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Thermal Denaturation of T4 Gene 32 Protein: Effects of Zinc Removal and Substitution†

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ABSTRACT: Gene 32 protein (g32P), the single-stranded (ss) DNA binding protein from bacteriophage T4, is a zinc metalloprotein. The intrinsic zinc is one of the factors required for the protein to bind cooperatively to a ssDNA lattice. We have used differential scanning calorimetry to determine how the thermodynamic parameters characterizing the denaturation of g32P are affected by removal or substitution of the intrinsic zinc. Over a wide concentration range (1–10 mg/mL), the native Zn(II) protein unfolds at a t_m of 55 °C with an associated mean enthalpy change of 139 kcal mol^{-1}. Under the same conditions, the metal-free apoprotein denatures over a relatively broader temperature range centered at 49 °C, with a mean enthalpy change of 84 kcal mol^{-1}. Substitution of Zn(II) in g32P by either Cd(II) or Co(II) does not significantly change the enthalpy of denaturation but does affect the thermal stability of the protein. All metallo forms of g32P when bound to poly(dT) undergo highly cooperative denaturational transitions characterized by asymmetric differential scanning calorimetry peaks with increases in t_m of 4–5 °C compared to the unliganded metalloprotein. Removal of the metal ion from g32P significantly reduces the cooperativity of binding to poly(dT) [Giedroc, D. P., Keating, K. M., Williams, K. R., & Coleman, J. E. (1987) *Biochemistry 26*, 5251–5259], and presumably as a consequence of this, apo-g32P shows no change in either the shape or the midpoint of the thermal transition on binding to poly(dT). Thus, a single metal ion tetrahedrally coordinated to each g32P monomer provides the largest increase in the thermodynamic stability when the protein is cooperatively complexed with ssDNA. In accord with calorimetric data, we suggest that a change in the conformation or a reduction in conformational flux must occur as a direct consequence of the association of zinc with g32P which in turn enables highly cooperative binding of the protein to ssDNA.

Gene 32 of bacteriophage T4 codes for a nucleic acid binding protein (g32P)[1] that is required in stoichiometric amounts for DNA replication, repair, and recombination [for a review, see Chase and Williams (1986)]. Approximately one-third of the overall free energy of g32P binding to single-stranded nucleic acids derives from cooperative protein–protein interactions between adjacent g32P molecules bound to the nucleic acid lattice (Kelly et al., 1976). These cooperative g32P–g32P interactions are dependent in part upon an intact NH_2-terminal "B" region (defined here as residues 1–21). Proteolytic removal of the B region reduces the cooperativity parameters for binding ssDNA from about 10^3 to 1 (Spicer et al., 1979).

We have recently demonstrated that g32P contains an intrinsic Zn(II) ion (Giedroc et al., 1986). The visible absorption spectrum of the Co(II)-substituted protein is suggestive of a rather regular tetrahedral geometry about the metal ion while the presence of the two intense sulfur to Co(II) charge-transfer bands in the near-ultraviolet indicates coordination to sulfur. Mercurial titrations suggest the presence of three cysteine-S^- ligands to the Zn(II) ion (Giedroc et al., 1986). Oligonucleotide binding studies indicate that while the removal of the metal ion from g32P has relatively little effect on the binding affinity of g32P for a nucleic acid lattice containing only one binding site, it significantly decreases the binding affinity for a lattice having two contiguous binding sites due to a reduction in the cooperativity parameter by more than 2 orders of magnitude (Giedroc et al., 1987). As a result, apo-g32P is significantly less effective than native g32P at destabilizing a partially double-stranded polynucleotide such as poly[d(A-T)], completely covering a ssDNA lattice, and protecting ssDNA from endonuclease digestion (Giedroc et al., 1987; Keating et al., 1987). The presence of Zn(II) sta-

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[1] Abbreviations: g32P, gene 32 protein; ss, single stranded; PMBS, *p*-(hydroxymercuri)benzenesulfonate; DSC, differential scanning calorimetry; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TNG, 10 mM Tris-HCl, 0.2 M NaCl, and 5% v/v glycerol, pH 8.

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bilizes the DNA binding core domain of g32P (residues 22–253) against proteolysis, as removal of the metal results in rapid degradation of the core protein by trypsin. Taken together, these data suggest that the zinc ion organizes a subdomain that is required for maintaining the protein–protein interactions necessary for the cooperative binding of g32P to a ssDNA lattice.

In an earlier study of g32P by differential scanning calorimetry (DSC), it was demonstrated that the thermal denaturation temperature, t_m, of g32P increased 4.5 °C on binding poly(dT) and the temperature range over which the transition occurred decreased to 60% of that seen with the protein alone (Williams et al., 1979). These changes in the denaturation profile were not observed when the protein was bound noncooperatively to $p(dT)_8$. In this study, we have used DSC to analyze the contribution of the intrinsic Zn(II) ion to the thermodynamic stability of g32P free in solution or when bound cooperatively to ssDNA.

Materials and Methods

Protein and Nucleic Acids. Isolation and purification of Zn(II) g32P from T4-infected *Escherichia coli* were carried out according to the method of Bittner et al. (1979). Protein concentrations were determined by the absorbance at 280 nm using the molar absorptivity of 3.7×10^4 M^{-1} cm^{-1}. Protein samples were dialyzed into metal-free 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 5% glycerol. Zinc contents of all proteins were determined by atomic absorption on an Instrumentation Laboratories IL157 spectrometer operating in a flame aspiration mode. Apo-g32P was produced with *p*-(hydroxymercuri)benzenesulfonate (PMBS) or *o*-phenanthroline as previously described and contained 0.03–0.05 mol of Zn(II)/mol. Zn(II) reconstitution of apo-g32P was accomplished as previously described (Giedroc et al., 1987). Cd(II) and Co(II) were directly exchanged with the intrinsic Zn(II) of g32P by dialysis against an excess of the metal chlorides followed by exhaustive dialysis against metal-free buffer. The Cd(II) and Co(II) contents were determined by atomic absorption as described. In addition, Co(II) in g32P was quantitated by visible absorption spectroscopy using a molar absorptivity of 660 M^{-1} cm^{-1} at 645 nm (Giedroc et al., 1986), and results showed excellent agreement with those obtained from atomic absorption. Metal-substituted g32P contained greater than 85% of the indicated metal in all cases with the remainder of the sites occupied by Zn(II). Poly(dT) was purchased from Pharmacia P-L Biochemicals. Nucleotide concentrations were determined spectrophotometrically using a molar absorptivity of 8100 M^{-1} cm^{-1} at 260 nm. A binding site size of seven nucleotides per g32P monomer was used for calculations of stoichiometry (Kowalczykowski et al., 1986).

Gel Filtration Chromatography. Sephadex G-25 was swollen in metal-free TNG. Small gel filtration columns (2 mL) were poured in 3-mL syringes and washed with buffer 3 times. Metallo-g32Ps were heated to 70 °C in a water bath for between 2 and 10 min in order to denature the protein. The samples were then immediately run over the gel filtration columns by centrifugation at ambient temperature. Postcolumn protein concentrations and metal contents were determined as described.

Differential Scanning Calorimetry. Calorimetric measurements were conducted in a DASM-4 differential scanning calorimeter (Privalov & Potekhin, 1986) at a scan rate of 1 K min^{-1}. Decreasing the scan rate to 0.5 K min^{-1} did not change the DSC profile of Zn(II) g32P. The enthalpy change associated with protein denaturation, ΔH_d, was determined from the areas under the curves as measured by planimetry with base lines estimated as previously described (Takahashi et al., 1981). Thermodynamic parameters were calculated by using the equations:

$$\Delta H_{cal} = \Delta H_d - \Delta C_p^d(T_{1/2} - T)$$

$$\Delta H_{vH} = 4RT_{1/2}^2 C_{ex,1/2}/\Delta H_d$$

$$\Delta S^\circ = \Delta H_d/T_{1/2} - \Delta C_p^d \ln (T_{1/2}/T)$$

$$\Delta G^\circ = \Delta H_{cal} - T\Delta S^\circ$$

where $T_{1/2}$ is the temperature at half-denaturation in degrees kelvin, T is the reference temperature, taken as 328.55 K [the mean denaturation temperature of Zn(II) g32P], ΔH_{vH} is the van't Hoff enthalpy, R is the gas constant (1.987 cal K^{-1} mol^{-1}), and $C_{ex,1/2}$ is the excess heat capacity at $T_{1/2}$ in kilocalories per degree kelvin per mole. The $t_{1/2}$ ($T_{1/2}$ in degrees centigrade) did not differ from the t_m, the temperature at maximum excess specific heat in degrees centigrade, by more than 0.5 °C. The permanent heat capacity changes, ΔC_p^d, showed significant variability between scans which appeared not to be related to the oxygen tension of the samples. Therefore, mean values for each form of g32P were used for calculations of thermodynamic data and are compiled in Tables I and II. The enthalpy of dissociation of poly(dT) given is the mean of the values for the different forms of the protein which were calculated by subtracting the enthalpy of denaturation of the protein alone from the enthalpy of denaturation of the protein complexed with poly(dT), with both enthalpies calculated to the reference temperature.

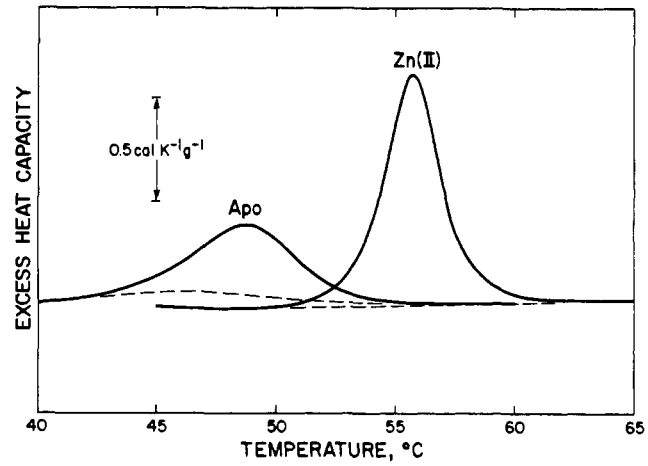

FIGURE 1: Effects of zinc(II) removal on the thermal denaturation of g32P. DSC scans of Zn(II) g32P and apo-g32P at 2 mg/mL are shown. The curves represent direct traces of the output signal from the calorimeter and illustrate the very high signal-to-noise ratios observed in this work.

Results

Thermal Denaturation of Zn(II) and Apo-g32P. A tracing of a DSC curve for the denaturation of Zn(II) g32P is shown in Figure 1. The Zn(II) protein undergoes a single symmetrical endothermic transition near 55 °C. The transition has been shown to be slowly reversible, with 23% of the initial enthalpy being recovered after 12 h (Williams et al., 1979). Since previous work has shown that even proteins that undergo apparently completely irreversible denaturations can nevertheless closely follow equilibrium thermodynamics (Edge et al., 1985), we have utilized the van't Hoff equation in analyzing g32P calorimetric data.

The thermodynamic data calculated from DSC scans on Zn(II) g32P solutions that ranged in concentration from 1 to

Table I: Effect of Metal Ion Removal and Substitution on the Thermodynamic Properties Characterizing the Thermal Unfolding of g32P[a]

g32P	no. of DSC scans	t_m (°C)	ΔC_p[d] (kcal K^{-1} mol^{-1})	ΔH_{cal} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	$\Delta G°$ (kcal mol^{-1})
native Zn(II)	10	55.4 ± 0.2	−0.77 ± 0.02	139 ± 5	1.8 ± 0.2	0
apo	14	49.3 ± 0.4	−1.11 ± 0.28	84 ± 7	2.6 ± 0.2	−1.7 ± 0.1
Cd(II)	7	53.3 ± 0.2	−2.87 ± 0.09	133 ± 10	1.7 ± 0.2	−0.9 ± 0.1
Co(II)	7	56.4 ± 0.2	−3.76 ± 0.10	138 ± 6	1.7 ± 0.1	0.4 ± 0.1
Zn(II)-R[b]	1	54.9	0	160	1.4	−0.2

[a] Thermodynamic parameters are means ± standard errors calculated at 55.4 °C by using the equations given under Materials and Methods. $\Delta G°$ indicates the free energy of unfolding calculated at the t_m of Zn(II) g32P (55.4 °C); i.e., $\Delta G° = 0$ for the native Zn(II) protein. The protein concentration ranged from 1 to 10 mg/mL for the Zn(II) and apo-g32P's, 1–6 mg/mL for the Cd(II) and Co(II) g32P's, and 3 mg/mL for the Zn(II)-R g32P. [b] Zn(II)-R g32P, apo-g32P reconstituted with 1 mol of Zn(II)/mol of g32P.

10 mg/mL are given in Table I. The mean value given for t_m reflects a small degree of variability between protein preparations, as well as a small variation in t_m with protein concentration which was not considered to be significant. This indicates there is little change in the degree of protein association over the course of the denaturation. The average number of molecules involved in an apparent denaturational cooperative unit, given by the ratio of the van't Hoff to calorimetric enthalpies, is 2. Thus, under these conditions, g32P appears to denature as a dimeric unit, remaining associated.[2]

Thermally denatured metallo-g32Ps, subjected to gel filtration to remove free metal ions, were found to contain 0.8 mol of metal/mol of g32P which suggests that during denaturation of g32P in the calorimeter metal ions do not dissociate from the protein.[3] Thus, the denatured states of the metallo and apo forms of g32P may not be precisely the same, and caution must be exercised in drawing conclusions from comparisons of denaturational properties of the various forms of the protein. However, the tetrahedral coordination geometry of the metal binding site in the folded protein is disrupted on denaturation, as indicated by the loss of the visible absorption spectrum of Co(II) g32P (data not shown). Given this result, a comparison of the denaturational properties of the Zn(II) protein with those of the metal-free protein must reflect the contribution that a single Zn(II) ion bound in tetrahedral configuration makes to the thermodynamic stability of g32P.

Removal of the Zn(II) from g32P lowers the denaturation temperature of the protein by 6 °C, as shown in Figure 1. This decrease is probably a consequence of a decrease in the stability of the folded form of the protein, though the possibility that it is due, at least in part, to an increase in the stability of the denatured form cannot be excluded. That Zn(II) primarily provides stabilization of the folded form of g32P is consistent with the fact that the Zn(II) protein as well as apoprotein reconstituted with stoichiometric Zn(II) exhibits a much greater resistance to proteolysis than apoprotein at ambient temperature (Giedroc et al., 1986, 1987).

Average thermodynamic parameters collected from the denaturation of three preparations of apo-g32P are given in Table I. Zinc dissociation accomplished by treatment of the protein with either PMBS or *o*-phenanthroline produced similar effects on t_m and ΔH_{cal}. As with the Zn(II) protein, t_m and ΔH_{cal} of the apo-g32P exhibit no significant concentration dependence, and the size of the apparent cooperative unit is

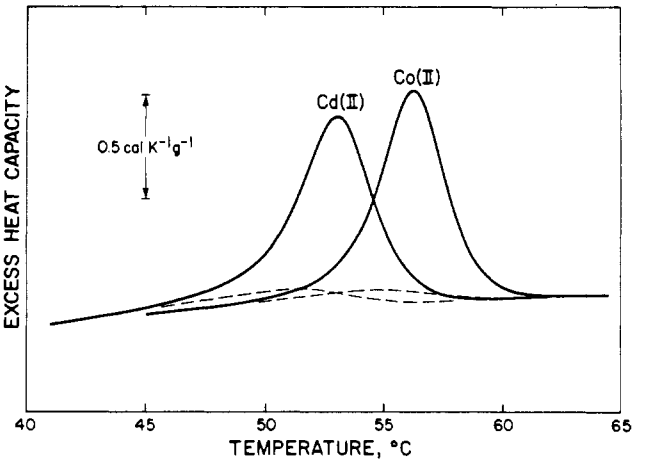

FIGURE 2: Effects of metal ion substitution on the thermal denaturation of g32P. DSC scans of Cd(II)-substituted g32P and Co(II)-substituted g32P at 2 mg/mL are shown.

two monomers. The average enthalpy change associated with the denaturation of apo-g32P is 84 kcal mol^{-1}, only 60% of that associated with Zn(II) g32P. Apo-g32P reconstituted with stoichiometric Zn(II) exhibits a t_m and ΔH_{cal} similar to those of the native Zn(II) protein (Table I).

Effect of Metal Substitution on the Thermal Denaturation of g32P. Representative DSC scans of g32P in which the intrinsic Zn(II) has been substituted with either Cd(II) or Co(II) are shown in Figure 2. As seen in Table I, the enthalpy changes associated with the denaturation of these metal-substituted forms of the protein are similar to that of the Zn(II) protein, while the transition temperatures vary. The Cd(II)-substituted protein is somewhat less stable, showing a 2 °C decrease in t_m relative to the Zn(II) g32P. In contrast, Co(II)-substituted g32P is marginally more stable than Zn(II) g32P with a t_m 1 °C higher.

The degree of stabilization or destabilization of g32P which accompanies metal ion removal or substitution is summarized in Table I in the values of $\Delta G°$, the free energy of denaturation defined at 55.4 °C, the temperature at which the Zn(II) protein is half-unfolded, i.e., $\Delta G° = 0$. The relative free energies parallel the relative t_m's for each g32P derivative as the apo-g32P is most destabilized compared to the Zn(II) protein, while the Cd(II) and Co(II) g32Ps are somewhat less and slightly more stable than the Zn(II) protein, respectively.

For both metallo- and apo-g32P, the process of denaturation is accompanied by a large negative change in heat capacity, on the order of 1 kcal K^{-1} mol^{-1}. This is unusual since most proteins exhibit a positive change in heat capacity, presumably due mostly to increased exposure of hydrophobic groups to the aqueous environment upon denaturation (Pfeil, 1986). Increased exposure of electrostatic charges to solvent can contribute to an overall heat capacity change which has a negative sign (Sturtevant, 1977).

[2] In the previous study on g32P (Williams et al., 1979), in which a different buffer was used, a ratio of 1 was reported. Association of free g32P monomers has been shown to be sensitive to both buffer conditions and temperature (Carroll et al., 1975).

[3] Approximately 70% of the Co(II) g32P precipitated during the gel filtration experiment, which involved a relatively rapid increase in temperature (see Experimental Procedures). In contrast, no precipitation was noticed after removing similar samples that had been subjected to a more gradual temperature increase in the calorimeter. The atomic absorption measurement was taken on the soluble Co(II) g32P.

Table II: Summary of the Thermodynamic Data Characterizing the Thermal Unfolding of Various g32Ps Complexed with Stoichiometric Poly(dT)[a]

g32P	no. of DSC scans	t_m (°C)	ΔC_p[d] (kcal K^{-1} mol^{-1})	ΔH_{cal} (kcal mol^{-1})	δt_m (°C)[b]	$\delta\Delta G°$[b] (kcal mol^{-1})
native Zn(II)	2	59.9 ± 0.2	−2.9 ± 0.2	152 ± 15	4.6	1.9
apo	3	49.8 ± 0.7	−0.46 ± 2.21	97 ± 22	0.5	0.0[c]
Cd(II)	2	57.3 ± 0.1	−1.36 ± 3.30	161 ± 2	4.0	1.7
Co(II)	2	60.9 ± 0.6	−2.16 ± 0.62	162 ± 3	4.5	2.1

[a] Thermodynamic parameters are means ± the average difference between the mean and individual experiments calculated to 55.4 °C by using the equations listed under Materials and Methods. The protein concentration in all cases was 2 mg/mL with stoichiometric poly(dT) present on the basis of a binding site size of seven bases per g32P monomer (Kowalczykowski et al., 1986). [b] These values represent the difference in t_m and $\Delta G°$ for the indicated protein in the presence and absence of poly(dT). [c] Within the error of the measurements, poly(dT) has essentially no effect on the free energy of unfolding of apo-g32P.

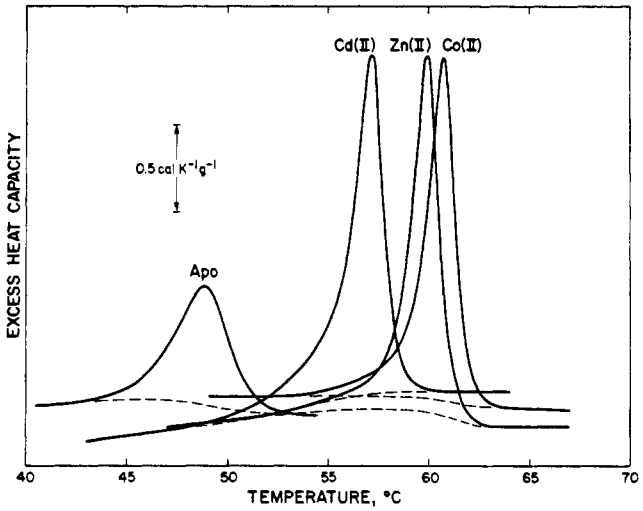

FIGURE 3: Effects of poly(dT) on the thermal denaturation of various g32Ps. DSC scans of apo, Cd(II), Zn(II), and Co(II) forms of g32P complexed with stoichiometric poly(dT) based on a binding site size of seven bases per g32P monomer are shown. The protein concentration in each case was 2 mg/mL.

Effects of Poly(dT) on the Thermal Denaturation of g32Ps. As reported previously, poly(dT) binding has a dramatic effect on the thermal denaturation of g32P (Williams et al., 1979). The effect of poly(dT) on the thermal denaturation of the apo, Zn(II), Cd(II), and Co(II) forms of g32P is compared in Figure 3. The most striking finding is the marked stabilization of the metalloproteins upon complexation with poly(dT) compared to the marginal stabilization afforded apoprotein under identical conditions. Thermodynamic parameters characterizing the denaturation of g32P in the presence of poly(dT) (Table II) show that the Zn(II) protein is stabilized by binding to poly(dT) with an increase in t_m of 4.6 °C to 59.9 °C. Increasing the concentration of poly(dT) to a 10-fold excess had little effect on the denaturation temperature of the protein (data not shown). The DSC peaks of the metallo forms of g32P become asymmetric on binding to poly(dT) which indicates that the denaturation is not a simple two-state process and that the van't Hoff equation in its simplest form is not applicable. Curve resolution of a representative DSC scan of Zn(II) g32P in the presence of poly(dT) indicates the denaturation occurs in two steps. This analysis also gave a value for ΔH_{vH} of 750 kcal mol^{-1}. This yields an apparent denaturational cooperative unit size of 5.5 molecules.

All metallo forms of g32P dissociate from polynucleotides on denaturation as demonstrated by the reannealing and subsequent characteristic helix–coil transition of poly[d(A-T)] above the t_m for each form of the protein (Giedroc et al., 1987). According to the isothermal scheme described in Edge et al. (1985), the difference in the enthalpies of denaturation of a protein in the presence and absence of a completely dissociable ligand is equal to the enthalpy of dissociation. The enthalpies of the denaturation of all g32Ps increase on addition of poly(dT) by an average of 20 ± 6 kcal (mol of g32P)$^{-1}$. This value is in excellent agreement with the association enthalpy estimated from the temperature dependence of binding of g32P to poly(ethenoadenylic acid) (Kowalczykowski et al., 1981).

The Cd(II)- and Co(II)-substituted forms of g32P undergo stabilization on binding to poly(dT) in a manner analogous to that of Zn(II) g32P, although there are some qualitative differences. Binding of the ssDNA to Co(II) g32P increases the t_m by 4.5 °C to 60.9 °C, making the Co(II) g32P complex the most thermally stable of the complexes (Figure 3). Just as Cd(II) g32P is the least stable of the metallo-g32Ps, the Cd(II) g32P–poly(dT) complex is the least stable of the metallo-g32P–ssDNA complexes (Figure 3). The t_m of Cd(II)-substituted g32P is increased by 4.0 °C upon binding poly(dT), concomitant with the indicated changes in curve symmetry.

The increased stabilization of the metalloproteins upon polynucleotide binding is also reflected in the positive $\delta\Delta G°$ values obtained by subtracting the free energy of denaturation associated with an unliganded protein from that of the cognate ssDNA complex (Table II). Although g32P is stabilized to varying degrees by the binding of Zn(II), Co(II), or Cd(II) at the intrinsic metal binding site (Table I), all metalloproteins are further stabilized upon binding to poly(dT).

In contrast to the metallo-g32Ps, poly(dT) binding has a marginal effect on the thermal transition of apo-g32P (Figures 1 and 3). The t_m of the apoprotein is increased by at most 0.5 °C, and the shape of the DSC peak remains unchanged even with a 10-fold excess of polynucleotide (data not shown). Apo-g32P is not significantly stabilized by the binding of poly(dT) as seen by the difference in denaturational free energy between the free and bound forms of close to zero (Table II).

Discussion

Differential scanning calorimetry of g32P shows that Zn(II) is an important structural element of the protein as zinc–ligand bond enthalpies contribute 55 of the total 139 kcal mol^{-1} enthalpy of denaturation. This increase in the enthalpy of denaturation on binding Zn(II) is comparable to that which is observed on Zn(II) binding to the apo forms of superoxide dismutase, alkaline phosphatase, and azurin (Table III). However, the 6 °C increase in t_m that accompanies Zn(II) binding to apo-g32P is relatively small in comparison to 20–28 °C increases observed in these other metalloproteins (Table III).

We suggest that some of the stabilization energy expected by the formation of the zinc chelate in g32P must be used to drive a significant negative change in the entropy of the folded protein. Consistent with this proposal, the average value for

Table III: Effect of Zinc on the Thermal Unfolding of Various Metalloproteins

protein	monomer M_r ($\times 10^{-3}$)	Zn(II) content (mol/mol of monomer)	t_m (°C)	δt_m[a] (°C)	ΔH_{cal} (kcal mol^{-1})	$\delta\Delta H_{cal}$[b] (kcal mol^{-1})	ref
gene 32 protein	33.5	1	55	6	139	55	this work
		0	49		84		
alkaline phosphatase (*E. coli*)	47	1	85	27	337	75	Chlebowski & Mabrey (1977)
		0	58		262		
superoxide dismutase[c] (bovine)	16.5	1	80	20	93	33	Lepock et al. (1985)
		0	60		60		
azurin (*P. aeruginosa*)	16.5	1	90	28	198	33	Engeseth & McMillin (1986)
		0	62, 86[d]		165		

[a] δt_m refers to the thermal denaturation temperature of the Zn(II) metalloprotein less that of the metal-free apoprotein. [b] $\delta\Delta H_{cal}$ refers to the enthalpy of denaturation of the Zn(II) metalloprotein less that of the metal-free apoprotein. [c] Superoxide dismutase contains one copper site and one zinc site per monomer. Neither of the above preparations contained copper. [d] Apoazurin is characterized by two endothermal transitions with a combined enthalpy of denaturation as given. The lower temperature transition merges with the higher temperature transition when azurin is reconstituted with zinc and gives rise to the increased enthalpy as shown.

the entropy of denaturation of Zn(II) g32P is 0.42 kcal K^{-1} mol^{-1} while that for the apo-g32P is 0.26 kcal K^{-1} mol^{-1}. If the assumption is made that the entropy levels of the denatured forms of the protein are comparable, then the folded form of the apoprotein does possess a higher degree of entropy than that of the Zn(II) protein.

The proposed reduction in the entropy of the folded protein that accompanies zinc binding may result from significant topological changes which signal a new average solution structure and/or a dynamic reduction in the amplitude of conformational fluctuations about the same structure. Differential ordering of solvent molecules about each of the folded structures will also contribute to differences in entropy. Although there is precedent for large changes in the polypeptide backbone structures occurring upon metal ion binding, e.g., one of the two high-affinity sites of troponin c goes from largely unfolded to helical upon binding of Ca^{2+} (Tsalkova & Privalov, 1985; Levine et al., 1977), in many instances metal ion binding often minimally perturbs the overall folding of a particular protein or metal ion binding domain in that detectable conformational changes are restricted to only those amino acid side chains in the immediate vicinity of the metal site (Wyckoff et al., 1983; Garrett et al., 1984). For example, Raman spectra of the apo and Zn(II) forms of superoxide dismutase suggest very small differences in their secondary structures (Lepock et al., 1985). In the case of g32P, far-ultraviolet circular dichroism experiments show that small but significant changes in backbone folding coupled to spatial rearrangement of one or more aromatic amino acid side chains occur upon metal ion [Zn(II), Cd(II)] reconstitution of apo-g32P (D. P. Giedroc and J. E. Coleman, unpublished results). Whatever the nature of these rather small changes in structure, they are nonetheless sufficient to increase significantly the resistance of this protein to proteolysis (Giedroc et al., 1986).

The enthalpy change observed on denaturation of g32P appears to be independent of the metal ion present within experimental error. This suggests that the conformations of the various metallo derivatives of g32P are similar. The resistance of all three metallo-g32Ps to proteolysis at room temperature supports this conclusion. The t_m and free energy estimate for the Cd(II) protein, however, show it to be less stable than the Zn(II) protein (Table I) despite the fact that Cd(II)–sulfur bonds in a small chelate complex are stronger than Zn(II)–sulfur bonds. Consistent with a lower thermodynamic stability, Cd(II) g32P is more susceptible to proteolysis at elevated temperatures than Zn(II) or Co(II) g32Ps (Giedroc et al., 1986). Since the denaturational enthalpies of all the metallo-g32Ps are similar, the reduced stability of Cd(II) g32P would appear to be predominantly entropic in origin and may be attributable to a subtle folding problem caused by the larger ionic radius of Cd(II) in a metal binding site designed to accommodate Zn(II).

A variety of data have shown that metal ion coordination is also an essential component of the cooperative binding mode of g32P (Giedroc et al., 1987). DSC has demonstrated that the thermodynamic stabilization of g32P which accompanies binding to poly(dT) absolutely requires the metal ion. Thus, the stabilization of unliganded g32P by Zn(II) is coupled to monomer–monomer interactions associated with cooperative binding to polynucleotides which results in further thermodynamic stabilization of the protein. Consequently, the difference in t_m between the apo-g32P–poly(dT) complex and the Zn(II) g32P–poly(dT) complex is 10 °C, some 4 °C greater than the difference in t_m between the proteins in the absence of ligand. The thermal transition is much sharper and more asymmetric for the Zn(II)–protein complex, presumably due to the cooperative nature of binding and denaturation (Figure 3). The other two metallo forms of g32P also bind cooperatively to poly(dT), resulting in similar increases in thermal stability and showing narrow asymmetric DSC peaks.

Since metallo-g32Ps appear to dissociate from polynucleotides on denaturation (Giedroc et al., 1987), the denatured forms of g32Ps are the same in the absence and presence of poly(dT) and the increases in t_m that accompany the addition of poly(dT) to the metallo-g32P's must result from the increased stability of the native forms of the protein. The apparent cooperative unit size of 5.5 Zn(II) g32P monomers derived from curve resolution may represent an underestimate of the actual number of monomers denaturing as a unit since the denatured form of the protein is presumably dimeric. The large cooperative unit size is consistent with electron micrographs of g32P bound to polynucleotides which show the protein to be highly oligomerized (Alberts & Frey, 1970). The asymmetry of the DSC peaks of the metallo-g32P–ssDNA complexes could result, in part, from a change in the degree of oligomerization of the protein during the transition.

In contrast to the metalated g32Ps, the DSC curve of apo-g32P complexed with poly(dT) is symmetric which suggests that a single-step transition occurs. The similarities between the ratios of van't Hoff to calorimetric enthalpies in the presence and absence of poly(dT) indicate that a large change in the degree of oligomerization of the protein does not occur on polynucleotide binding. This is consistent with the proposal that the protein is less oligomerized than metallo-g32Ps on the polynucleotide lattice due to defects in cooperative interactions. Electron microscopy studies of apo-g32P have shown that, at conditions below saturation, the apo form of g32P appears defective in its ability to bind in long

stretches along ssDNA (Keating et al., 1987).

To summarize, while the binding of Zn(II), Co(II), and Cd(II) stabilizes g32P by varying degrees (Table I), all three metal ions confer a conformation whereby the binding of poly(dT) results in a large thermal stabilization of the protein (Table II). In contrast, the binding of poly(dT) has little effect on the free energy of denaturation of apo-g32P (Table II). Since earlier work has clearly shown that the metal ion makes a substantial contribution toward the ability of g32P to bind cooperatively to ssDNA (Giedroc et al., 1987; Keating et al., 1987), the thermodynamic stability of Zn(II) g32P relative to apo-g32P must reflect stabilization by zinc of a particular structure that is intimately involved in establishing the protein–protein and protein–nucleic acid interfaces that are formed in the cooperative mode of binding to ssDNA.

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