Site-Specific Mutagenesis of T4 Gene 32: The Role of Tyrosine Residues in Protein·Nucleic Acid Interactions†

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ABSTRACT: Bacteriophage T4 gene 32 encodes a single-stranded DNA (ssDNA) binding protein (gp32) required for T4 DNA replication, recombination, and repair. Previous physicochemical studies on gp32 and other ssDNA binding proteins have suggested that binding may involve hydrophobic interactions that result from the close approach of several aromatic amino acid side chains with the nucleic acid bases. In the case of gp32, five tyrosines and two phenylalanines have previously been implicated in gp32·ssDNA complex formation. Site-directed mutagenesis of T4 gene 32 was employed to produce a set of eight gp32 mutant proteins, each of which encoded a single substitution at one of the eight tyrosine residues within gp32. The mutant gp32 proteins were then subjected to physicochemical analysis to evaluate the role of each tyrosine residue in gp32 structure and function. Oligonucleotide binding studies suggest that tyrosine residues 84, 99, 106, 115, and 186 each contribute from 0.3 to 0.7 kcal/mol to ssDNA binding, which corresponds to 3–7% of the overall binding energy for gp32·ssDNA complex formation. Replacement of tyrosine residues 73 and 92 appears to lead to large structural changes that may be the result of disrupting the zinc binding subdomain within gp32.

Bacteriophage T4 gene 32 protein (gp32) has served as a prototype for a class of proteins that bind preferentially to single-stranded nucleic acids in a non-sequence-specific fashion. Gp32 binds tightly ($K_a \approx 10^9$ M^{-1}) and cooperatively to single-stranded DNA (ssDNA) and is essential to T4 DNA replication, recombination, and repair [for a review see Chase and Williams (1986)]. Current models of T4 DNA replication propose that gp32 binds stretches of ssDNA at the replication fork, thereby removing adventitious secondary structure features from the ssDNA while protecting it from nucleases. It has been suggested that aromatic amino acids might be important to single-stranded nucleic acid binding through a mechanism in which these side chains participate in hydrophobic interactions with nucleic acid bases. Studies using model peptides with positively charged and aromatic amino acids such as Lys-Tyr-Lys and Lys-Phe-Lys demonstrate that even these short tripeptides have a marked preference for binding ssDNA (Dimicoli & Helene, 1974; Mayer et al., 1979). To study the mechanism of gp32 binding to ssDNA, a variety of physicochemical approaches including ^{1}H NMR, chemical modification, fluorescence quenching, and differential scanning calorimetry have been used.

Gp32 has 301 amino acids (M_r = 33 487) (Williams et al., 1980; Krisch & Allet, 1982) and a binding site size of about 6–7 nucleotides/monomer (Kelly & Von Hippel, 1976; Kelley et al., 1976). Gp32 is also a zinc metalloprotein containing one zinc atom tetrahedrally coordinated to three cysteines and one histidine (Giedroc et al., 1986, 1989). Removal of the zinc ion from gp32 decreases the overall free energy of gp32 binding to $p(dT)_8$, which is too short to allow cooperative binding, by 13% (Giedroc et al., 1987). Since the overall free energy of binding of the apo-gp32 to $p(dT)_{16}$, which is long enough to allow two bound gp32 molecules, is 30% less than that of the corresponding gp32 complex, it appears that the Zn(II) ion makes some contribution to both gp32·DNA and gp32·gp32 cooperative protein interactions (Giedroc et al., 1987; Shamoo et al., 1989). Differential scanning microcalorimetry of gp32 demonstrates that Zn(II) is also a very important structural element of the protein as zinc–ligand bond enthalpies contribute 40% of the overall enthalpy of denaturation of gp32 (Keating et al., 1988).

Limited proteolysis of gp32 has indicated that gp32 is made up of three domains (Hosoda et al., 1974; Williams & Konigsberg, 1978). Characterization of the partial proteolysis products of gp32 revealed that removal of the C-terminal A domain (residues 254–301) to give gp32*-A results in a fragment that is unable to bind other T4 DNA replication proteins (Hosoda et al., 1980). Removal of the N-terminal B domain (residues 1–21) (gp32*-B) abolishes gp32·gp32 interactions that are responsible for the cooperativity of gp32 binding to ssDNA (Hosoda et al., 1980). If both the A and B domains are removed, a core gp32*-(A+B) is obtained, which has the same intrinsic affinity for ssDNA as the intact protein (Spicer et al., 1979). Chemical modification with tetranitromethane provided the first evidence that several tyrosine residues might be directly involved in gp32 binding to ssDNA (Anderson & Coleman, 1975). Prigodich et al. (1984, 1986), using ^{1}H NMR with core gp32*-(A+B)·oligonucleotide complexes, have demonstrated that resonances corresponding to five tyrosine and two phenylalanine residues are shifted upon complex formation. They also showed that the number and magnitude of the shifts associated with ssDNA binding increased with the length of oligonucleotide until the site size of gp32 was reached, after which no further changes were observed (Prigodich et al., 1984). Recently, we have shown that core gp32*-(A+B) could be photochemically cross-linked at Phe-183 to oligonucleotide $p(dT)_8$, suggesting that this aromatic residue is situated at the gp32·ssDNA interface (Shamoo et al., 1988). Taken together, these studies demonstrate that the ssDNA binding site of gp32 is contained within residues 22–253, which comprise the core gp32*-(A+B) fragment. Since tyrosine represents one of the few amino acids

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that have been directly implicated in gp32 binding to ssDNA and since there are only eight tyrosines in gp32, these residues offer an excellent choice for site-directed mutagenesis. In the following study, systematic substitution of each tyrosine residue by a nonaromatic amino acid has been used to develop a family of singly substituted gp32 mutants that were examined in terms of their structural and functional characteristics. The physicochemical data acquired from this set of gp32 mutants allowed us to test current models for the involvement of aromatic amino acids in non-sequence-specific nucleic acid binding.

Materials and Methods

Materials. Poly[d(AT)], poly(dT), $p(dT)_8$, and $p(dT)_{16}$ were purchased from Pharmacia; [γ-^{32}P]ATP and site-directed mutagenesis kits were obtained from Amersham. All DNA modification enzymes were purchased from either New England Biolabs or Boehringer-Mannheim. TPCK-treated trypsin was purchased from Cooper Biomedical. Apo-gp32 was made by using the technique of Giedroc et al. (1986).

Site-Directed Mutagenesis of Gene 32. By use of the methods of Gillam and Smith (1979) or Taylor et al. (1985) in vitro site-directed mutagenesis was performed on M13 constructs containing gene 32 [designated pYS5 and described in Shamoo et al. (1986)]. Oligodeoxynucleotides used as mutagenic primers in the procedure were synthesized at either the Albert Einstein College of Medicine DNA Synthesis Facility or the Yale School of Medicine Protein and Nucleic Acid Chemistry Facility. Oligonucleotides were HPLC purified by using a Nucleogen DEAE 60-7 column and the manufacturer's protocol. Gp32ser73 (a tyrosine to serine substitution at position 73),[1] gp32ser115, and gp32ser137 were made by using techniques described by Gillam and Smith (1979). The mutagenic oligonucleotides were used as primers on pYS 5 ssDNA for the large fragment of DNA polymerase I and the resulting mutant/parental heteroduplex ligated with T4 DNA ligase. *Escherichia coli* JM103 were transfected with the heteroduplex and the resulting plaques screened by in vitro hybridization on nitrocellulose filters by using ^{32}P-labeled mutagenic oligonucleotides as the probe (Mark et al., 1984). Final confirmation of the mutation was achieved by rescreening plaques for the introduction of a new restriction site that was diagnostic for the particular substitution (Prigodich et al., 1986).

Mutants gp32ala84, gp32leu92, gp32val99, gp32leu106, and gp32ile186 were made by using the technique of Taylor et al. (1985), which is commercially available as a kit from Amersham. Following the manufacturer's protocols pYS 5 ssDNA was annealed to a mutagenic oligonucleotide. The primed template was then extended by using the large fragment of DNA polymerase I in the presence of 2 mM dATP, dGTP, dTTP, and dCTPαS. Incorporation of thionucleotide into the mutant strand allows endonuclease *Nci*I to generate nicks within the parental strand, the majority of which can then be removed by using exonuclease III. Removal of most of the parental strand is then followed with another extension reaction using DNA polymerase I to create a mutant/mutant duplex DNA. The mutant/mutant DNA was then transfected into *E. coli* TG 1 and either directly screened for the incorporation of a new restriction site or sequenced by the technique of Sanger et al. (1977).

Overexpression and Purification of Mutant Proteins. Inserts containing gene 32 mutants gp32ser73, gp32ser115, and gp32ser137 were released from pYS 5 by digesting the plasmid with *Eco*R1 and *Bam*H1 to release a 1.0-kb DNA fragment that was then isolated; the termini were filled in to create blunt ends, and the resulting DNA was ligated to *Hpa*1-cleaved pKC30 (Shamoo et al., 1986). The other mutants were subcloned in similar fashion except only the *Eco*R1 site was filled and the fragment ligated into *Hpa*1- and *Bam*H1-cleaved pKC30. Nalidixic acid induction of the constructs and purification of mutant gp32 proteins gp32ala84, gp32val99, gp32leu106, gp32ser115, gp32ser137, and gp32ile186 were performed by the methods described by Shamoo et al. (1986). Because gp32ser73 did not bind to ssDNA cellulose, an alternative protocol was employed that used anion-exchange HPLC with a Pharmacia Mono Q column. Gp32ser73 was loaded into the column containing 10 mM Tris, pH 8.0, containing 50 mM NaCl, 1 mM EDTA, and 0.1 mM β-mercaptoethanol and eluted by using a linear gradient from 0.05 to 1.0 M NaCl. Limited trypsin proteolysis of gp32 and mutants to core gp32*-(A+B) was done by the method of Williams et al. (1981).

Protein Chemistry. Gp32 was quantitated spectrophotometrically by using a molar absorptivity of 3.7×10^4 M^{-1} cm^{-1} at 280 nm or by amino acid analysis on a Beckman 7300. Mutant gp32leu92 was electroblotted from an SDS–polyacrylamide gel onto a poly(vinylidene difluoride) (PVDF) membrane (Millipore) by using the method of Matsudaira (1987). The band of interest was then cut from the membrane and sequenced directly.

Poly[d(AT)] Melting. Wild-type and mutant gp32 induced melting of the alternating poly[d(AT)] copolymer was monitored by the resulting hyperchromicity at 260 nm. Except where indicated, protein and poly[d(AT)] were added to a final concentration of 12 and 84 μM, respectively, where the poly[d(AT)] concentration was in terms of phosphate, to prechilled (10 °C) 0.5-mL cuvettes containing 0.3 mL of 150 mM NaCl, 10 mM Tris, pH 8.0, and 0.1 mM EDTA. The net absorbance at 260 nm versus a protein blank was monitored as a function of temperature by using a Gilford 2600 UV/vis spectrophotometer connected to a Gilford 2527 thermoprogrammer. The poly[d(AT)] melting temperature was determined from the maxima in a first derivative plot of the absorbance data. Calculation of K_c was carried out by using (Crothers, 1971; Giedroc et al., 1987)

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

where ΔH is the heat of formation for double-stranded poly[d(AT)] [$\approx$–8000 cal/mol of base pairs (Crothers, 1971)], B_c is the density of binding sites on double-stranded DNA (0.333), T_m and T_m' are the melting temperature of poly[d(AT)] in the absence and presence of protein, respectively ($\Delta T_m = T_m' - T_m$), R is the gas constant (1.987 cal mol^{-1} deg^{-1}), and a is the "free" concentration of gp32 at T_m, approximately equal to the total gp32 concentration minus half the poly[d(AT)] concentration in terms of binding site assuming that gp32 covers six nucleotides.

Fluorescence Quenching of Gp32 and Mutant Proteins with Poly(dT), $p(dT)_8$, and $p(dT)_{16}$. Fluorescence quenching experiments were performed on an SLM Model 8000 spectrofluorometer connected to an IBM XT computer. Experiments were done at a protein concentration of 1×10^{-6} M^{-1} in 10 mM Tris, pH 8.0, containing 50 mM NaCl and 0.1 mM EDTA. Data were acquired by excitation at 282 nm and monitoring emission at 347 nm. The effects of dilution, photobleaching, and screening caused by addition of polynucleotide were corrected for. The gp32 site size (n) was determined from stoichiometric binding curves obtained from

[1] Gp32 mutant proteins are denoted by either single- or triple-letter abbreviation for the amino acid substitution and the position number within the amino acid sequence; i.e., gp32s73 and gp32ser73 both indicate a single tyrosine to serine change at position 73.

titration of protein with poly(dT). The apparent gp32 affinity for $p(dT)_{16}$ was determined by using

$$K_a = [\text{gp32}\cdot p(dT)_{16}\text{complex}]/[\text{free gp32}]^2 \quad (2)$$

The resulting K_a includes contributions from both direct gp32·nucleic acid interactions as well as cooperative gp32·gp32 interactions between adjacent gp32 molecules bound to $p(dT)_{16}$. The latter interactions are quantitated in terms of the unitless cooperativity parameter ω, which is equal to K_a/K_{int}. Values of K_{int} were determined from $p(dT)_8$ titrations as the slope of the plot of $1/\Delta F$ (change in fluorescence) vs $1/[p(dT)_8]_{free}$ (Kelly et al., 1976). Cooperativity (ω) was then calculated from $K_a = (S_2\omega)^{1/2}K_{int}$, where S_2 is a statistical factor that takes into account the number of possible ways in which a gp32 molecule can bind a finite lattice (Kelly et al., 1976).

Determination of Zinc Content in Gp32 and Mutant Proteins. Protein samples were dialyzed against metal-free 10 mM Tris, pH 8.0, 50 mM NaCl, and 0.1 M EDTA, and zinc content was assayed by using an Instrumentation Laboratories IL 157 atomic absorption spectrometer.

Circular Dichroism of Gp32 and Mutant Proteins. Protein samples (5 μM) were prepared in 10 mM Tris, pH 8.0, containing 50 mM NaCl and 0.1 mM EDTA. Aliquots of 1.2 mL were placed in VWR CD cuvettes with a path length of 0.1 cm. Data were collected on an Aviv circular dichroism spectropolarimeter Model 60DS from 300 to 200 nm at 1-nm intervals and the spectra fitted as a polynomial function.

Differential Scanning Calorimetry. Samples of between 0.7 and 1.2 mg mL^{-1} in 10 mM Tris, pH 8.0, containing 150 mM NaCl and 0.1 mM EDTA were subjected to differential scanning calorimetry in either a DASM-4 or Microcal MC-2 calorimeter at a scan rate of 1 K min^{-1}. Changes in excess specific heat were monitored as a function of temperature and the resulting curves integrated by using planimetry (Keating et al., 1988) to determine the enthalpy change associated with protein denaturation in the presence and absence of poly(dT). Thermodynamic parameters were calculated by using

$$\Delta H_{cal} = \Delta H_d - \Delta C_p^d(T_{1/2} - T) \quad (3)$$

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

$$\Delta S^\circ = \Delta H_d/T_{1/2} - \Delta C_p^d \ln (T_{1/2}/T) \quad (5)$$

$$\Delta G^\circ = \Delta H_{cal} - T\Delta S^\circ \quad (6)$$

where $T_{1/2}$ is the temperature at half-denaturation in degrees kelvin, T is the value of $T_{1/2}$ for gp32 denaturation (328.45 K), ΔH_{vH} is the van't Hoff enthalpy, R is the gas constant, and $C_{ex,1/2}$ is the excess specific heat at $T_{1/2}$. Equations 5 and 6 are an expression of the Gibbs–Helmholtz equation.

Results

Expression and Purification of Gene 32 Mutants in E. coli. Gene 32 mutants were constructed as described under Materials and Methods and introduced into *E. coli* AR120. Nalidixic acid was used to induce the expression of the gene 32 mutants and generally resulted in synthesis of large amounts of altered gp32, accounting for about 10% of the total cellular protein (data not shown).

Mutants gp32ala84 (Tyr 84 to ala), gp32val99, gp32leu106, gp32ser115, gp32ser137, and gp32ile186 were all isolated by using the method of Shamoo et al. (1986) since all six of these proteins bound tightly to ssDNA cellulose (data not shown). Mutant gp32ser73 was isolated by a slightly different protocol (see Materials and Methods) because it did not bind to ssDNA cellulose. Plasmids carrying gene 32 that encoded a mutation for tyrosine 92 to leucine (gp32leu92) did not produce protein of the expected molecular weight but instead produced a few small molecular weight peptides (<16 000 daltons). Direct amino-terminal sequencing of the largest of these peptides isolated from crude cellular extracts run on SDS–polyacrylamide gels indicated that the fragment was the amino terminus of gp32 (data not shown). Reconstruction of the plasmid encoding gp32leu92 and induction at different temperatures yielded identical results, suggesting that substitution of tyrosine 92 has rendered the protein susceptible to the action of host cell proteases, and thus the only mutant protein that could not be isolated was that corresponding to a tyrosine to leucine substitution at position 92.

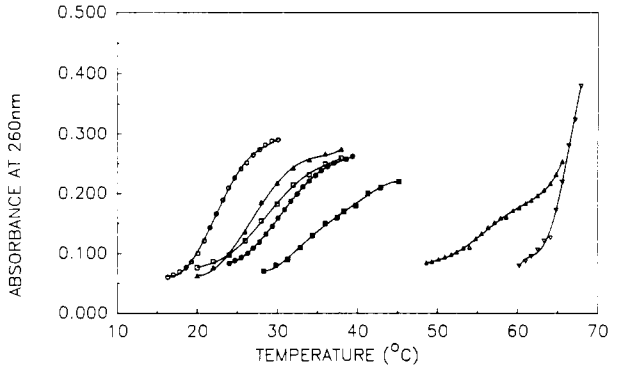

FIGURE 1: Melting of the alternating poly[d(AT)] copolymer by gp32 and mutant proteins. The observed first transitions for each of the mutant proteins with poly[d(AT)] in 10 mM Tris, pH 8.0, containing 150 mM NaCl and 0.1 mM EDTA were plotted as a function of temperature. The net increase in absorbance at 260 nm is plotted after correction for a protein blank. Gp32ser73 and gp32val99 do not appear in the figure because gp32ser73 did not induce a transition and gp32val99 precipitated at low temperature in the presence of poly[d(AT)]. The gp32, gp32ser115, gp32ser137, and gp32ala84 proteins all reached the same final extent of hyperchromicity. In contrast, the poly[d(AT)] in the gp32ile186 and gp32leu106 experiments was not completely melted due to the onset of protein denaturation. (○) gp32; (●) gp32ala84; (△) gp32leu106; (▲) gp32ser115; (□) gp32ser137; (■) gp32ile186; (▽) DNA alone.

Thermal Denaturation of Poly[d(AT)] by Gp32 and Mutant Proteins. Previous studies by Alberts and Frey (1970) have shown that gp32 is able to substantially reduce the melting temperature of the partially double-stranded alternating poly[d(AT] copolymer. The change in melting temperature (T_m) is the result of stabilization of the single-stranded form of poly[d(AT)] by gp32 during the helix (double-stranded) to coil (single-stranded) transition. The ability of gp32 to reduce the T_m of poly[d(AT)] is directly related to its ability to bind preferentially to ssDNA as opposed to dsDNA and can be expressed as a coil association constant (K_c) (see Materials and Methods) (Crothers, 1971).

The curves in Figure 1 compare the thermal transition for poly[d(AT)] with that of wild-type gp32 as well as with those of various mutants. Under the conditions used in Figure 1, wild-type gp32 reduces the T_m of poly[d(AT)] from 65 °C to approximately 20 °C. As shown in Table I, the mutant proteins varied considerably in their ability to act as helix-destabilizing proteins. Whereas the gp32ser115 protein lowered the T_m by 38 °C, the gp32leu106 protein lowered the T_m by only 23 °C. Gp32ser73 did not appear to destabilize poly[d(AT)], since no transition below that normally seen for poly[d(AT)] was observed (data not shown). Mutant gp32val99 was not included in Figure 1 since it precipitated in the presence of poly[d(AT)] between 33 and 37 °C.

Values of K_c derived from the poly[d(AT)] melting experiments by using eq (1) are summarized in Table I. The

Table I: Summary for Poly[d(AT)] Melting Experiments with Gp32 and Mutant Proteins

protein	$t_{m,DNA}$[a] (°C)	$t_{m,gp32}$[b] (°C)	K_c (M^{-1})
gp32	21.8	55.0	7.3×10^7
gp32ser73	ND[c]	ND[c]	
gp32ala84	33.5	54.0	8.8×10^6
gp32leu106[d]	>42.0	53.0	$<7.7 \times 10^5$
gp32ser115	27.0	44.0	2.0×10^7
gp32ser137	29.0	46.0	1.5×10^7
gp32ile186[e]	>36.0	54.0	$<5.8 \times 10^6$
DNA only	65.0		

[a] The temperature at which the first transition (protein-induced helix to coil) occurs. [b] The temperature at which thermal protein denaturation occurs allowing poly[d(AT)] to reanneal. [c] ND = not detected. [d] The protein concentration in the gp32leu106 experiment was increased by 2-fold to 24 μM to lower the poly[d(AT)] melting temperature. By using this approach a greater extent of poly[d(AT)] melting occurred prior to the onset of protein denaturation. Even with this change, it appears from the final extent of hyperchromicity that only about 36% of the poly[d(AT)] was melted in this experiment. The poly[d(AT)] t_m given above is therefore clearly a minimum estimate, and conversely the calculated K_c is a maximum estimate. [e] The final extent of poly[d(AT)] melting with gp32ile186 protein was only about 60% relative to that reached with gp32 due to the onset of protein denaturation. As a result, the t_m given above represents a minimum estimate, and the K_c is a maximum estimate.

mutant gp32 proteins show reductions in K_c from 4- to nearly 100-fold compared to wild-type gp32, indicating that mutations at positions 84, 106, 115, 137, and 186 result in decreased helix destabilizing ability. Since gp32ser73 did not reduce the T_m of poly[d(AT)], it is likely that the mutation at tyrosine 73 has abolished gp32 binding to poly[d(AT)].

Binding of Gp32 and Mutant Proteins to Poly(dT), $p(dT)_8$, and $p(dT)_{16}$. Fluorescence quenching can be used to determine the apparent binding constant (K_a), the intrinsic binding constant for a single site (K_{int}) and cooperativity (ω) (see Materials and Methods). Previous studies by Kelly et al. (Kelly & Von Hippel, 1976; Kelly et al., 1976), Spicer et al. (1979), and Giedroc et al. (1987) have estimated the gp32 site size at 6–7 ± 1 nucleotides/monomer, which is in good agreement with the value of 6.3 ± 1 nucleotides/monomer that we observed for wild-type gp32 binding to poly(dT) (see Table II). Figure 2 shows binding isotherms for each mutant with poly(dT) and $p(dT)_{16}$ and indicates that, with the exception of gp32ser73, the maximum percent quenching and site size of the mutant proteins are within experimental uncertainty of those observed for wild-type gp32 (see Table II). The fact that under conditions where stoichiometric binding of wild-type gp32 to poly(dT) is observed, gp32ser73 fluorescence is not quenched is in agreement with the conclusion from the poly[d(AT)] melting experiments that the mutation at position 73 has abolished ssDNA binding.

To determine whether the decreases in K_a measured for $p(dT)_{16}$ binding were the result of decreases in intrinsic affinity for ssDNA and/or in gp32·gp32 cooperativity, we measured the affinity of the gp32 mutants for $p(dT)_8$ (see Figure 3 and Table II). Since $p(dT)_8$ corresponds to only one site size, the

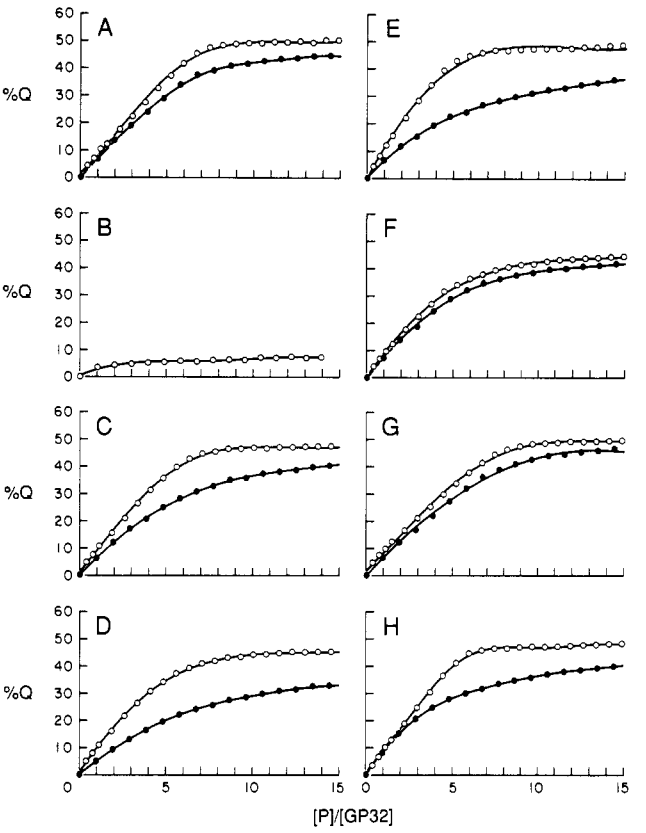

FIGURE 2: Fluorescence titration curves of gp32 and mutant proteins with poly(dT) and $p(dT)_{16}$. The percent quenching (%Q) of the original protein fluorescence is plotted as a function of the ratio of added poly- or oligonucleotide phosphate to gp32. All proteins were at a concentration of 1×10^{-6} M^{-1} in 50 mM NaCl, 10 mM Tris, pH 8.0, and 0.1 mM EDTA. Poly(dT) and $p(dT)_{16}$ titrations are represented as open and solid circles, respectively. (A) gp32; (B) gp32ser73; (C) gp32ala84; (D) gp32val99; (E) gp32leu106; (F) gp32ser115; (G) gp32ser137; (H) gp32ile186.

binding constants measured for gp32 and the mutants to $p(dT)_8$ (Table II) should reflect only the intrinsic affinity of the protein for ssDNA. Overall, the intrinsic association constant obtained for wild-type gp32 [$(3.7 \pm 0.7) \times 10^5$ M^{-1}] is in excellent agreement with the values reported by Kelly et al. (1976) (3.3×10^5 M^{-1}), Spicer et al. (1979) (6×10^5 M^{-1}), and Kowalczykowski et al. (1981) (3.9×10^5 M^{-1}). The relatively small decreases in K_{int} for the mutants that have substitutions at positions 84, 99, 106, 115, and 186 reflect the loss of gp32·ssDNA interactions as opposed to gp32·gp32 cooperative protein interactions.

To evaluate the effect of the tyrosine mutations on cooperative gp32·gp32 interactions, we have calculated cooperativity parameters for each protein by comparing K_a values obtained from $p(dT)_8$ and $p(dT)_{16}$ titrations. The gp32·gp32 cooperativity parameter has been estimated by Von Hippel and co-workers (Von Hippel et al., 1982) to be equal to about 1×10^3, which is in reasonable agreement with our estimate

Table II: Summary for Fluorescence Quenching Experiments with Gp32 and Mutant Proteins

protein	site size	$p(dT)_8$ (K_{int}) (M^{-1})	$p(dT)_{16}$ (K_a) (M^{-1})	ω[a]	$K_{int}\omega$ (M^{-1})
gp32	6.3	3.7×10^5	1.5×10^7	410	1.5×10^8
gp32ala84	6.0	1.6×10^5	7.6×10^6	590	9.5×10^7
gp32val99	5.9	1.2×10^5	4.8×10^6	430	5.2×10^7
gp32leu106	5.0	1.8×10^5	4.2×10^6	130	2.4×10^7
gp32ser115	5.8	2.2×10^5	9.2×10^6	460	1.0×10^8
gp32ser137	6.4	4.1×10^5	1.7×10^7	410	1.7×10^8
gp32ile186	5.8	1.2×10^5	4.4×10^6	340	4.1×10^7

[a] Calculated by using association constants measured for $p(dT)_8$ and $p(dT)_{16}$ as described under Materials and Methods.

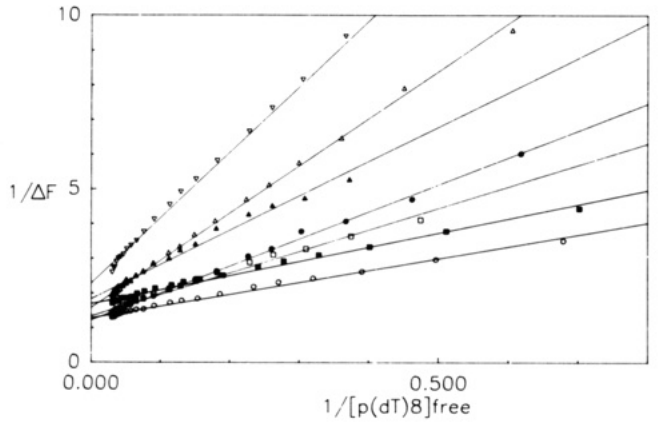

FIGURE 3: Double-reciprocal plot of the extent fluorescence quenching obtained for gp32 and mutant proteins upon the addition of $p(dT)_8$. Protein concentrations were 1×10^{-6} M^{-1} in 50 mM NaCl, 10 mM Tris, pH 8.0, and 0.1 mM EDTA. Lines were fitted by using a least-squares algorithm. (○) gp32; (●) gp32ala84; (△) gp32val99; (▲) gp32leu106; (□) gp32ser115; (■) gp32ser137; (▽) gp32ile186.

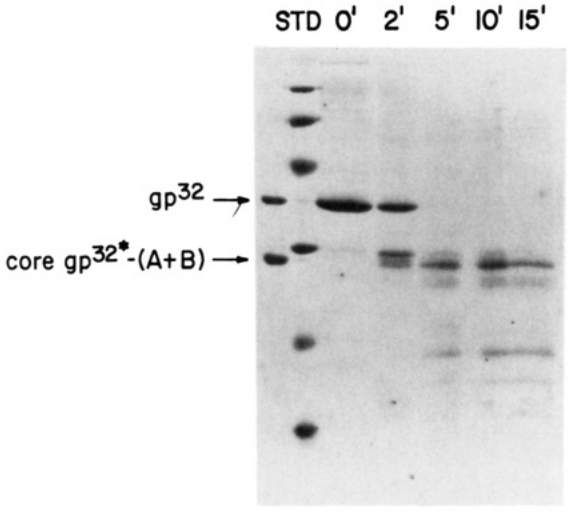

FIGURE 4: Partial trypsin proteolysis of gp32ser73 to core gp32ser73*-(A+B) at 20 °C. Five-microgram aliquots of gp32ser73 were digested with a 1:20 (w/w) ratio (trypsin:gp32) of trypsin in 50 mM ammonium bicarbonate (pH 8.0). A sample was removed before digestion, and thereafter samples were removed at the times indicated. Upon removal, digestion was stopped by boiling the sample for 3 min in sample buffer and storage on ice. Samples were electrophoresed on a 15% SDS–polyacrylamide gel and stained with Coomassie blue. (Lane 1) Molecular weight standards; (lanes 2–6) time course of trypsin proteolysis at 0, 2, 5, 10, and 15 min, respectively.

of approximately 4×10^2 (Table II). Values of ω estimated in this way (Table II) suggest that only the mutation at position 106 resulted in some significant loss in cooperativity.

In general, therefore, the decreases in K_a for the mutations at positions 84, 99, 106, 115, and 186 arise from decreases in the intrinsic affinity of the mutant proteins for ssDNA, except for position 106, where losses in intrinsic affinity are accompanied by a decrease in protein cooperativity.

Partial Trypsin Proteolysis of Gp32 and Mutants to Core Gp32-(A+B).* Partial trypsin proteolysis of gp32 to core gp32*-(A+B) (data not shown) has provided an important approach for probing the domain structure in gene 32 mutant proteins. Limited proteolysis with trypsin removes the N-terminal B (residues 1–21) and C-terminal A domains (residues 254–301) to produce core gp32*-(A+B). Under the conditions used in Figure 4, there is normally no proteolysis beyond the 26000-dalton core gp32*-(A+B). Failure to obtain the stable core gp32*-(A+B) can be interpreted in terms of improper folding. Of the mutants isolated here, only gp32ser73 failed to yield a stable core protein (Figure 4). Despite trying other proteases and lowering the digest temperature to 4 °C, no proteolytically stable gp32ser73 core was obtained.

Table III: Zinc Content Gp32 and Mutant Proteins from Atomic Absorption Studies

protein	mol of Zn^{2+}/ mol of protein	protein	mol of Zn^{2+}/ mol of protein
gp32	0.9	gp32leu106	1.3
gp32ser73	<0.1	gp32ser115	0.9
gp32ala84	1.0	gp32ser137	1.0
gp32val99	0.9	gp32ile186	0.9

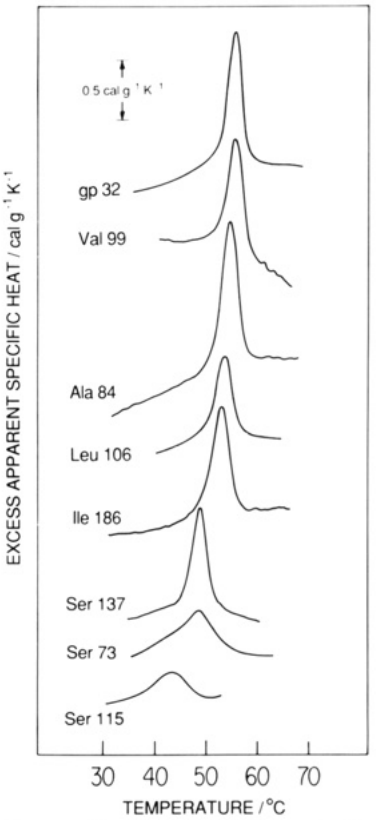

FIGURE 5: DSC scans of the thermal unfolding of gp32 and its mutant forms. Protein concentrations were 1–2 mg mL^{-1} in 10 mM Tris, pH 8.0, containing 150 mM NaCl and 0.1 mM EDTA. All curves are actual tracings illustrating the noise to signal ratios encountered in this work, with the instrumental base line deducted.

Zinc Content in Gp32 and Mutant Proteins. Giedroc et al. (1986, 1987, 1989) and Keating et al. (1988) have suggested that a zinc ion organizes a subdomain important to gp32 cooperativity. The zinc content of each of the mutant proteins was measured by using atomic absorption. Of all the mutants listed in Table III, only gp32ser73 lacked zinc, suggesting that the change of tyrosine 73 to serine has disrupted elements of the zinc binding subdomain.

Differential Scanning Calorimetry of Gp32 and Mutants. Differential scanning calorimetry of gp32 has been used to measure the thermostability and excess specific heat capacity of wild-type gp32 and apo-gp32 (gp32 without zinc) in the presence and absence of ssDNA (Williams et al., 1979; Keating et al., 1988).

Figure 5 and the accompanying results in Table IV confirm the prior studies of Williams et al. (1979) indicating that gp32 undergoes a single transition at 55.3 °C with a denaturational enthalpy of 123 kcal/mol in 10 mM Tris, pH 8.0, containing 150 mM NaCl and 0.1 mM EDTA. Similarly, all mutant proteins appear to denature in a single two-state step but in

Table IV: Summary of Thermodynamic Parameters for the Thermal Unfolding of Gp32 and Its Mutant Proteins[a]

protein	no. of scans	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	$\Delta S°$ (kcal K^{-1} mol^{-1})	$\Delta G°$ (kcal mol^{-1})
gp32	6	55.3 ± 0.2	123 ± 8	−1.3 ± 1.7	2.0 ± 0.1	0.37	0
gp32ser73	1	48.3	48	−6.7	2.4	0.15	−1.1
gp32ala84	3	54.4 ± 0.1	147 ± 5	+1.7 ± 1.0	1.5 ± 0.1	0.45	−0.4
gp32val99	2	55.0 ± 0.3	111 ± 2	−1.7 ± 1.0	2.1 ± 0.1	0.34	−0.1
gp32leu106	3	52.9 ± 0.5	92 ± 15	−3.0 ± 2.4	2.2 ± 0.5	0.28	−0.7
gp32ser115	3	43.3 ± 0.4	45 ± 2	−2.4 ± 1.3	2.3 ± 0.1	0.15	−1.7
gp32ser137	2	48.8 ± 0.1	107 ± 16	−1.7 ± 1.3	2.2 ± 0.3	0.33	−2.2
gp32ile186	3	52.6 ± 0.3	148 ± 5	+3.7 ± 1.7	1.2 ± 0.1	0.46	−1.2
apo-gp32[b]	14	49.3 ± 0.4	84 ± 7	−1.11	2.6 ± 0.2		−1.7 ± 0.1

[a] Values for $t_{1/2}$, ΔH_{cal}, $\Delta H_{vH}/\Delta H_{cal}$, and ΔC_p^d are means with standard errors calculated at 55.3 °C, the $t_{1/2}$ of wild-type gp32. $\Delta S°$ and $\Delta G°$ are values calculated by eq 5 and 6 at 55.3 °C. ΔC_p^d is the permanent change in excess specific heat of the protein. [b] Keating et al. (1988).

most cases with smaller denaturational enthalpies than wild-type gp32. Despite showing irreversible denaturation, the van't Hoff equation is used in analyzing the thermodynamic data, as previous work has shown that proteins that undergo apparently irreversible denaturation still follow equilibrium thermodynamics (Edge et al., 1985). The apparent cooperative unit size (see $\Delta H_{vH}/\Delta H_{cal}$ in Table IV) suggests that gp32 and its mutant forms denature in solution as dimeric forms except gp32ile186, which denatures as a monomer. Gp32ala84, gp32val99, gp32leu106, and gp32ile186 all have small changes in apparent thermostability (1–3 °C) compared to wild type, whereas gp32ser73, gp32ser115, and gp32ser137 have significantly larger decreases (6.5–12 °C). Mutant proteins gp32ser73, gp32ser115, gp32ser137, and gp32ile186 have significant decreases in denaturational free energy (see Table IV) compared to the wild-type protein. The lack of correlation that is evident in Table IV between enthalpy and free energy changes, with respect to both magnitude and sign, has been observed in many other systems and is necessarily accompanied by compensating changes in entropy.

Differential Scanning Calorimetry of Gp32 and Mutant Proteins in the Presence of Poly(dT). Figure 6 shows the effects of poly(dT) addition on gp32 and mutant gp32 thermostability. Addition of poly(dT) to gp32 at a ratio of 1:1 with respect to binding sites resulted in apparent stabilization of gp32 such that the denaturation temperature increased from 55.3 to 59.6 °C as previously observed (Keating et al., 1988). The mutant gp32 proteins included in Table V were all found to be thermally stabilized by the addition of poly(dT), though not as much as wild-type gp32 (see Table V). All denaturations are well approximated by a single cooperative system with the cooperativity increased upon poly(dT) binding [$\Delta H_{vH}/\Delta H_{cal}$ data for protein–poly(dT) complexes not shown in Table V]. In the presence of poly(dT) four of the mutants, gp32ala84, gp32val99, gp32leu106, and gp32ile186, had apparent transition temperatures that were within 5 °C of that of wild-type gp32. A striking feature of the gp32ser115 protein is the large extent to which this mutant is stabilized by the addition of poly(dT) (see Table V). The free energy of stabilization ($\Delta G°_1$) resulting from the addition of poly(dT) to the gp32ser115 mutant is actually twice that for wild-type gp32, that is, 3.4 compared to 1.7 kcal/mol. This result suggests that some of the binding energy is being used to "drive" the gp32ser115 protein into a conformation that more closely approximates that of the wild-type gp32.

Circular Dichroism of Gp32 and Mutants. Circular dichroism spectra were used as an indicator of overall polypeptide backbone conformation. Previous studies by Anderson and Coleman (1975) suggest that gp32 contains approximately 20% α helix and 20% β sheet. Spectra collected on wild-type gp32 and the resulting secondary structure estimates made by using the methods of Greenfeld and Fasman (1969) were in good agreement with the values given by Anderson and Coleman. Comparison of circular dichroism spectra for wild-type and mutant gp32 proteins reveals that of the mutants

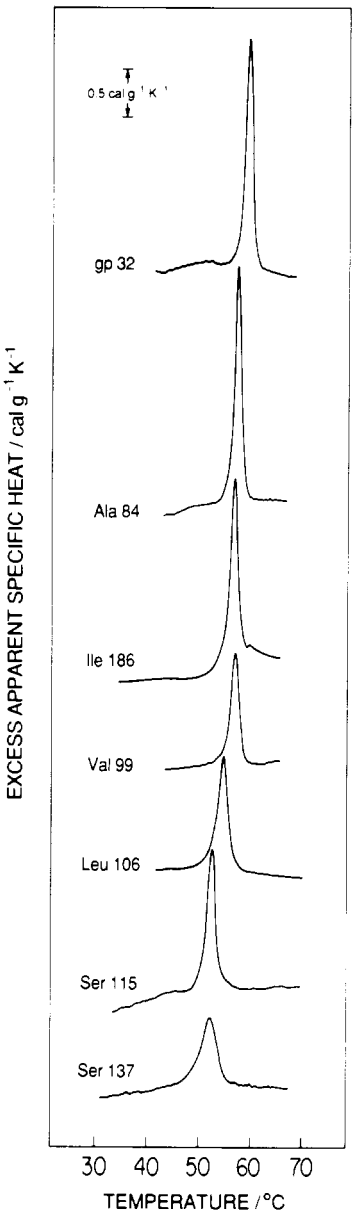

FIGURE 6: DSC scans of the thermal unfolding of gp32 and its mutant proteins in the presence of a 2-fold excess (in terms of binding sites) of poly(dT). By use of K_a values determined from fluorescence quenching, more than 95% of the protein should be complexed to poly(dT). Gp32ser73 was not tested because it had no measureable affinity for poly(dT). Protein concentrations were 1 mg mL^{-1} except gp32ser115, which was 0.75 mg mL^{-1} in 10 mM Tris, pH 8.0, containing 150 mM NaCl and 0.1 mM EDTA.

Table V: Summary of Thermodynamic Parameters Evaluated from the Thermal Unfolding of Gp32 and Its Mutant Proteins in the Presence of Poly(dT)[a]

protein	no. of scans	$t_{1/2}$ (°C)		ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	$\Delta G°_1$ (kcal mol^{-1})	$\Delta G°_2$ (kcal mol^{-1})
gp32	2	59.6	4.3	131	−1.0	1.7	0
gp32ala84	1	57.5	3.2	145	−1.8	1.3	−0.9
gp32val99	1	57.0	2.3	97	−3.2	0.6	−0.8
gp32leu106	1	54.8	2.0	104	−2.5	0.6	−1.5
gp32ser115	1	52.5	9.2	120	−0.2	3.4	−2.6
gp32ser137	1	51.8	3.4	86	−1.5	0.8	−1.9
gp32ile186	1	57.0	4.3	160	+7.4	2.2	−1.2
apo-gp32	3	49.8	0.5	97	−0.5	0.0	

[a] $t_{1/2}$ is the increase in $t_{1/2}$ of the various mutants of gp32 in the presence of poly(dT). $\Delta G°_1$ is the free energy of denaturation of the protein–poly(dT) complex calculated to the $t_{1/2}$ of the protein in the absence of poly(dT) and is a measure of the apparent stabilization caused by the binding to poly(dT). $\Delta G°_2$ is the free energy of denaturation of the protein–poly(dT) complex calculated to the $t_{1/2}$ of the gp32–poly(dT) complex and is a measure of the apparent destabilization caused by the mutation. ΔC_p^d is the permanent change in excess specific heat of the protein.

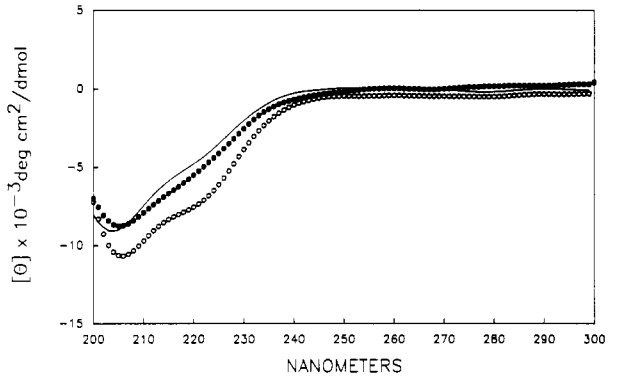

FIGURE 7: Circular dichroism spectra of gp32, gp32ser73, and apo-gp32. Protein samples at 5 × 10^{-6} M^{-1} were prepared in 150 mM NaCl, 10 mM Tris, pH 8.0, and 0.1 mM EDTA. (○) gp32; (●) apo-gp32; (—) gp32ser73.

tested only gp32ser73 has large perturbations in its polypeptide backbone (see Figure 7) that are similar to changes seen upon removal of the zinc ion from wild-type gp32.

Discussion

Tyrosines 73 and 92 Are Essential for Maintaining the Overall Gp32 Native Structure. In evaluating the results of in vitro mutagenesis experiments on proteins, the first question that must always be addressed is what effect the particular amino acid substitution has on the overall conformation of the protein. In this regard our data clearly indicate that substitution of serine for tyrosine 73 and leucine for tyrosine 92 results in profound changes in protein folding. The gp32ser73 protein lacks the intrinsic Zn(II) that is normally present in the wild-type gp32 and is unable to form a protease-resistant gp32*-(A+B) core. On the basis of the results of DSC this mutant has a transition temperature that is 7 °C lower than and an associated enthalpy that is only equal to 40% that seen with native gp32. Similar thermodynamic parameters were obtained with apo-gp32 (Keating et al., 1988). The changes in secondary structure accompanying the serine 73 mutation are sufficiently global that they can be easily detected by circular dichroism. Although the extreme protease sensitivity of the serine 73 mutant protein is similar to that for apo-gp32, circular dichroism spectra suggest that the loss of secondary structure in the serine 73 mutant is more pronounced that in the case of apo-gp32. It would appear therefore that the structural changes induced in the gp32ser73 mutant protein are too severe to be totally accounted for on the basis of loss of the intrinsic zinc ion alone. Although the leucine 92 protein could not be isolated, its hypersensitivity to host cell protease action strongly suggests a defect in folding and once again is reminiscent of the increased protease sensitivity of the apo-

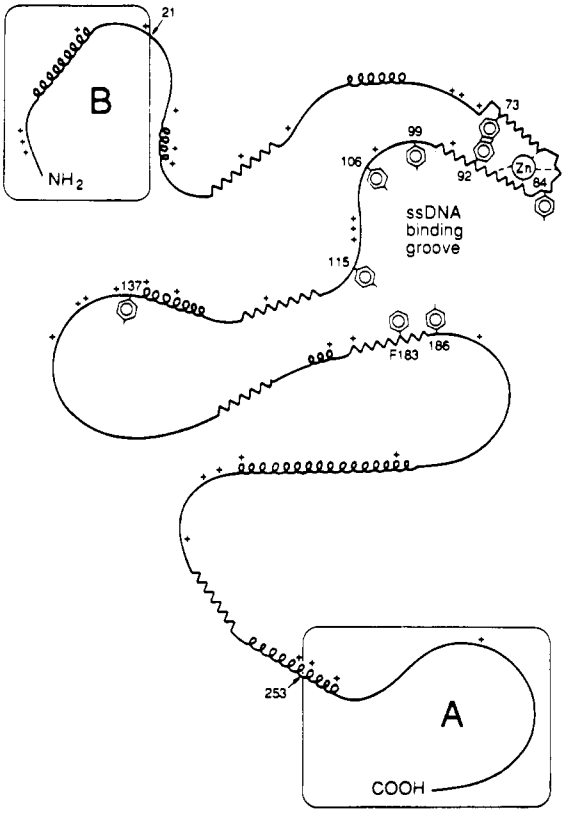

FIGURE 8: Model of gp32 incorporating secondary structure features predicted by Garnier predictions and circular dichroism spectra, as well as the data obtained from mutagenesis of tyrosines in gp32. The tyrosines and phenylalanine 183, which has previously been shown by photo-cross-linking to be at the core gp32*·dT$_8$ interface, are represented schematically by their side chains. Tyrosines 73 and 92 are shown away from the putative ssDNA binding groove and are believed to constitute important structural determinants in the zinc binding subdomain. Tyrosines 84, 99, 106, 115, and 186 as well as phenylalanine 183 are depicted as clustering into a region responsible for binding ssDNA. Tyrosine 137 is shown away from the cluster since the data for that mutation were more ambiguous.

gp32. Taken together with secondary structure predictions based on the amino acid sequence of gp32 (Shamoo et al., 1989) and distance geometry modeling of the Zn(II) binding sphere (Giedroc et al., 1989) it is tempting to speculate that, as depicted in Figure 8, tyrosines 73 and 92 are important for stabilizing a β-sheet region that is in the postulated Zn(II) binding loop formed by residues 76–91. Disruption of the predicted β sheet extending into and out of the Zn(II) coordination sphere might alter the structure of the zinc binding subdomain in such a way as to prevent the incorporation of Zn(II) into gp32, thus resulting in many of the structural alterations that are seen in the serine 73 protein and that we

believe also occur in the leucine 92 mutant protein. Consistent with our results on the gp32ala84 protein, molecular modeling studies suggest that tyrosine 84, which is thought to be in the middle of a short loop that links the halves of the gp32 Zn(II) coordination sphere, is not directly involved in stabilizing the zinc binding subdomain. Rather, tyrosine 84 is pictured as pointing away from the Zn(II) ion and in Figure 8 is actually at the opposite end of the metal binding loop. If tyrosines 73 and 92 are in fact important for stabilizing a β-sheet region that leads into and out of a gp32 Zn(II) binding loop, then they would be somewhat analogous to the two highly conserved phenylalanine resdues that are thought to stabilize a β-stranded region that leads into and out of a short loop that contains two cysteine residues involved in metal chelation in proteins containing "zinc binding fingers" (Berg et al., 1988).

Tyrosine 137 Does Not Appear To Be Directly Involved in the Gp32·ssDNA Interaction or in Stabilizing the Overall Gp32 Structure. Mutation of Tyr-137 to serine results in a protein that behaves like wild type with respect to partial proteolysis, incorporation of zinc, and circular dichroism spectra. Although the gp32ser137 mutant protein is less thermostable than wild-type gp32, its overall structure appears similar to that of native gp32 at 25 °C as judged by the previously mentioned criteria. Likewise, substitution of serine for Tyr-137 does not result in a significant decrease in intrinsic affinity for ssDNA as measured by fluorescence quenching studies with oligonucleotides $p(dT)_8$ and $p(dT)_{16}$. In contrast, poly[d(AT)] melting studies suggest that wild-type gp32 has a 5-fold higher affinity than the gp32ser137 mutant protein for ssDNA. This apparent discrepancy may be due to the inability of a two-binding-site lattice such as $p(dT)_{16}$ to model adequately events occurring on an indefinite ssDNA lattice where each gp32 molecule would be involved in two as opposed to one gp32·gp32 cooperative interaction. The nearly indistinguishable values of the $p(dT)_8$ binding constants for the wild-type and gp32ser137 proteins argues against the direct involvement of tyrosine 137 in the gp32·oligonucleotide interaction. If tyrosine 137 does contribute to the overall gp32 affinity for polynucleotides, it probably does so by enhancing the cooperativity of binding to long ssDNA lattices. Since the K_a calculated from poly[d(AT)] melting data depends on the ratio of the affinities of gp32 for ssDNA compared to dsDNA, it is also possible that the decreased helix-destabilizing activity of the gp32ser137 mutant could be due to a relative *increase* in affinity for dsDNA rather than to a relative *decrease* in affinity for ssDNA.

Five of the Eight Tyrosine Residues in Gp32 Appear To Contribute Directly to ssDNA Binding. In contrast to the three mutants already discussed, mutations at tyrosines 84, 99, 106, 115, and 186 all showed a significant decrease in intrinsic affinity for ssDNA while maintaining "native " gp32 structure at 25 °C. If each of the mutants is considered separately, their individual energetic contributions to ssDNA binding range from 0.3 to 0.7 kcal/mol, which constitutes 3–7% of the overall binding energy of gp32 for $p(dT)_{16}$. The in toto energetic contribution of tyrosines 84, 99, 106, 115, and 186 could therefore account for 26% of the total free energy of binding to $p(dT)_{16}$. Since gp32·gp32 cooperatively is responsible for about 36% of the overall free energy of binding to $p(dT)_{16}$, the contribution of the proposed hydrophobic interactions between tyrosines 84, 99, 106, 115, and 186 and nucleic acid bases might be as much as 50% of the *intrinsic* affinity of gp32 for $p(dT)_{16}$. That the individual contributions of these tyrosine residues to the energy of binding is rather small is in qualitative agreement with previous ^{1}H NMR studies by Prigodich et al. (1986), which show that gp32·ssDNA complex formation gives shifts in the aromatic region of only about 0.06–0.4 ppm, well below those expected if full intercalation between tyrosine side chains and nucleic acid bases occurred (Giessner-Prettre & Pullman, 1971, 1976). Instead, it is more likely that tyrosine residues 84, 99, 106, 115, and 186 may be clustered in a gp32 "ssDNA binding groove" and that they participate in protein·nucleic acid interactions that contribute to ssDNA binding energy and specificity through more distant hydrophobic contacts between the tyrosine side chains and nucleic acid bases. Although ^{1}H NMR studies have not yet been carried out on the remaining gp32 mutant proteins, previous studies on the gp32ser115 protein are in agreement that Tyr-115 is at the interface of the gp32·ssDNA complex and that resonances corresponding to the 2,6 and 3,5 protons of Tyr-115 are shifted upfield during complexation with ssDNA (Prigodich et al., 1986). The previous finding that ^{1}H NMR spectra for the native and gp32ser115 proteins are so similar that the tyrosine 115 resonances are able to be identified also supports our contention that even though the gp32ser115 mutant is less thermostable than wild-type gp32, this amino acid substitution does not significantly perturb the overall "native" structure at 25 °C.

Our in vitro analysis of gp32 mutant proteins differs in at least one respect from the conclusions reached in a recent in vivo study. Gauss et al. (1987) made the interesting observation that the phenotypic effects of several different amino acid substitutions throughout the core gp32*-(A+B) region could be suppressed in a Tab32-4 bacterial host by increasing the Zn(II) concentration in the medium. As a result, it appeared from these in vivo data that as many as 25 different amino acid substitutions all resulted in a decreased gp32 affinity for Zn(II). Gauss et al. (1987) thus concluded that the entire central domain (residues 22–253) interacts directly or indirectly with zinc. In contrast to these in vivo results, we found that at least six of the mutant proteins examined in our study, including one, gp32ala84, that is within the Zn(II) binding domain that spans residues 77–90 (Giedroc et al., 1986, 1989), contain tightly bound Zn(II) that is not removed by extensive dialysis versus EDTA. Our in vitro results suggest that numerous amino acid substitutions can be tolerated throughout the central domain of gp32 without affecting its avidity for Zn(II).

Although elucidation of the exact mechanism of gp32 binding to single-stranded nucleic acids will have to await results from X-ray diffraction analysis of gp32·ssDNA cocrystals, it is important, particularly in regard to in vitro mutagenesis of other ssDNA binding proteins, to note that at least in the case of gp32 no single tyrosine residue appears to contribute more than 8% of the overall binding energy. The real importance of the aromatic amino acid side chain·nucleic acid base interactions may lie more in the specificity they seem to impart with respect to the ability of these proteins to bind single-stranded rather than double-stranded nucleic acids and perhaps in the configuration that they impose onto the ssDNA rather than in their direct contribution to binding affinity per se. It has previously been proposed that gp32 imposes an extended "ladderlike" structure onto the ssDNA such that it is both protected from nucleases and aligned properly for T4 replication, recombination, and repair processes. Hydrodynamic studies by Scheerhagen et al. (1985) and electron microscopy of gp32·ssDNA complexes by Delius et al. (1972) are in general agreement that gp32 extends and imposes a regular structure upon the lattice. Since aromatic amino acids also appear to be involved in the binding of numerous other

single-stranded nucleic acid binding proteins such as bacteriophage fd gene 5 (Anderson et al., 1975) *E. coli* SSB (Merrill et al., 1984), and rat A1 hnRNP (Merrill et al., 1988). This aspect of gp32 binding may turn out to be a general feature shared by many prokaryotic and eukaryotic proteins that interact with single-stranded nucleic acids.

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