emission maximum at 331 nm with a shoulder positioned at 380 nm. The intensities of the emission maxima characteristic of each metal-substituted g32P relative to the native Zn(II) protein are as follows: Zn(II), 1.00%; Cd(II), 0.96; Co(II), 0.55; Apo, 0.59. Percent quenching (% Q) of the initial protein fluorescence by increasing amounts (moles of P_i per mole of g32P) of $d(pT)_8$ (▲) (left panels), $d(pT)_{16}$ (●), and poly(dT) (△) (right panels) for (A) native Zn(II) g32P, (B) Cd(II) g32P, (C) Co(II) g32P, and (D) apo-g32P. g32P concentrations in all cases were 1.0×10^{-6} M.

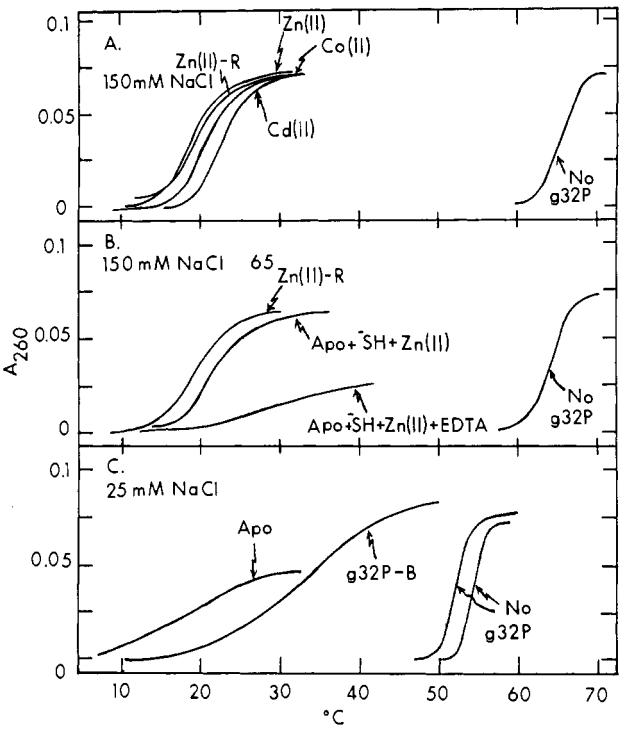

FIGURE 4: Thermal denaturation of poly[d(A-T)] induced by various g32Ps. The thermal denaturation of double-stranded poly[d(A-T)] was monitored by hyperchromism at 260 nm. Unless otherwise indicated, 1 mM EDTA was present in the standard reaction mixture while g32P was present at 2-fold over the concentration of binding sites (n = 7) (3.20×10^{-6} M). (A) Melting of poly[d(A-T)] with no added protein and in the presence of Zn(II), Cd(II), Co(II), and Zn(II)-R g32Ps at 150 mM NaCl; (B) in the presence of ^{65}Zn(II)-R g32P, apo-g32P [0.16 mol of Zn(II)] + 10 mol equiv of $ZnCl_2$ + EDTA, apo-g32P + 10 mol equiv of $ZnCl_2$ with no EDTA present; (C) melting of poly[d(A-T)] at 25 mM NaCl in the presence of apo-g32P and g32P-B. In panel C, g32P-B was present at 4.5-fold excess over the concentration of g32P binding sites (5.37×10^{-6} M). Sample cuvettes were preequilibrated at 12 °C for the melting experiments described in panels A and B, while an equilibration temperature of 10 °C was used for the experiments in panel C.

only by a factor of ~4, consistent with an ω value of ~3.7 or essentially noncooperative binding to the two-site lattice. Thus, a major effect of metal ion removal is to compromise the ability of g32P to bind cooperatively to polynucleotides. Binding to poly(dT) provides a significant further enhancement of the binding affinity shown by the apoprotein (Table II). It should be noted that K_a for the apoprotein binding noncooperatively to $d(pT)_8$ is only 4–5-fold less than that observed for the metalloproteins.

The K_a values given in Table II for the metallo-g32P–poly(dT) complexes represent minimum values (Kelly et al., 1976). The "apparent site size" for the apo-g32P (as measured by protein fluorescence quenching titrations) decreases drastically as the apo-g32P concentration is decreased below about 1 μM (Giedroc et al., 1986). To avoid this phenomenon, all of the binding isotherms shown in Figure 3 were carried out at a g32P concentration of 1 μM.[2] At this concentration of metalated g32Ps, however, there is too little free g32P present at any point in the binding isotherm to be able to measure an affinity higher than that shown; thus they are minimum estimates (Table II). However, the K_a of 2.6×10^7 M^{-1} for the apo-g32P–poly(dT) complex should be accurate (see below). As described in the following section, this problem can be circumvented and an estimate of the true g32P–polynucleotide binding constant obtained by the use of poly[d(A-T)] melting studies.

Thermal Denaturation of Poly[d(A-T)] Induced by Apo-g32P and Metal-Substituted g32P. While an apparent kinetic block prevents g32P from melting native dsDNA (Jensen et al., 1976), g32P can completely melt the synthetic alternating copolymer poly[d(A-T)] (Greve et al., 1978). The difference in the T_m's observed for this homopolymer in the presence and absence of g32P is directly related to the apparent binding affinity of the protein for this lattice. In addition, there exists a linear relationship for log[NaCl] vs. log K_a, enabling one to extrapolate K_a values to NaCl concentrations where melting cannot be observed (Jensen et al., 1976).

A representative group of melting transitions characteristic of the native Zn(II), Co(II), Cd(II), and Zn(II)-R g32P derivatives at 150 mM NaCl observed with the increase in optical density at 260 nm accompanying melting as the assay is shown in Figure 4A. All metalated proteins depress the T_m for poly[d(A-T)] by 43–47 °C, with effectiveness in reducing T_m's increasing in the order Zn(II) ≅ Zn(II)-R > Co(II) > Cd(II). Identical trends were observed at 200 and 250 mM NaCl (data

[2] One possible explanation for the anomalous nucleotide titration behavior of apo-g32P at low concentration is that the apo-g32P denatures at protein concentrations too low to permit indefinite aggregation (and presumably, stabilization) to occur. Apo-g32Ps are prepared with PMBS, a mercurial reagent that reversibly reacts with g32P cysteine residues, which causes expulsion of the Zn(II) ion (Giedroc et al., 1986). In a study prior to the realization that the protein contained Zn(II), Carroll et al. (1975) had shown that reaction of g32P with a mercurial reagent significantly perturbs self-association of g32P monomers.

Table III: Association Constants (M^{-1}) for the Binding of Apo- and Metallo-g32Ps to Poly[d(A-T)][a] Derived from T_m Depression Experiments

g32P	K_a (M^{-1}) 150 mM NaCl	K_a (M^{-1}) 25 mM NaCl	x-fold apo
Zn(II)	1.7×10^8	4.8×10^{10}	1100
Cd(II)	8.4×10^7	1.3×10^{11}	3000
Co(II)	1.2×10^8	2.4×10^{11}	5600
Zn(II)-R	1.4×10^8	2.1×10^{11}	5000
^{65}Zn(II)-R	1.6×10^8		
apo + $ZnCl_2$	9.1×10^7		
apo	ND[b]	4.3×10^7	1
g32P-B	ND[b]	7.1×10^5	0.02

[a] Derived according to the procedure outlined under Materials and Methods from the data presented in Figure 4. [b] ND, none detected.

not shown). K_a values calculated from the T_m depression experiments according to published procedures indicate $K_a \sim 10^8$ M^{-1} in 150 mM NaCl and $\sim 10^{11}$ M^{-1} in 25 mM NaCl for all metal-substituted g32Ps (Table III).

At 150 mM NaCl, apo-g32P is unable to depress the T_m for poly[d(A-T)]. Slight transitions observed correspond to the amount of Zn(II) contaminating the apo-g32P preparations. At 150 mM NaCl apo-g32P in its folded form might reduce the melting temperature of poly[d(A-T)] moderately, but this cannot be tested, since the apoprotein begins to unfold at ~44 °C (Keating et al., 1987). When greater than stoichiometric Zn(II) is added to the apoprotein in standard reaction cocktails containing 1 mM EDTA, melting of poly[d(A-T)] is not restored; however, if the EDTA is removed and Zn(II) added to the apoprotein, the reconstituted protein melts poly[d(A-T)] at ~22 °C (Figure 4B). A similar melting temperature depression is characteristic of the ^{65}Zn(II)-R g32P as shown (T_m = 19.0 °C).

If the double helix of the DNA is made less stable by lowering the salt concentration, apo-g32P can be observed to reduce the T_m of poly[d(A-T)], since some protein-induced DNA melting can be observed prior to thermal denaturation of the protein (Figure 4C and below). At a NaCl concentration of 25 mM the apparent T_m lowering by the apoprotein is ~36 °C. However, complete melting of the double-stranded polymer by the apoprotein even at 25 mM NaCl is not attained before the protein denatures. Thus, the midpoint of this transition is a moderate overestimate of the melting point depression by apo-g32P. For comparison purposes, the DNA melting curve induced by the proteolytic product, g32P-B, in which the amino-terminal domain is removed is also presented in Figure 4C; a T_m reduction of ~20 °C is observed. The melting transitions induced by both apo-g32P and g32P-B are extremely broad. K_a values for poly[d(A-T)] characteristic of apo-g32P and g32P-B at 25 mM NaCl are compared to those extrapolated for the metal-substituted g32P derivatives at 25 mM NaCl in Table III.

As expected, the K_a's for the metal-substituted g32P polynucleotide complexes (10^{11} M^{-1}) are considerably higher than the minimum estimates of $\sim 3 \times 10^8$ M^{-1} obtained by protein fluorescence quenching studies (Table II), while K_a's obtained for the apoprotein are similar ($\sim 3 \times 10^7$ M^{-1}) by use of either technique. Metal-substituted g32Ps exhibit K_a's on the average some 3 orders of magnitude greater than those of the apoprotein under the same conditions, while g32P-B exhibits an even lower K_a than the metal-free protein.

Extended Melting Curves for Poly[d(A-T)] in the Presence of Apo-g32P and Metal-Substituted g32P. The optical density changes throughout the temperature range extending from the g32P-induced melting of poly[d(A-T)] to the melting temperature of the free polymer include not only the formation

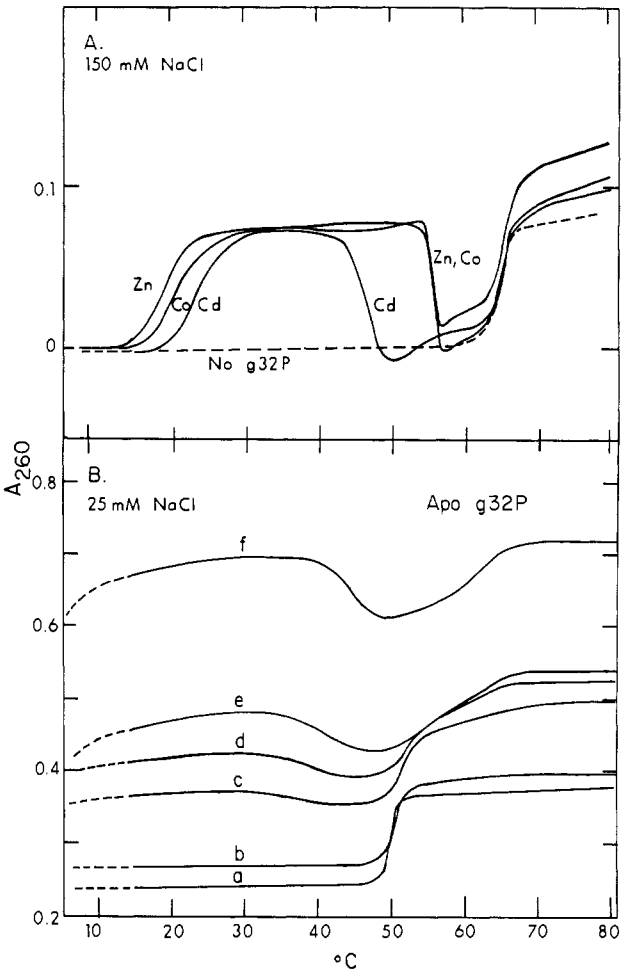

FIGURE 5: Extended melting profiles of poly[d(A-T)] in the presence of apo- and metallo-g32Ps. (A) Complete melting transitions of poly[d(A-T)] alone and in the presence of Zn(II), Cd(II), and Co(II) g32Ps. Experimental conditions as outlined in Figure 4A. (B) Complete melting transitions of poly[d(A-T)] (6.4×10^{-6} M g32P binding sites, n = 7) in 25 mM NaCl with no added g32P (curve a) and in the presence of 1.6×10^{-6} (curve b), 3.2×10^{-6} (curve c), 4.8×10^{-6} (curve d), 6.4×10^{-6} (curve e), and 1.28×10^{-5} M (curve f) apo-g32P. Reaction mixtures were prepared on ice and loaded into cuvettes preequilibrated at 16 °C. Optical density was determined starting immediately and recorded for 5 min before starting temperature program. (---) Represents apo-g32P-induced DNA melting between 5 and 16 °C before the time program was initiated. After this time, a 1 °C/min temperature rise was initiated and absorbance recorded (—).

of the g32P–ssDNA complex but also the subsequent thermal unfolding of the protein and dissociation of the ssDNA complex. These thermal transitions, at a saturating concentration of g32P, are shown in Figure 5A for the Zn(II), Co(II), and Cd(II) proteins. While all metal-substituted g32Ps melt poly[d(A-T)] completely below 40 °C, above 40 °C melting profiles undergo steep dips corresponding to the thermal unfolding of the proteins, confirmed by differential scanning calorimetry (in preparation). The Cd(II) derivative is somewhat less thermally stable than the Zn(II) or Co(II) proteins (Figure 5A). The dips above 40 °C must also be associated with dissociation of the proteins–ssDNA complex and reformation of the DNA helix, since a subsequent melting of the poly[d(A-T)] is observed at its normal melting temperature (Figure 5A).

Complete thermal transitions at 25 mM NaCl characteristic of the apo g32P–poly[d(A-T)] complex as a function of protein concentration are presented in Figure 5B. At low ionic strength (25 mM NaCl) and at the protein concentrations

employed in Figure 5A, the apoprotein melts a significant portion of the poly[d(A-T)] at 16 °C, the cuvette equilibration temperature used in these experiments (Figure 5B, curve f). Following completion of the apoprotein-induced melting of ssDNA, a broad negative thermal transition occurs which is concentration dependent and must correspond to gradual unfolding of the apoprotein. In addition, the denatured apo-g32P appears to act as a helix stabilizer (McGhee, 1976) as it significantly perturbs the shape of the helix–coil transition of the DNA itself upon dissociation of the complex (Figure 5B). Two points are relevant from these data. (1) The degree of lattice saturation, unlike that for the Zn(II) protein (not shown), is substantially less than expected on the basis of input apo-g32P concentration and is not quite saturated (~86%) even at an apo g32P–ssDNA ratio of 2:1 (Figure 5B, curve f). This indicates that the association constant, K_a, for this interaction is in the concentration range shown; i.e., it is estimated to be 5.5×10^5 M^{-1} at stoichiometric apo-g32P concentration (curve e). (2) The shape of the negative thermal transition corresponding to dissociation (denaturation) of the g32P–ssDNA complexes in each case suggests the cooperative melting unit is larger in the Zn(II) g32P complex than in the apo-g32P complex, consistent with scanning calorimetry experiments (Keating et al., 1987). Both observations taken together suggest that the metal-free g32P exhibits relatively weak binding to poly[d(A-T)], largely as a result of radically reduced cooperativity of binding.

Endonuclease Digestion of g32P–fd DNA Complexes. A single-stranded endonuclease can be used to probe for differences in structure or stability between the complexes of fd DNA with Zn(II) g32P and apo-g32P, since native g32P completely covering the fd DNA circle protects the single strand from endonuclease cleavage [cf. Curtis and Alberts (1976)].

Both the apo-g32P and the Zn(II) protein possess significant affinity for fd DNA and bind stoichiometrically. As previously noted for poly(dT), the site size observed for apo-g32P on fd DNA is ~70% of that for the native protein (data not shown). To assay for digestion of fd DNA, the purified DNA products following treatment with nuclease for set time periods were electrophoresed through an alkaline agarose gel. A preliminary experiment showed that, at a single time of endonuclease digestion (5–6 min), significantly greater than stoichiometric amounts (~10 per site) of apoprotein were required to obtain quantitative protection from nuclease action, while only 1–2 site size equivalents of native g32P were needed to afford the same protection.

A time course of endonuclease digestion in the presence of a single concentration of apo-g32P and Zn(II) g32P was performed to assess any differences in the off rate of the two proteins. Significant DNA digestion is apparent only after 30 min of exposure to endonuclease when native g32P is used to form the complex (Figure 6). On the other hand, DNA covered with apo-g32P is nicked to this degree after just 2 min of exposure to nuclease and is subsequently degraded to progressively smaller fragments (Figure 6). These results suggest an off rate some 10–15-fold greater for the apo-g32P–fd DNA complex relative to the native g32P–fd DNA complex.

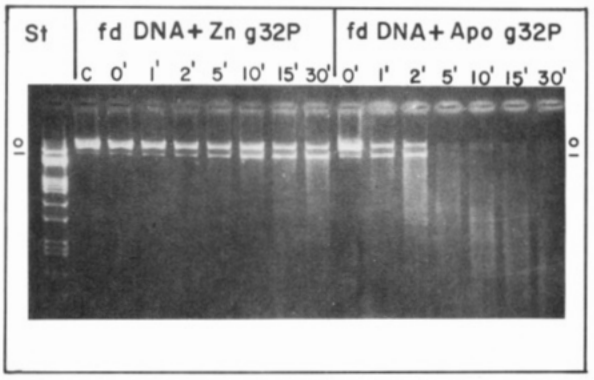

FIGURE 6: Endonuclease digestion of g32P–fd DNA complexes as monitored by agarose gel electrophoresis. Digestions by *N. crassa* endonuclease of g32P–fd DNA complexes formed with native Zn(II) g32P or apo-g32P were carried out for 1, 2, 5, 10, 15, and 30 min. The migration of intact closed circular and that of once-nicked fd DNA species are designated by a circle and a line as shown. St, dsDNA molecular weight ladder derived from digestion of T7 DNA with *Hpa*I; C, fd DNA standard; 0′, DNAs recovered from 30 min, 37 °C, incubation of fd DNA with Zn(II) g32P or apo-g32P as indicated with no exogenous nuclease added.

Discussion

What is the function of Zn(II) in gene 32 protein? The first role discovered for Zn(II) in proteins was an enzymatic one where Zn(II) either coordinates and activates a solvent molecule or provides an open coordination site for formation of a mixed complex between protein-bound metal ion and a substrate atom (Chlebowski & Coleman, 1976). On the basis of the characteristics of the Zn(II) complex in gene 32 protein, all of which suggest a S^-,S^-,S^-,N set of donors creating a tetrahedral complex, Zn(II) is not likely to expand its coordination sphere or exchange ligands readily (Giedroc & Coleman, 1986; Giedroc et al., 1986). Since Zn(II) does not appear to greatly affect either the qualitative or quantitative binding of the protein to a single site on a nucleotide lattice (Figure 3), the metal ion would not appear to provide a bridge between protein and nucleotide. This conclusion is supported by difference visible absorption spectra of Co(II) g32P with and without poly(dT) which show little if any change in either the positions or intensities of "d–d" electronic transitions when poly(dT) binds (D. Giedroc and J. Coleman, unpublished results). This finding is consistent with the absence of any large conformational changes or ligand exchanges affecting the geometry of the metal site upon complexation with a cooperatively binding ligand.

The presence of a Zn(II) complex embedded in a protein is a significant structural element, and the free energy of formation of the complex should significantly alter the free energy of stabilization of the folded protein. For example, in alkaline phosphatase, a Zn(II) metalloenzyme in which the effect of Zn(II) binding on the free energy of stabilization of the molecule has been examined in detail by differential scanning calorimetry, the binding of a single Zn(II) ion to the A site of one subunit increases the negative free energy of stabilization of the folded protein by ~35 kcal mol^{-1} (Chlebowski & Mabrey, 1977). Since the folded form of the apoprotein is only ~20 kcal mol^{-1} more stable than the unfolded form, Zn(II) makes a relatively dramatic contribution to the stability of this metalloprotein. Differential scanning calorimetry demonstrates that the Zn(II) increases the T_m for unfolding of g32P by 7 deg and the thermal unfolding transition becomes much more cooperative (Keating et al., 1987). The presence of poly(dT) makes this increase in stabilization and cooperativity of unfolding even more dramatic, but this occurs only if the Zn(II) ion is present.

A conservative estimate for the association constant for Zn(II) binding to g32P is 10^{10}; hence, a free energy factor of at least 13–14 kcal mol^{-1} is associated with formation of the Zn(II) complex. While all this binding energy may not appear directly as stabilization of the protein if otherwise unstable conformations of the polypeptide are induced by Zn(II)

binding, there must be reasonably large local effects of Zn(II) coordination. Once such an exclusively structural role has been assigned to Zn(II) and the expected thermodynamic consequences observed including the altered transition curves for the g32P-induced melting of dsDNA (Figures 4 and 5), which in large part reflect the decreased thermal stability of the protein, the molecular structure surrounding the Zn(II) complex and its effects on the function of g32P are less easy to probe in precise detail.

The effect such a bound ion has on reducing the amplitudes of the fluctations of the protein structure, i.e., the temperature factors, within the Zn(II) binding domain must be considerable. The complete resistance of the Zn(II)-containing DNA-binding core of g32P to proteolysis (Figure 1) must be a reflection of this increased rigidity. Significant reduction in the amplitude of polypeptide motions may be very important in some binding reactions, especially where protein–protein or protein–nucleotide interactions are involved. One well-documented example of Zn(II) playing such a role is its function in aspartate transcarbamoylase where its binding to four S^- ligands, although located completely within the regulatory subunits, is nevertheless necessary for the association of the regulatory with the catalytic subunits, a protein–protein interaction that does not use the Zn(II) directly as a bridging element (Monaco et al., 1978). The Zn(II)-coordinated Cys residues in the regulatory subunit define the ends of peptide loops which directly interact with the surface of the catalytic subunit (Monaco et al., 1978). The Cys–Zn(II) complex must control the precise conformation of these surface loops and thereby indirectly controls the magnitude of the R–C interactions. An analogous structural role for the Zn(II) complex in g32P might be to potentiate monomer–monomer interactions which may control the magnitude of the cooperative binding interactions between molecules of g32P bound to ssDNA.

Since the in vivo function of g32P and similar ssDNA binding proteins appears to depend in considerable measure on the ability of these proteins to bind cooperatively to ssDNA, we employed assays that compare the cooperative binding of apo- and metallo-g32P to the nucleotide lattice. It is in fact in the cooperative binding of g32P to DNA that Zn(II) appears to play its essential role. The apoprotein has lost most if not all of its ability to bind cooperatively to ssDNA (Table II, Figure 3). Since the cooperativity contributes a factor of $\sim 10^3$ to the binding constant of metallo-g32P for ssDNA, it is not surprising to find that the lifetime of the apoprotein–fd DNA complex is shortened to the extent that it no longer effectively protects the DNA from nuclease digestion (Figure 6). It may be difficult but not impossible to test the physiological counterpart of this result by examining the efficiency of T4 DNA replication in relatively "Zn-free" *E. coli*, an experimental system that has resulted in the synthesis of Zn-free proteins (Harris & Coleman, 1968).

Since a three-dimensional structure for g32P is not available, it is impossible even to speculate on the Zn-induced structure responsible for the protein–protein interactions leading to cooperative binding to DNA. Previous work has shown the domain formed by the amino-terminal 21 amino acids must be present for the cooperative form of the binding isotherm to appear (Spicer et al., 1979), a feature corroborated by the melting curve of poly[d(A-T)] in the presence of g32P-B (Figure 4C). Since removal of Zn(II) from the holoprotein, leaving the polypeptide intact, has an almost identical result, i.e., abolishing cooperativity (Table II, Figure 4), the Zn(II) domain and the amino-terminal B domain must both be necessary but not sufficient to establish cooperativity.

Removal of the metal ion from native g32P is a reversible process, especially when rebinding is aided by the presence of thiol (Figures 1 and 4B). In terms of restoring DNA binding, both Co(II) and Cd(II) can obviously substitute adequately for the native Zn(II) ion (Figures 2–4). Zn(II) and Co(II) have similar ionic radii, and in a tetrahedral ligand field proposed to consist of three sulfur and one nitrogen atoms as ligands, it is not surprising that Co(II) and Zn(II) act equally well to stabilize the gene 32 protein (Figure 5A). Ordinarily, the stability of a Cd(II) complex with sulfur ligands would be expected to be thermodynamically more stable than the Zn(II) complex. The larger Cd(II) ion, however, while adequately restoring the DNA binding properties does render the protein more sensitive to thermal denaturation (Figure 5A) (Giedroc et al., 1986), suggesting that the precise configuration of this metal ion complex plays a significant role in the folding and packing of the protein. Thus Zn(II) appears to organize a subdomain within the core (residues 22–253) of g32P which is essential along with the amino-terminal domain (residues 1-21) in maintaining the protein–protein interactions necessary for the cooperative binding of g32P to a ssDNA lattice.

Registry No. Poly[d(A-T)], 26966-61-0; $d(pT)_8$, 54284-61-6; $d(pT)_{16}$, 78005-39-7; poly(dT), 25086-81-1; Zn, 7440-66-6; Cd, 7440-43-9; Co, 7440-48-4; polyriboethenoadenylic acid, 41911-88-0.

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Characterization of the M1(Ala^{213}) Type of α1-Antitrypsin, a Newly Recognized, Common "Normal" α1-Antitrypsin Haplotype

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ABSTRACT: α1-Antitrypsin (α1AT) is a highly pleomorphic 52-kDa serum glycoprotein that functions as the major inhibitor of neutrophil elastase. Of these, the most common normal α1AT haplotypes identified by isoelectric focusing (IEF) of serum are those of the M family, including M1, M2, and M3. In the course of studying the α1AT type Z gene, we identified a restriction endonuclease *Bst*EII polymorphism in the M1 gene that predicted the existence of a previously unidentified, but relatively common, haplotype of M, referred to as M1(Ala^{213}) [Nukiwa, T., Satoh, K., Brantly, M. L., Ogushi, F., Fells, G. A., Courtney, M., & Crystal, R. G. (1986) *J. Biol. Chem. 261*, 15989–15994]. In this study we have cloned both α1AT genes from an individual heterozygous for the M1(Ala^{213}) and M1(Val^{213}) haplotypes. Sequencing of the coding exons of both demonstrated that they are identical except for the Ala–Val difference at residue 213. The codominant transmission of the M1(Ala^{213}) gene was demonstrated in a family study. Evaluation of 39 genomic samples of Caucasians with the IEF haplotype M1 demonstrated haplotype frequencies of 68% for M1(Val^{213}) and 32% for M1(Ala^{213}). α1AT serum levels of individuals inheriting the M1(Ala^{213}) gene in a homozygous fashion were in the same range as those for homozygous M1(Val^{213}) as was the rate of association of the M1(Ala^{213}) protein with neutrophil elastase. Interestingly, comparison of the M1(Ala^{213}) gene sequence to all of the known α1AT sequences at the gene, cDNA, and protein levels demonstrated that M1(Ala^{213}) is the closest to the baboon α1AT coding exons, suggesting that M1(Ala^{213}) is the "oldest" type human α1AT known.

α1-Antitrypsin (α1AT, also referred to as α1-antiprotease or α1-antiproteinase) is a 52-kDa glycoprotein produced and secreted by hepatocytes and mononuclear phagocytes (Gadek & Crystal, 1982; Travis & Salvesen, 1983; Carrell, 1986). The mature protein is comprised of a single polypeptide chain of 394 amino acids and three, *N*-asparaginyl-linked complex carbohydrate side chains (Carrell et al., 1982; Long et al., 1984; Mega et al., 1980). In normal individuals, α1AT comprises approximately 90% of the α1-globulin band of conventional serum protein electrophoretic pattern (Gadek & Crystal, 1982), and the serum levels of α1AT are 150–350 mg/dL (Gadek & Crystal, 1982; Keuppers, 1978). Although α1AT is a broad-spectrum antiprotease capable of complexing with and inhibiting a variety of serine proteases, its major function in the human is as an inhibitor of neutrophil elastase (EC 3.4.21.11) (Travis & Salvesen, 1983), an omnivorous protease capable of cleaving many proteins, including most proteins that comprise the structural backbone of tissues (Bieth, 1986). α1AT interacts with neutrophil elastase through the Met^{358}–Ser^{359} residues of α1AT (Travis & Salvesen, 1983; Johnson & Travis, 1978), a sequence that resides on the outside of the molecule (Loeberman et al., 1984). In the normal form of α1AT, this interaction occurs with an association rate constant (K_a) of approximately 10^7 M^{-1} s^{-1} (Beatty et al., 1980). Because the off-rate is so slow, this interaction is essentially irreversible, and neither the α1AT nor the elastase is capable of further function (Travis & Salvesen, 1983; Beatty et al., 1980).

α1AT is coded for by a single gene comprised of five exons and four introns encompassing approximately 10 kb of chromosome 14 (Long et al., 1984; Schroeder et al., 1985). More than 30 haplotypes of α1AT have been described; conven-

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