Energetics of Arginine-4 Substitution Mutants in the N-Terminal Cooperativity Domain of T4 Gene 32 Protein†

Jana L. Villemain and David P. Giedroc*

Department of Biochemistry and Biophysics, Center for Macromolecular Design, Institute of Biosciences and Technology, Texas A&M University, College Station, Texas 77843-2128

Received February 19, 1993; Revised Manuscript Received July 6, 1993®

ABSTRACT: Gene 32 protein (gp32) from bacteriophage T4 is a sequence-nonspecific single-strand (ss) nucleic acid binding protein which binds highly cooperatively to ss nucleic acids. The N-terminal "B" or basic domain (residues 1–21) is known to be required for highly cooperative binding by gp32 (where $K_{app} = K_{int}\omega$, $\omega \geq 500$), since its removal results in a protein which binds ss nucleic acids noncooperatively ($\omega = 1$). In this paper, we probe the molecular details of cooperative binding by gp32 by physicochemical characterization of a set of four single amino acid substitution mutants of Arg^4: Lys^4 (R4K gp32), Gln^4 (R4Q gp32), Thr^4 (R4T gp32), and Gly^4 (R4G gp32). The qualitative ranking of binding affinities to poly(A) is wild-type ≥ R4K > R4Q > R4T > R4G > gp32-B (gp32 lacking the first 21 amino acids). The occluded site size is $n_{app} = 7.5 \pm 0.5$ for all gp32s. Resolution of K_{int} and ω for wild-type, R4K, R4Q, and R4T gp32s was estimated under conditions of low lattice saturation ($\nu \leq 0.011$) using multiple reverse fluorescence titrations collected at 10 mM Tris-HCl, pH 8.1, 20 °C, and a NaCl concentration where K_{app} was (2–4) × 10^6 M^{-1} for each gp32 on the ribohomopolymer poly(A). Binding parameters for all gp32s were obtained directly or compared by conservative extrapolation of the [NaCl] dependence of K_{app} to 0.20 M NaCl, 20 °C, pH 8.1. The magnitude of ω was then assumed not to vary with [NaCl] (shown for R4T gp32), allowing estimation of K_{int} at 0.20 M NaCl. We find that R4K gp32 binds to poly(A) with an overall affinity (K_{app}) which is 2–3-fold lower than wild-type gp32, while ω for each molecule seems indistinguishable (wild-type gp32, $\omega \approx$ 800–1300; R4K gp32, $\omega \approx$ 600–1200). Surprisingly, R4Q gp32 is characterized by an ω also not readily distinguishable from the wild-type and R4K proteins ($\omega \approx$ 800–4400), while K_{app} is reduced about 10-fold. This mutant also shows a significantly reduced [NaCl] dependence of the binding to poly(A). R4T gp32 binds about 10-fold weaker than the Q mutant. It exhibits an ω ranging from 300 to 700 and a substantially reduced [NaCl] dependence ($\partial \log K_{int}/\partial \log [NaCl] = -1.4$ from 0.10 to 0.20 M NaCl), indicative of significant perturbations in both K_{int} and ω terms. R4G gp32 binds with a K_{app} about 20-fold reduced from R4T gp32, with most of this difference at 0.20 M NaCl apparently residing in the ω term ($\omega \approx$ 5–35 from multiple titrations). Finally, gp32-B appears to bind with a ≈10-fold lower affinity than R4G gp32, consistent with the finding of residual cooperativity in R4G gp32. The overall trend in equilibrium affinities of mutant gp32s for poly(A) parallels their relative helix-destabilizing activities as measured with the partially double-stranded alternating DNA copolymer poly[d(A-T)]. We conclude that the positive charge of Arg^4 is critical for maintenance of cooperative binding and helix-destabilizing activity of gp32 in a manner which is dependent upon the nature of the substitution. Further, these substitutions appear to give rise to a more deleterious effect on the [NaCl] dependence and overall affinity of gp32 for single-stranded nucleic acids than might have been anticipated from the loss of a single side chain in the N-terminal domain.

Bacteriophage T4 gene 32 protein (gp32)[1] is the prototype sequence-nonspecific single-strand (ss) DNA binding protein which plays a structural role in DNA replication, recombination, and repair [for a review, see Karpel (1990)]. It is the paradigm for helix-destabilizing proteins which bind with an "unlimited" type of cooperativity to ssDNA segments transiently formed during these processes (Lohman et al., 1988). Formation of such clusters of gp32 monomers directly derives from the high cooperativity of binding and probably imparts on the ssDNA a particular conformation which both is readily utilized by the DNA polymerase or recombinase and also serves a protective role against the action of intracellular nucleases. In both DNA replication and recombination, it is also clear that apart from interactions with ssDNA, one or more gp32 monomers also engage in functional heterologous contacts with other proteins of the multienzyme complexes which carry out these processes [for reviews, see Young et al. (1992) and Nossal (1992)].

T4 gp32 has a defined primary structure (301 amino acids) (Williams et al., 1981) and unknown tertiary structure. Limited trypsinolysis experiments generally reveal that gene 32 protein contains three functional domains which map to distinct regions of the primary structure (Spicer et al., 1979).

† This work was supported by NIH Grant GM42569, American Cancer Society Junior Faculty Research Award JFRA-270 (to D.P.G.), and a Research Enhancement Grant from the Texas Agricultural Experiment Station. This work is in partial fulfillment of the requirements of the Ph.D. degree at Texas A&M University (to J.L.V.).

* Address correspondence to this author. Telephone: 409-845-4231. FAX: 409-845-9274.

® Abstract published in *Advance ACS Abstracts*, September 15, 1993.

[1] Abbreviations: gp32, T4 gene 32 protein (previously g32P); gp32-B, N-terminal deletion gp32 molecule expressed from a plasmid which encodes gp32 residues 22–301; gp32-(A+B), gene 32 protein residues 22–253 (lacking the A and B domains); ss, single-stranded.

0006-2960/93/0432-11235$04.00/0

Two terminal domains encompassing residues 1–21 (the N-terminal "B" or basic domain) and the C-terminal "A" or acidic domain (residues 254–301) are apparently exposed to solvent and are thought to be structural appendices on the ssDNA binding core domain (residues 22–253) (Williams & Konigsberg, 1978). The core domain contains an intrinsic Zn(II) ion (Giedroc et al., 1986) and appears to contain major determinants for ss nucleic acid binding. Although the details are not yet known, the Zn(II) ion is essential to maintaining the structural integrity of the core fragment (Giedroc et al., 1986, 1987). It now appears well established that the "A" domain plays a functionally important role in heterologous protein–protein interactions which are requisite in the formation of multiprotein complexes which carry out replication (Nossal, 1992; Hurley et al., 1993) and recombination (Jiang et al., 1993).

A simplified view of the three-domain model of gp32 has the "B" domain participating in contiguous monomer–monomer interactions on the ssDNA while the gp32 monomer–ssDNA interface is functionally and physicially separate (Newport et al., 1981). Extensive investigation of the interaction of the core fragment with ssDNA has been used as a model for the protein–nucleic acid interactions attendant in the cooperative protein–ssDNA complex (Prigodich et al., 1986; Pan et al., 1989), although the core fragment itself binds without cooperativity to polynucleotides and natural ssDNAs (Lonberg et al., 1981). On this basis as well as characterization of recombinant gp32-B (gp32 lacking the N-terminal 21 residues), the N-terminal "B" domain has been shown to be required for cooperativity of binding, although the molecular details remain obscure. It is this domain which also mediates gp32 self-association in the absence of nucleic acids (Giedroc et al., 1990). Recent experiments suggest that the C-terminal "A" domain is inhibitory to the noncooperative binding by a "B"-domain deletion of gp32 (gp32-B), particularly at low [NaCl] (Giedroc et al., 1990). This is consistent with the picture that at least some of the additional binding free energy derived from "B"-domain-dependent cooperative interactions is used to facilitate an "A"-domain conformational change such that the structural transitions or roles of the two terminal domains are energetically coupled (Giedroc et al., 1990).

In this paper, we describe experiments which begin to allow us to identify the functionally important amino acids of the N-terminal domain and directly assess the functional interdependence of the interactions which govern molecular cooperativity (embodied in ω) and the intrinsic protein–ssDNA binding events. We accomplish this through resolution of the intrinsic binding constant (K_{int}) and cooperativity parameter (ω) for each of four amino acid substitution mutants of Arg^4 (R4K, R4Q, R4T, and R4G gp32s) which vary widely in overall binding affinity for poly(A). We find that the positive charge at amino acid 4 appears necessary for maintenance of cooperative binding and helix-destabilizing activity of gp32. Further, each single amino acid mutation appears to globally destabilize the complex, since the reduction of ω in each case cannot fully account for the change in overall binding affinity ($K_{app} = K_{int}\omega$) for poly(A). In addition, all mutants, with the possible exception of the R4K gp32, appear to be characterized by a reduced $\partial \log K_{int}/\partial \log$ [NaCl], further evidence for a perturbation in K_{int}. Our data appear to require that the N-terminal "B" domain participates in cooperative binding by controlling the magnitude and energetics of both ω and K_{int} terms, a finding unanticipated from simple models of cooperative binding by gp32.

MATERIALS AND METHODS

Materials

All buffers were prepared with doubly-distilled Milli-Q water. Buffer salts were obtained from Sigma. Poly(A) was obtained form Midland Certified Reagent Co. (Midland, TX) and was fractionated on Sepharose S-200 (Pharmacia); the void peak was collected and dialyzed exhaustively against T/0.10 M NaCl. The concentration of poly(A) nucleotides was determined with duplicate dilutions in the this buffer using ϵ = 10 300 M (nucleotide)$^{-1}$·cm^{-1} at 260 nm (Kowalczykowski et al., 1981b). The fractionated poly(A) was polydisperse and migrated roughly alongside a ≈500 bp duplex DNA fragment on a 1% agarose gel (TBE). Poly[d(A-T)] was obtained from Midland and used following extensive dialysis against T/0.05 M NaCl. The concentration of poly[d(A-T)] nucleotides was determined using ϵ = 6700 M (nucleotide)$^{-1}$·cm^{-1} at 260 nm. Poly(dT) was obtained and used as described (Giedroc et al., 1990).

Methods

Buffers. T buffer is 0.01 M Tris-HCl/0.1 mM Na_3EDTA, pH 8.1 at 20 °C. To make the buffers for binding measurements, a 10× stock solution of T buffer was mixed with the appropriate amount of 3.94 M NaCl and the volume made up with doubly-distilled water. The stock NaCl concentration was determined by refractive index. The conductivity of all buffer solutions was also determined at ambient temperature to ensure their relative consistency of [NaCl]. Other buffers used for protein purification were made as described (Giedroc et al., 1992).

Construction of Overexpressing Plasmids for Mutant Gene 32 Proteins. All standard molecular biological methods were carried out according to Maniatus et al. (1982). The phagemid p32NB.6211 (Giedroc et al., 1992) has a 1.1-kb insert which is flanked by unique 5′ *Nhe*I and 3′ *Bam*HI restriction sites containing the T4 gene 32 coding sequences. This insert also contains a unique *Nde*I site at the +1 Met and a unique translationally silent *Sac*I site at Ala^8-Glu^9 [see Giedroc et al. (1992) for details]. pT7g32.wt (5.5 kb) was constructed by ligation of the 240 bp *Nde*I–*Nco*I fragment from p32NB.6211 into pT7g32-B.wt (Giedroc et al., 1990) similarly digested and purified. pT7g32.R4G was similarly constructed with the analogous 240 bp *Nde*I–*Nco*I fragment from p32NB.6110 (Giedroc et al., 1992). pT7g32-B.wt and pT7g32.R4G were used to express gp32-B and R4G gp32, respectively, as described previously (Giedroc et al., 1990). The unique *Nde*I and *Sac*I restriction sites of pT7g32.wt allowed for insertion of a synthetic double-stranded "cassette" which contains a random mutation at Arg^4. The cassette was synthesized with 25% A, T, C, and G in the first two positions of codon 4 and with 50% C and G in the third position on the top strand. Three inosine residues were present at codon 4 on the bottom strand (Knight & Sauer, 1989). The two strands of the cassette were annealed by heating briefly to 90 °C, followed by slow-cooling for 30 min at room temperature. The double-stranded cassette, containing 5′ *Nde*I and 3′ *Sac*I ends, was ligated with T4 ligase (16 °C, 16-h incubation, 100-fold molar excess cassette over vector) into *Nde*I–*Sac*I doubly-digested pT7g32.R4G. The ligation mixture was transformed into calcium-competent *Escherichia coli* TB1 cells and plated onto LB/ampicillin (100 μg/mL) plates. Isolated colonies were chosen, and crude plasmids obtained from alkaline-lysis minipreps were subjected to double-stranded dideoxy DNA sequencing to determine the mutation

present at codon 4. Four mutant plasmids encoding R4K (designated pT7g32.R4K), R4Q (pT7g32.R4Q), and R4T (pT7g32.R4T) gp32s were chosen for further study. It subsequently proved impossible to transform *E. coli* BL21-(DE3)/pLysS, the host which provides T7 RNA polymerase from the inducible *lac*UV5 promoter, with pT7g32.wt. We attribute this to cell death by constitutively expressed, active gp32 since transformation with pT7g32.R4G, which would express the much less active R4G gp32 (see below), works efficiently and is nonlethal. We therefore switched to the tightly regulated phage λP_L system which has been shown to be very stable and give high levels of expression of intact g32P (Giedroc et al., 1989). We subcloned each 1.3-kb *Xba*I–*Bam*HI fragment obtained from pT7g32.wt, pT7g32.R4K, pT7g32.R4Q, and pT7g32.R4T into pTL9W (Rush et al., 1989) digested with *Nhe*I and *Bam*HI and purified. This gives $pP_L\phi g32$.wt, $pP_L\phi g32$.R4K, $pP_L\phi g32$.R4Q, and $pP_L\phi g32$.R4T (6.7 kb), which when transformed into *E. coli* TB1 provides for gp32 expression by derepression of λP_L *cI*857-mediated repression of the P_L promoter upon shift to nonpermissive (40 °C) temperature. Each $pP_L\phi g32$.nnn plasmid also contains the coding sequences of the *cI*857 repressor expressed constitutively, ensuring tight regulation under permissive temperatures (Rush et al., 1989). Translational control of $pP_L\phi g32$.nnn is provided by the phage T7 gene 10 ribosome binding site carried from the original pET-3b (Studier et al., 1990) vector. Protein expression was achieved by growing *E. coli* TB1 cells containing $pP_L\phi g32$.nnn to an OD_{600} of $\approx$1 at 30 °C before rapidly raising the temperature to 39 °C and disabling the mutant cI repressor. The temperature was kept at 39 °C for approximately 2 h before cells were harvested at 4 °C.

Protein Purification. Wild-type gp32 was prepared from an overproducing strain of *E. coli* TB1 transformed with $pP_L\phi g32$.wt as previously described using the three-column procedure of DE-52, ssDNA–cellulose, and phenyl-Sepharose chromatographies (Giedroc et al., 1992). Gene 32 protein mutants were purified in exactly the same fashion, except that several mutants eluted differently from the ssDNA–cellulose column. In all cases, a [NaCl] step gradient was applied to the ssDNA–cellulose column (typically 2.5 × 30 cm) with steps at 0.05 M (equilibration buffer), 0.15 M, 0.5 M, and 2.0 M NaCl in 10 mM Tris-HCl, 1 mM Na_3EDTA, 1 mM β-mercaptoethanol, and 5% (v/v) glycerol, pH 8.0. The wild-type R4K, R4Q gp32s eluted at the 2.0 M NaCl step, while the R4T and R4G mutants were eluted at the 0.5 M NaCl step. A different batch of ssDNA–cellulose was used to purify each mutant protein. Peak fractions containing gp32 were pooled and further purified on phenyl-Sepharose (Pharmacia), exactly as described previously (Giedroc et al., 1989). Protein preparations were in all cases greater than 95% homogeneous on overloaded SDS–PAGE gels, with the major impurity being a trace of the gp32-(A+B) core fragment, which either copurified or was generated upon storage. The relative amount of this contaminant never exceeded 5% in any protein preparation used for binding measurements. Proteins were stored at –20 or –80 °C without significant degradation.

Limited Proteolysis Experiments. Two hundred microliter samples containing 1.25 μM wild-type or R4G gp32 in the presence or absence of a saturating concentration of poly(dT) (13.5 μM nucleotide) were preincubated at 16 °C in 10 mM Tris-HCl, 50 mM NaCl, 1 mM Na_3EDTA, 1 mM β-mercaptoethanol, and 5% (v/v) glycerol, pH 8. Under comparable solution conditions, all added protein was shown to be bound to the poly(dT) (data not shown). At time t = 0 min, 0.01 mg/mL trypsin TPCK (about 4:1 w/w trypsin:gp32 ratio) was added and the incubation continued. At the indicated times, 15-μL aliquots were withdrawn, mixed with 7.5 μL of 3× ice-cold SDS–PAGE loading buffer, heated to 75 °C for 5 min, and returned to ice until electrophoresis. Electrophoresis was carried out on a 12.5% SDS–PAGE slab gel and stained with Coomassie blue to visualize the products.

Fluorescence Binding Experiments. The fluorescence of gp32 derivatives was monitored with an SLM 8000 spectrofluorometer by excitation at 292 or 296 nm (excitation band-pass = 2 nm) and emission at 347 nm (band-pass = 4 nm). The temperature was maintained with a thermostated sample compartment at 20 ± 0.1 °C. Protein samples, prepared at the indicated concentrations in 2.0 mL of buffer, were continuously stirred gently throughout the course of titration with small (2–5 μL) aliquots of a concentrated stock solution of polynucleotide. The measured fluorescence values (F_i) were converted to corrected fluorescence values ($F_{corr,i}$) after accounting for dilution, inner filter correction (Giedroc et al., 1990), and photobleaching, the latter obtained by simply monitoring the protein fluorescence without additions as a function of time through the course of the companion titration. The extent of photobleaching was always less than 5% under the excitation conditions used. Q_{obs} is given by $Q_{obs} = (F_{corr,max} - F_{corr,i})/F_{corr,max}$. Salt-back titrations were performed exactly as described (Giedroc et al., 1990) with small aliquots (5–25 μL) of a concentrated salt solution (3.94 M NaCl) being added and the fluorescence being measured after equilibrium had been reestablished (evidenced by no time-dependent change in the fluorescence signal). We have determined that the binding density obtained from the degree of quenching, $\nu_Q = L_B/D_T$ (D_T = [poly(A)]$_T$), directly reflects the true binding density, ν, using a general method of analysis outlined in Bujalowski and Lohman (1987). Briefly, $Q_{obs}(L_T/D_T)$ vs log D_T was plotted for each reverse titration and a unique Q_{obs} value extracted for each D_T value. Then Q_{obs} vs L_B/L_T was plotted to determine if the relationship $Q_{obs}/Q_{max} = L_B/L_T$ was linear for each mutant; the value of Q at $L_B/L_T = 1$ is Q_{max}. Using this method, we determined Q_{max} and confirmed that $Q_{obs}/Q_{max} = L_B/L_T$ over the binding density range used to make estimates of thermodynamic parameters. Since this latter relation holds, individual or multiple titrations were subjected to a nonlinear least-squares minimization routine (JANA) created for NONLIN by Dr. M. L. Johnson. This algorithm was used to extract unique values of K_{int} and ω with n fixed at 7.5 from the McGhee–von Hippel closed-form expression for the cooperative large-ligand overlap binding model (McGhee & von Hippel, 1974):

$$Q_{obs}/Q_{max} = L_B/L_T \quad (1)$$

$$\nu_Q = (Q_{obs}/Q_{max})(L_T/D_T) \quad (2)$$

$$L_F = (1 - Q_{obs}/Q_{max})L_T \quad (3)$$

$$\nu_Q/L_F = K_{int}(1 - n\nu_Q)\{[2\omega(1 - n\nu_Q)]/[(2\omega - 1)(1 - n\nu_Q) + \nu_Q + R]\}^{n-1} \times \{[1 - (n + 1)\nu_Q + R]/[2(1 - n\nu_Q)]\}^2 \quad (4)$$

where $R = \{[1 - (n + 1)\nu_Q]^2 + 4\omega\nu_Q(1 - n\nu_Q)\}^{1/2}$. In this analysis, the values of L_B, L_F, and D_T were calculated from the fluorescence titration data using eqs 1–3. These values (where $\nu_Q = L_B/D_T$) were used as input into JANA to solve for L_B numerically at iteratively adjusted K_{int} and ω such that the sum of the residuals between calculated and observed L_B

is minimized. Analysis of individual titrations as well as a simultaneous analysis of multiple titrations obtained under the same solution conditions (e.g., obtained at different L_T) was performed. In general, the simultaneous analysis was less meaningful (sum of the residuals squared was much greater than that from individual titrations) due to the range of parameter values obtained from fits of individual titrations. Therefore, the results of fitting individual titrations are reported. Only those data points where $\nu \leq 0.011$ were used in any analysis; these data points were weighted equally.

Poly[d(A-T)] Melting Experiments. Stock solutions of gp32s in 10 mM Tris-HCl, 50 mM NaCl, and 5% v/v glycerol, pH 8, and poly[d(A-T)] in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.1 M NaCl were diluted into ice-cold T buffer with the appropriate [NaCl] to a volume of 400 μL. These solutions were loaded into cuvettes preequilibrated at 5 °C. The solutions were allowed to equilibrate another 5–10 min after which time a 0.5 °C/min temperature increase was initiated. Melting profiles were determined at a fixed wavelength (260 nm) on a Cary 1 scanning spectrophotometer equipped with a temperature controller. 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)] is ≈–8000 cal/mol of base pairs (Crothers, 1971), T_m' and T_m refer to the melting temperatures of poly[d(A-T)] in the presence and absence of gp32, respectively, $\Delta T_m = T_m' - T_m$, R, the gas constant, is 1.987 cal deg^{-1} mol^{-1}, a is the free gp32 concentration at T_m', and B_c is the density of protein binding sites on the dsDNA. Assuming a site size of 7.5 for the monomer of gp32, $B_c = 1/(7.5/2) = 2/7.5 = 0.267$ (Giedroc et al., 1987), while a = [gp32] – 0.5[poly[d(A-T)] binding sites]. Note that this relation does not take into account the enthalpy of formation of the protein–nucleic acid complex, which may well be different for each gp32 derivative. This relation also assumes that all available nucleotide binding sites are filled by gp32 at 5 °C.

RESULTS

Limited Proteolysis of B-Domain Mutant gp32–Poly(dT) Complexes. The model for gp32 structure suggests that the N-terminal "B" domain is an essentially solvent-exposed appendage on the globular core domain (residues 21–253) of the molecule. It is unknown as yet whether this short domain has a defined conformation. Mutations here are expected to introduce little perturbation of the core domain of the protein. Partial proteolysis of gene 32 protein in the presence and absence of polynucleotide can be used as a sensitive assay for the structural integrity of the core region of the molecule (Williams & Konigsberg, 1978; Giedroc et al., 1986). In the absence of ss nucleic acids, the terminal "A" and "B" domains are quantitatively released from the ssDNA binding core domain, leaving a 25 026-Da trypsin-resistant fragment (Spicer et al., 1979). This protection from proteolysis absolutely requires the intrinsic Zn(II) ion bound to the core (Giedroc et al., 1986). Genetic removal of the entire "B"-domain region (residues 1–21) does not appreciably destabilize the core domain as evidenced by limited proteolysis (Giedroc et al., 1990). Similarly, the R4G substitution has no detectable effect of the structural integrity of the core domain (Figure 1B, lanes 2–6) since, like the wild-type protein (Figure 1A, lanes 2–6), smaller fragments do not appear to be generated upon extensive incubation with trypsin.

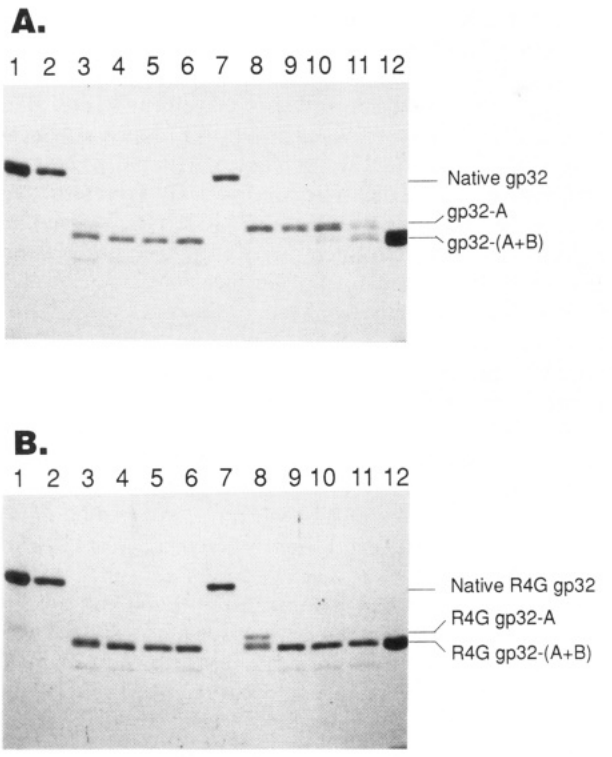

FIGURE 1: Limited proteolysis of (A) 1.25 μM wild-type gp32 and (B) R4G gp32 in the absence (lanes 2–7) and presence (lanes 7–11) of saturating 13.5 μM nucleotide poly(dT). Aliquots were withdrawn at 0 min (lanes 2 and 7), 5 min (lanes 3 and 8), 15 min (lanes 4 and 9), 30 min (lanes 5 and 10), and 60 min (lanes 6 and 11) following trypsin addition. The samples were processed and electrophoresced on a 15% SDS–PAGE gel as described under Materials and Methods. The migration of intact gp32 (lane 1), gp32-A, and gp32-B(A+B) (lane 12) is indicated.

In the presence of saturating poly(dT), Williams and Konigsberg (1978) showed that the N-terminal "B" fragment is protected from proteolytic removal in the cooperative binding conformation. In contrast, the C-terminal "A" domain is removed at a slightly faster rate in this binding conformation. This means that the gp32-A (residues 1–253) will build up appreciably prior to its conversion to the core fragment when bound to polynucleotides. This is what we observe for wild-type gp32 (Figure 1B, lanes 7–11). When gp32 is bound to a single-site-size oligonucleotide of insufficient length to accommodate contiguously bound gp32 monomers, the "B" domain is rapidly proteolyzed. This suggests that this protection from proteolysis requires contiguously bound gp32 monomers in the cooperative binding mode. In contrast to the behavior of the wild-type protein (Figure 1A, lanes 7–11), the saturated poly(dT) complex formed with R4G gp32 gives only little enhanced protection from cleavage of the "B" domain (Figure 1B, lanes 7–11) relative to the free protein (lanes 2–6). This is consistent with formation of a structurally perturbed but saturated complex with R4G gp32. The data below reveal that the R4G substitution results in a dramatic loss of cooperativity of binding of gp32.

Equilibrium Binding of Select Arg^A Mutants of gp32 to the Model RNA Homopolynucleotide Poly(A). The limiting model for the cooperative binding conformation of gp32 predicts that mutations deposited in the N-terminal domain would have a large effect on the cooperativity of binding (ω) with little or no effect on the apparent affinity of the gp32 monomer for an isolated lattice site, K_{int}, where $K_{app} = K_{int}\omega$ (Newport et al., 1981; Casas-Finet et al., 1992). The binding of gp32 to ss nucleic acids shows a steep salt concentration dependence with $\partial \log K_{app}/\partial \log$ [NaCl] = –6 ± 1, relatively

Table I: Equilibrium Binding Parameters Obtained for the Wild-Type and Arg[4] Gene 32 Protein Mutants with Poly(A) at pH 8.1, 20 °C, and the Indicated [NaCl][a]

protein	Q_{max}[b]	[NaCl] (M)	K_{app}[c] ($M^{-1} \times 10^{-6}$)	ω[c]	K_{int} (M^{-1})[c]	ν (range)[d]
wild-type	0.29	0.275	3.7–4.5	800–1300	3400–4500	0.011→0.00077
R4K	0.26	0.25	2.2–3.3	550–1200	1800–4800	0.011→0.00096
R4Q	0.31	0.16	3.7–5.9	850–4400	1400–4400	0.011→0.00071
R4T	0.29	0.05	1.8–3.8	300–750	5100–5600	0.036→1.1×10^{-5}
R4G	0.29	0.05	0.08–0.12	5–35[e]	5400–8000[f]	0.0022→9.6×10^{-5}
apo	0.115	0.25	0.73–1.0	120–670	1100–9200	0.12→0.0087

[a] Determined from a set of reverse fluorescence titrations like those shown in Figure 2 in T buffer, pH 8.1, 20 °C, with the indicated [NaCl]. Input protein concentration varied from 1.5×10^{-7} to 3.5×10^{-7} M for the wild-type, R4K, R4Q, R4T, and R4G gp32s and from 1.5×10^{-7} to 2.5×10^{-6} M for apo-gp32. [b] Determined from the general method of analysis (Bujalowski & Lohman, 1987) and from titrations carried out under tight binding conditions, except for R4T and R4G gp32s which were determined directly by carrying out titrations to nucleic acid excess. The apparent site size, n_{app}, was determined to be 7.5 ● 0.5 for the wild-type, R4K, and R4Q gp32s (see Materials and Methods). $n_{app} = 7.0$ for apo-gp32. [c] Range of parameter values determined from nonlinear least-squares parameter estimation of individual reverse titrations with $n_{app} = 7.5$ (see Figure 2). [d] Data points used in the parameter estimation over all titrations (Figure 2) taken together. [e] Determined from a general method of analysis of four titrations of R4G gp32 (ranging from 3.5×10^{-8} to 3.2×10^{-7} M protein; $Q_{max} = 0.40$) with poly(dT) under the conditions indicated. K_{app} was found to be 32 (20–40) $\times 10^6$ M^{-1}. [f] Determined from five poly(A) titrations (0.1–0.3 μM R4G gp32) and analyzed with fixed $\omega = 10$ (see txt for details).

independent of lattice type (Newport et al., 1981). All or nearly all of this salt dependence resides in the K_{int} term, with ω being essentially independent of [NaCl] (Newport et al., 1981). Resolution of the parameters K_{int} and ω according to McGhee–von Hippel linear lattice theory (McGhee & von Hippel, 1974) for the binding of individual mutant proteins to a model polynucleotide is required to determine how a particular mutation might influence the magnitudes of these parameters. The RNA homopolymer poly(A) was chosen for these experiments since it binds gp32 with a moderate affinity at moderate NaCl concentrations (Newport et al., 1981). In addition, a large body of data exists for the binding of wild-type gp32 to this polynucleotide (Kowalczykowski et al., 1981b; Newport et al., 1981).

The interaction of gp32 with poly(A) was monitored by measuring the decrease in the intrinsic protein (Trp) fluorescence which occurs upon RNA binding. All binding measurements were carried out in the "reverse" mode, in which a fixed input concentration of ligand gp32 (L_T) is titrated with lattice poly(A). At the beginning of the titration, ligand is at excess, and thus binding proceeds from high to low binding density on the poly(A). All measurements unless otherwise noted were carried out at pH 8.1 in 10 mM Tris-HCl/0.1 mM Na_3EDTA (T buffer) with the indicated concentration of NaCl at 20 °C.

Independent determination of the apparent site-size n_{app}, the number of occluded nucleotides (n) per gp32 monomer, was carried out under low-salt conditions (T/0.05 M NaCl). The binding was shown to be stoichiometric (tight) for the wild-type, R4K, and R4Q mutants on poly(A) under these conditions since titrations performed over a 2–3-fold range in [protein] result in an invariant determination of n_{app}. n_{app} was found to be ≈7.5 (±0.5) nucleotides for these three proteins, consistent with literature values of n_{app} which cluster around 7–8 from reverse titrations (Spicer et al., 1979; Karpel, 1990). The binding of R4T and R4G gp32s to poly(A) was not stoichiometric under any conditions tested, and thus it was assumed that $n_{app} = 7.5$. The maximal extent of quenching of the Trp fluorescence (Q_{max}) was also determined for these tight binding proteins from these titrations (Table I). Q_{max} was determined for the R4T and R4G gp32s by measuring the extent of quenching at a large excess of poly(A) and was found to be invariant with changes in L_T. Q_{max} was also determined from a simultaneous analysis of three to five titrations using a general method of analysis (Bujalowski & Lohman, 1987) for the wild-type, R4K, and R4Q mutants and was found to agree very well with the determination of Q_{max} made from stoichiometric reverse titrations (within 5%) (see Materials and Methods). The values of Q_{max} obtained for the wild-type and mutant proteins are summarized in Table I.

Solution conditions were then identified for each protein under which nonstoichiometric binding could be readily detected in reverse fluorescence titrations. Initial experiments, carried out in T buffer/0.25 M NaCl, pH 8.1 and 20 °C, revealed that whereas the binding of the wild-type and R4K proteins could be readily detected and distinguished from one another under these conditions, none of the other proteins bound to an appreciable extent. This suggested qualitatively that the Q, T, and G mutants were significantly perturbed in their binding to poly(A). Since the binding of gp32 to ss nucleic acids is known to have an electrostatic component (Kowalczykowski et al., 1981b; Newport et al., 1981), preliminary salt-induced dissociation experiments were done with the use of "salt-back" titrations (Giedroc et al., 1990) as described under Materials and Methods. Here, the aim was to identify a range of [NaCl] which could be used to monitor complex formation with each mutant protein under comparable total protein and lattice poly(A) concentrations. Initial experiments showed a clear trend in the overall binding affinity ($K_{app} = K_{int}\omega$) of wild-type > R4K > R4Q > R4T > R4G gp32 (data not shown; to be further expanded upon below), as well as identifying favorable solution conditions.

Figure 2 shows representative binding isotherms obtained for wild-type gp32 (Figure 2A) and each Arg[4] mutant protein (panels B–E). In all cases, the total protein concentration was kept below 3.5×10^{-7} M, conditions under which the wild-type protein shows little or no ligand aggregation (Carroll et al., 1975; Giedroc et al., 1990). R4G gp32, which binds weakest to polynucleotides, was shown to be monomeric by gel filtration chromatography up to a concentration of 2 μM (data not shown). It was therefore assumed that R4K, R4Q, and R4T gp32s would exhibit the same or less self-association as the wild-type protein. This suggests that at $L_T \leq 3.5 \times 10^{-7}$ M, all gp32s would be expected to be monomeric. In panels A–D, the [NaCl] was chosen to obtain a binding constant ($K_{app} = K_{int}\omega$) ranging from 2×10^6 to 4×10^6 M^{-1}, facilitating visual comparison of sets of isotherms over the same range (0.1–0.35 μM) of total protein concentration, as well as similar binding density ranges. This creates conditions where $(K_{int}\omega)^{-1} \geq L_T$, the desired situation. Note that for the wild-type, R4K, and R4Q gp32s (panels A–C), the apparent plateau in Q_{obs} for individual isotherms (apparent maximum of L_B) is far lower than Q_{max} and appears dependent upon the total input ligand concentration. This behavior is diagnostic of protein ligands which bind with high unlimited cooperatively to

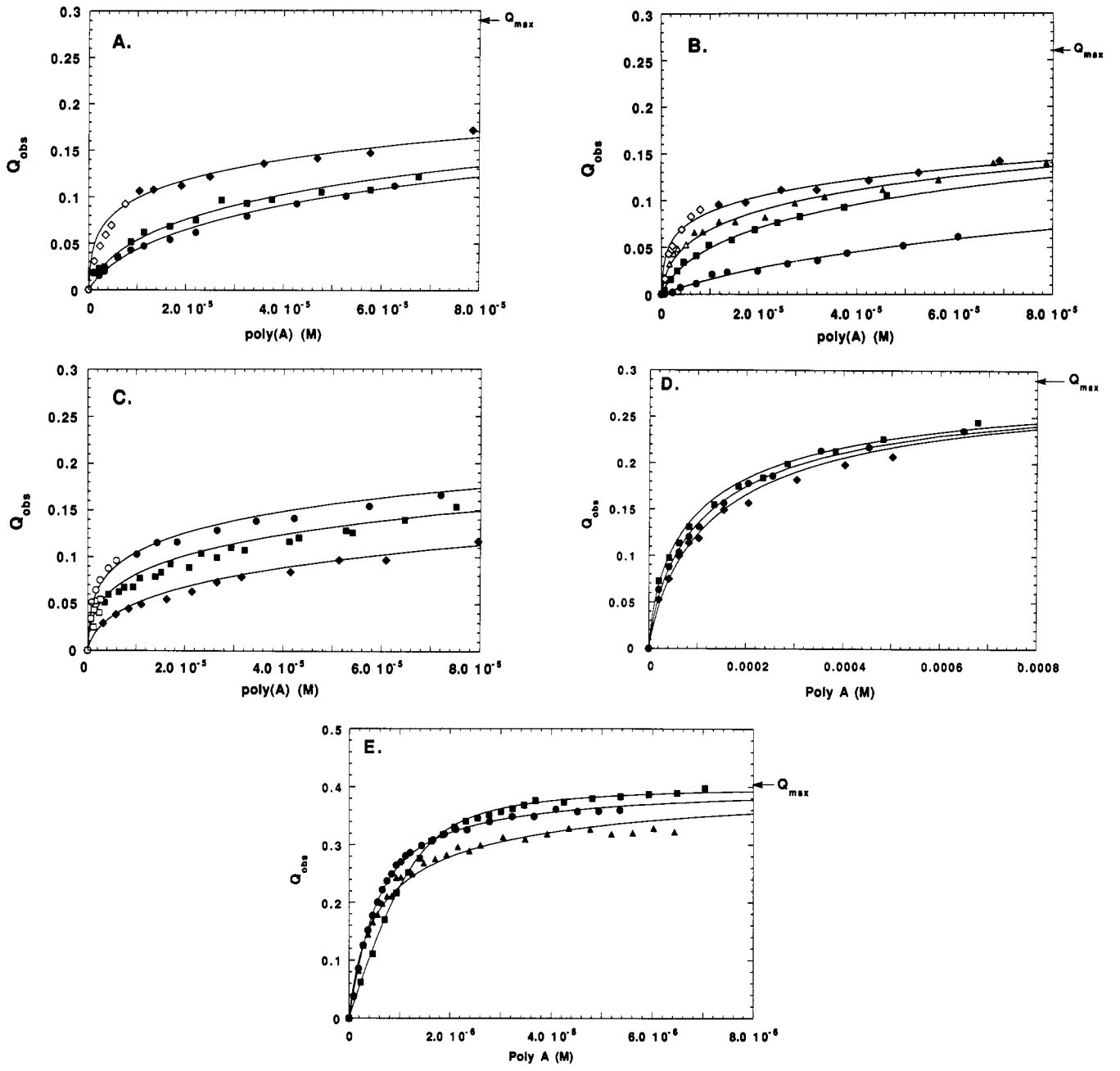

FIGURE 2: Equilibrium binding isotherms obtained for (A) wild-type gp32 on poly(A), T/0.275 M NaCl, 20 °C; (B) R4K gp32 on poly(A), T/0.25 M NaCl, 20 °C; (C) R4Q gp32 on poly(A), T/0.16 M NaCl, 20 °C; (D) R4T gp32 on poly(A), T/0.05 M NaCl, 20 °C; and (E) R4G gp32 on poly(dT), T/0.05 M NaCl, 20 °C. The solid line through each set of experimental data describes theoretical isotherms for the McGhee–von Hippel linear lattice binding model as described by parameters (K_{int} and ω with n_{app} fixed at 7.5) obtained by nonlinear least-squares estimation. The filled symbols represent data points obtained at $\nu \leq 0.011$ used in the estimation of parameters. The ν range analyzed in each titration is indicated. The open symbols ($\nu \geq 0.011$) were not used in the analysis (see text for details). L_t (μM), K_{int} (M^{-1}), ω, and range in ν are respectively given for each data set. (A) (♦) L_t = 0.30 μM, K_{int} = 4500 M^{-1}, ω = 820, ν = 0.0089→0.00077; (■) 0.20, 3900, 960, 0.011→0.0011; (●) 0.15, 3400, 1300, 0.010→0.0017; (B) (♦) L_t = 0.35 μM, K_{int} = 3700 M^{-1}, ω = 900, ν = 0.011→0.0023; (▲) 0.31, 4500, 660, 0.012→0.0021; (■) 0.27, 4800, 550, 0.0064→0.0024; (●) 0.23, 1800, 1200, 0.0085→0.00096; (C) (●) L_t = 0.30 μM, K_{int} = 4100 M^{-1}, ω = 850, ν = 0.010→0.0017; (■) duplicate titrations of different R4Q gp32 preparations at 0.23, 2700, 1600, 0.011→0.0012; (♦) 0.15, 1400, 4100, 0.0043→0.00071; (D) (■) L_t = 0.30 μM, K_{int} = 5600 M^{-1}, ω = 320, ν = 0.0036→0.00070; (●) 0.15, 5400, 500, 0.0016→0.00015; (♦) 0.078, 5100, 750, 0.00069→1.1 × 10^{-5}; (E) all data points were used in the analysis of each titration; (■) L_t = 0.15 μM, K_{int} = 4.7 × 10^6 M^{-1}, ω = 4, ν = 0.10→0.021; (●) 0.075, 1.6 × 10^6, 15, 0.059→0.012; (▲) 0.035, 2.1 × 10^6, 35, 0.035→0.0043.

polynucleotides (Kowalczykowski et al., 1986). This is also the case for R4T gp32 over the same range of [poly(A)] used in panels A–C but becomes visually less apparent at the [poly(A)] used to obtain saturation of the ligand (panel D). Only with R4G gp32 is this not the case. Thus, R4K, R4Q, and R4T gp32s, like the wild-type protein, bind with significant cooperativity. The R4G mutant, on the other hand, appears to bind with less cooperativity.

Resolution of K_{int} and ω for gp32s. From the data shown in Figure 2, attempts were then made to resolve K_{int} from ω for the wild-type protein at 0.275 M NaCl, R4K gp32 at 0.25 M NaCl, R4Q gp32 at 0.16 M NaCl, and R4T gp32 at 0.05 M NaCl. We generally found that the magnitude of K_{app} for each gp32 derivative is well-defined, independent of input L_T, and consistent between different preparations of protein (Table I, data not shown). It is important to note, however, that ω and K_{int} are highly mathematically correlated in the closed-formed expression which describes the McGhee–von Hippel overlap model of linear lattice binding by large ($n > 1$) ligands.[2] This makes their absolute resolution experimentally difficult

since a decrease in the value of ω can often be compensated by a corresponding increase in the value of K_{int}, with little or no effect on the value of K_{app} or the variance of fit. In addition, we consistently observed that for any one gp32 species, nonlinear least-squares resolution of K_{int} and ω from reverse titration data revealed that ω varied inversely with input L_T and thus the binding density range spanned in multiple titrations (Figure 2, Table I). This was found to be the case even though replicate titrations done under identical solution conditions gave very good agreement and overlapping isotherms (Figure 2). Cluster size simulations [cf. Kowalczykowski et al. (1986)] using the parameters obtained for individual gp32s suggest that this apparent anomaly might derive from finite lattice end effects which become nonnegligible even at moderately high binding densities (e.g., at the beginning of a reverse titration) in a highly cooperative equilibria on our poly(A). Under even the most favorable circumstances, only a range of ω and K_{int} can be obtained from multiple titrations, from which qualitative trends in the data should become apparent. We therefore took the following steps in the data analysis to ensure that K_{int} and ω obtained for distinct gp32 species could be qualitatively compared. Multiple titrations for each gp32 derivative spanned overlapping ranges of binding density (ν) or polynucleotide lattice saturation (θ) (indicated in the legend to Figure 2 and Table I) since K_{app} was constrained to be (2–4) $\times$ 10^6 M^{-1}. To minimize any lattice end effects, only those data points where $\nu \leq 0.011$ (or $\theta \leq 0.083$) were considered in an analysis, thus removing points at the beginning of some reverse titrations at higher L_T (see *open* symbols in Figure 2). The range in the values of K_{int} and ω obtained from a nonlinear least-squares parameter optimization of *individual* titrations, some of which are shown in Figure 2 as best-fit theoretical isotherms superimposed on the data, is reported in Table I. As can be seen, these considerations still result in a modest (2–5-fold) range of the value of ω obtained in multiple titrations (Table I), a range, however, comparable to previous reports (Newport et al., 1981).

In order then to compare the magnitudes of K_{int} and ω for individual proteins under one set of solution conditions, the following steps were taken. First, it was necessary to obtain the [NaCl] dependence of K_{app} ($\partial \log K_{app}/\partial \log$ [NaCl]) for each gp32 derivative. For illustration, representative binding isotherms obtained at different [NaCl] are shown for R4T gp32 in Figure 3, with the $\partial \log K_{app}/\partial \log$ [NaCl] (log–log) plots shown in Figure 4. It was then assumed that ω is invariant (or well within the range of ω values) with [NaCl], in agreement with that previously found for the wild-type protein (Kowalczykowski et al., 1981b) and found to be the case for R4T gp32 (Figure 3). The range of ω values shown in Table I was then used to estimate the range of K_{int} at 0.20 M NaCl, given K_{app} obtained by linear extrapolation of the log–log slope *or* direct measurement, from $K_{int} = K_{app}/\omega$. These steps were necessary since, with the exception of R4T gp32, ω could not be estimated directly at 0.20 M NaCl since the binding was either too tight or too weak. These data are tabulated for each gp32 derivative in Table II; 0.20 M NaCl was also chosen since it approximates physiological conditions and minimizes for (wild-type and R4K gp32s) or eliminates (for R4Q and R4T gp32s) the extrapolation of the log–log plots (Figure 4) required to compare the proteins.

[2] An independent and direct determination of K_{int} from single-site-sized oligonucleotides [analogous to reduced-valancy operator experiments in repressor binding studies (cf. Brenowitz et al., 1990)] is not possible since previous findings show that this binding process is thermodynamically distinct from that which describes K_{int} for the gp32 monomer on long polynucleotides (Giedroc et al., 1990; Newport et al., 1981).

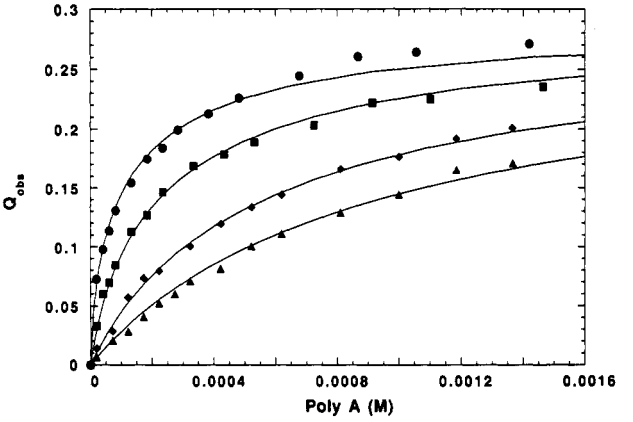

FIGURE 3: [NaCl] dependence of the binding of R4T gp32 to poly(A) in T buffer, 20 °C. Solid curves are theoretical isotherms with ω fixed at 300 and n_{app} = 7.5. The parameters obtained when both K_{int} and ω are permitted to float are indicated parenthetically. $\nu \leq 0.004$ for all data points. (●) 0.05 M NaCl, L_t = 0.32 μM, K_{int} = 5500 M^{-1} (K_{int} = 6100 M^{-1}, ω = 225); (■) 0.10 NaCl, L_t = 0.30 μM, K_{int} = 3100 M^{-1} (K_{int} = 2600 M^{-1}, ω = 500); (♦) 0.15 M NaCl, L_t = 0.30 μM, K_{int} = 1450 M^{-1} (K_{int} = 1470 M^{-1}, ω = 270); (▲) 0.20 M NaCl, L_t = 0.32 μM, K_{int} = 900 M^{-1} (K_{int} = 900 M^{-1}, ω = 300).

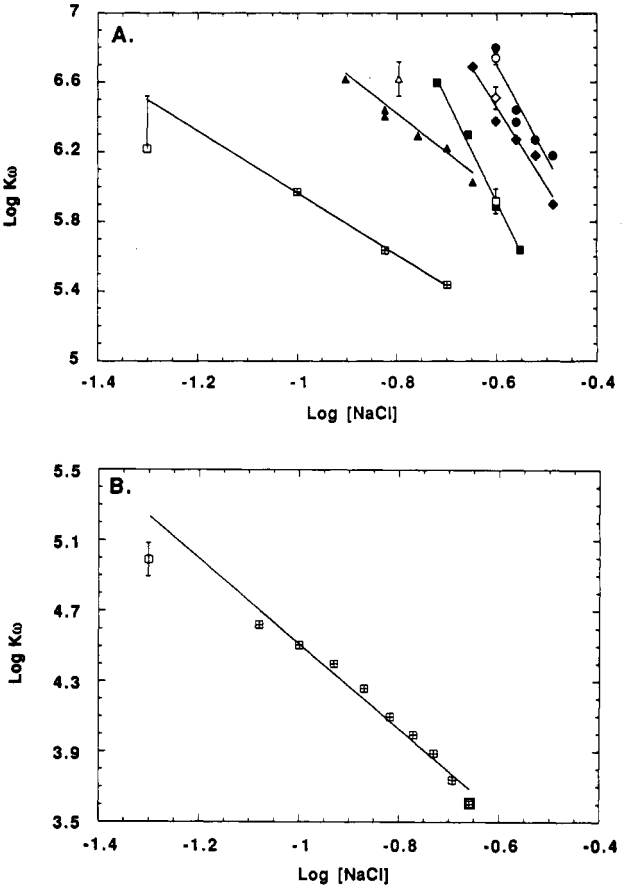

FIGURE 4: [NaCl] dependence of the binding of all gp32 derivatives to poly(A) in T buffer, 20 °C, expressed in a $\partial \log K_{int}\omega/\partial \log$ [NaCl] (log–log) plot. The closed symbols represent nonlinear least-squares estimation of K_{app} from individual reverse titrations while the open symbols derive from a simultaneous analysis of three to six titrations at different L_t, some of which are shown in Figure 2. (A) (●, ○) Wild-type gp32, $\partial \log K_{int}\omega/\partial \log$ [NaCl] (slope) = –5.3 (±1.6); (♦, ◇) R4K gp32, slope = –4.6 (±0.9); (■, □) metal-free apo-gp32, slope = –5.9 (±0.3); (▲, △) R4Q gp32, slope = –2.2 (±0.8); (⊞, □) R4T gp32, slope = –1.5 (±0.2). (B) R4G gp32, slope = –2.4 (±0.3), estimated using the salt-back titration method.

Table II: Comparison of the Binding Parameters Obtained for Wild-Type and Arg[4] Gene 32 Protein Mutants on Poly(A) by Extrapolation to 0.20 M NaCl, pH 8.1, 20 °C[a]

protein	$\partial \log K_{app}/\partial \log$ [NaCl]	[NaCl] range (M)	K_{app} (M^{-1})[b]	K_{int} (M^{-1})[c]
wild-type	−5.3 (±1.6)	0.25–0.325	1.6×10^7	10000–20000
R4K	−4.6 (±0.9)	0.225–0.325	8.3×10^6	7000–12000
R4Q	−2.2 (±0.8)	0.125–0.22	1.6×10^6	400–2000
R4T	−1.8 (±0.2)	0.10–0.20	$1.9\ (\pm 0.7) \times 10^5$	250–700
R4G[d]	−2.5 (±0.2)	0.08–0.22	6.1×10^3	300–1200
gp32-B[e]	−2.2 (±0.2)	0.10–0.20	1×10^3	1000
apo	−5.0 (±0.5)	0.19–0.28	3.2×10^6	5000–25000

[a] Determined from a comprehensive analysis of individual reverse titrations carried out at 6.75×10^{-7} M total [protein] and other data shown in Figure 2 as described in the legends to Figures 3 and 4. [b] Determined from extrapolation of the log–log plots on poly(A) for wild-type and R4K gp32s, while the values for the other gp32s were measured directly (Figures 4A). [c] Obtained by dividing K_{app} by the lower and upper limits of ω shown in Table I to give lower and upper limits of K_{int}. [d] Determined for R4G gp32 on poly(A) using the salt-back fluorescence titration method with ω held constant at 15 (see Materials and Methods; Table I). [e] Poly(U) data taken from Giedroc et al. (1990) using the salt-back technique.

The binding properties of each gp32 mutant are considered below.

R4K gp32. Inspection of Tables I and II reveals largely overlapping ranges in ω for this mutant and the wild-type protein. The range in ω for the wild-type protein encompasses previous determinations which tend to cluster around 1000 (Kowalczykowski et al., 1986). However, R4K gp32 appears to bind with an apparent affinity about 2–3-fold-lower than that of the wild-type protein (Table I), suggesting that at 0.20 M NaCl, K_{int} is perhaps reduced about 2-fold (Table II). The [NaCl] dependence of R4K gp32 binding reveals that it may be only slightly less negative than the wild-type protein (Figure 4, Table II). Substitution of Arg[4] with Lys[4] therefore results in a small but easily measurable reduction in the binding equilibria.

Metal-Free Apo-gp32. Although not the major subject of this report, we wished to compare this gp32 derivative to the "B"-domain mutants since previous data suggested that Zn(II) removal with the organomercurial *p*-mercuriphenylsulfonic acid (Giedroc et al., 1986) gives rise to a protein which binds polynucleotides with a reduced cooperativity (Giedroc et al., 1987; Keating et al., 1987). Table I shows that the apoprotein at T/0.25 M NaCl, 20 °C, binds to poly(A) with an $K_{app} \approx$ 5-fold-reduced relative to the Zn(II)-containing wild-type protein. Table II suggests that at 0.20 M NaCl, 20 °C, this difference in K_{app} largely derives from the reduced magnitude of ω, which is about 3–5-fold lower under these conditions.

R4Q gp32. This mutant binds to poly(A) with the next highest overall affinity with K_{int} and ω resolved at 0.16 M NaCl, conditions which give $K_{app} = 4 \times 10^6$ M^{-1} (Table I). In multiple titrations, the Q mutant was found to bind with a cooperativity comparable to the wild-type protein and the R4K mutant, with a range of 800–4400 obtained for this protein.[3] At 0.20 M NaCl, R4Q gp32 binds about 10-fold weaker than gp32 with a large fraction of this difference falling in the K_{int} term. Thus, this substitution of the Arg[4] side chain for Gln appears to result in a negligible change in the magnitude of ω with most of the perturbation residing in the K_{int} term (Table II). This perturbation also produces a decreased slope ($\partial \log K_{app}/\partial \log$ [NaCl] ≈ -2.2) in the [NaCl] dependence of the binding of R4Q gp32 to poly(A) in log–log plots (over 0.12–0.225 M NaCl) relative to the wild-type and R4K proteins (over 0.225–0.325 M NaCl) (Figure 4, Table II).

R4T gp32. The effect of the substitution of Arg[4] for Thr is easily measured, as binding under experimentally determined tight binding conditions for the other proteins (T/0.05 M NaCl, 20 °C) reveals nonstoichiometric binding by this mutant. Under these conditions, R4T gp32 binds with a K_{app} of (1.8–3.8) $\times 10^6$ M^{-1} to poly(A). Although the binding of the wild-type, R4K, and R4Q mutants is too tight to measure here, K_{app} can conservatively be estimated to exceed 10^8 M^{-1} for the wild-type protein (J. L. Villemain, unpublished simulations). The cooperativity parameter obtained in multiple titrations was found to range from ω = 300 to 750 (Table I), corresponding to only a ≈2–5-fold drop in the magnitude of ω relative to wild-type gp32. This mutant protein shows a significantly reduced [NaCl] dependence ($\partial \log K_{app}/\partial \log$ [NaCl] ≈ -1.8 from 0.05 to 0.20 M NaCl) (Table II), such that at 0.20 M NaCl, the magnitude of K_{app} is reduced to a greater extent, about 80-fold. Individual reverse titrations of R4T gp32 with poly(A) collected at different [NaCl] can be fit with essentially the same ω (ω = 300; Figure 3), so that the reduced [NaCl] dependence of the binding of R4T gp32 relative to the wild-type protein derives from a similarly diminished salt sensitivity of the K_{int} term. Nonlinear least-squares estimation of K_{int} and ω from the four titrations shown in Figure 3 reveals that ω varies from 225 to 550 in a nonsystematic manner relative to the [NaCl], while $\partial \log K_{int}/\partial \log$ [NaCl] ≈ -1.4, comparable to the dependence of K_{app} on [NaCl] (−1.8). This suggests that the interactions which govern the magnitudes of K_{int} and ω in gp32 are *both* perturbed by this substitution.

R4G gp32. The binding of R4G gp32 to poly(A) was shown to be nonstoichiometric in T/0.05 M NaCl, 20 °C, and is significantly weaker than the binding by the R4T mutant. As with R4T gp32, [poly(A)] in excess of 1 mM was required to effect saturation of R4G gp32 (data not shown). Determination of ω was not possible for R4G gp32 on poly(A), since the binding is so weak and the binding density so low that the amount of bound protein depends only on the value of K_{int}. K_{int} is in fact defined quite well in multiple titrations ($K_{int} = 6700 \pm 1300$, Table I), but the goodness of fit of these titrations is insensitive to the value of ω between 1 and 100. To overcome this, estimation of K_{int} and ω was carried out with poly(dT), due to its tighter binding, on the four titrations shown in Figure 2E. This permitted a much larger region of the binding density range to be investigated, and therefore formation of potential cooperative interactions. Potential lattice end effects are much less important in this weakly cooperative equilibria, thus justifying this method of analysis for R4G gp32. For illustration, when R4T gp32 titrations are carried out on poly(A) such that the entire binding density range could be similarly analyzed, we find $\omega \approx 180$. ω was

[3] A determination of K_{int} and ω for wild-type, R4K, R4Q, and R4T gp32s has been made from duplicate forward titrations of poly(A) using circular dichroism (CD) spectroscopy (Khan & Giedroc, 1992) and considering only data points where $\theta \leq 0.20$. For wild-type gp32, $K_{app} = (2.4 \pm 0.5) \times 10^5$ M^{-1}, ω = 500–700 in T/0.40 M NaCl, pH 8.1, 20 °C. For R4K gp32, $K_{app} = (6.2 \pm 0.5) \times 10^5$ M^{-1}, ω = 200–280 in T/0.35 M NaCl, pH 8.1, 20 °C. For R4Q gp32, $K_{app} = (2.7 \pm 0.3) \times 10^5$ M^{-1}, ω = 370–580 in T/0.30 M NaCl, pH 8.1, 20 °C. For R4T gp32, $K_{app} = (5.4 \pm 0.3) \times 10^5$ M^{-1}, ω = 280–350 in T/0.125 M NaCl, pH 8.1, 20 °C. The determination of ω for the wild-type gp32 is consistent with the analysis of a large number of forward CD titrations obtained for the wild-type protein over a wide range of temperature (20–42 °C) and [NaCl] in 10 mM HEPES, pH 7.7 (H. Qiu and D. P. Giedroc, manuscript in preparation). These ω values are just on the low side of the range reported from reverse titrations (Table I), but required the use of data points at higher binding densities to estimate parameters. These findings provide additional evidence that ω values for the wild-type gp32 and "B"-domain mutants are more similar than different.

found to be ≈10–15 from a simultaneous analysis of all R4G titrations on poly(dT), ranging from 5 to 35 from the fitting of individual titrations (Table I). The value of $\omega = 10$ was then used to extract K_{int} from individual titrations of R4G gp32 with poly(A). At 0.20 M NaCl, $K_{app} = 6100\ M^{-1}$, corresponding to approximately 50–100-fold decreases in both ω and K_{int}, with K_{app} reduced by some 2500-fold (Table II). The qualitative finding is that ω for the T and G proteins differs significantly, with little or no difference in K_{int} (Table II). The $\partial \log K_{app}/\partial \log$ [NaCl] of ≈ −2.5 (from 0.08 to 0.22 M NaCl) is approximately half of the value of the wild-type protein and in the range observed for the Q and T mutants over the same [NaCl] (Table II, Figure 4B). Thus, a large partial defect in ω appears accompanied by a similarly large perturbation in K_{int} for this mutant, relative to wild-type gp32.

gp32-B. For comparison, previously published data obtained for the gp32 deletion molecule lacking all 21 amino-terminal amino acids, gp32-B, are also summarized in Table II (Giedroc et al., 1990). gp32-B binds to poly(U) with a $K_{app} = 1000\ M^{-1}$ with $\omega = 1$ at T/0.20 M NaCl, 20 °C (Giedroc et al., 1990), or ≈6-fold lower than R4G gp32. The affinities of gp32 for poly(A) and poly(U) are comparable [they vary by at most a factor of 2 (Newport et al., 1981)], permitting an approximate comparison between these parameters with those obtained on poly(A). These data are consistent with the finding that R4G gp32 does indeed exhibit residual cooperativity (Table II), relative to a mutant which completely lacks the entire N-terminal domain. The overall reduction in K_{app} for gp32-B at 0.20 M NaCl is 16 000-fold, with the [NaCl] dependence of the binding falling in the range exhibited by R4Q, R4T, and R4G mutants, rather than the wild-type protein (Table II).

Helix-Destabilizing Activity of Arg⁴ gp32 Mutants. Single-strand-specific binding proteins can perturb the nucleic acid helix-to-coil transition by virtue of their binding to, and stabilization of, the ss coil form (McGhee, 1976). This "melting" activity is conveniently assayed with the model-alternating DNA copolymer poly[d(A-T)] (Giedroc et al., 1987). Poly[d(A-T)] is partially double-stranded in solution and therefore undergoes a helix-to-coil transition upon thermal denaturation, easily monitored by measuring the polynucleotide absorption as a function of temperature. The melting temperature (T_m) at which this transition occurs is a reflection of the stability of the helix under defined solution conditions. The difference between the T_m obtained in the presence and absence of saturating gp32 is determined by the apparent binding affinity, K_{app}, as well as the enthalpy of formation of the complex (see Materials and Methods). It reflects the approximate ability of each protein to destabilize the helical form of poly[d(A-T)].

In Figure 5A are shown complete melting transitions obtained for the wild-type, R4K, and R4Q gp32s at T/0.15 M NaCl, pH 8.1. Under these conditions, the free copolymer melts with a T_m of 65.1 °C. In the presence of saturating wild-type protein, the T_m is reduced to 13.4 °C, while for R4K gp32 the T_m is slightly higher at 16.0 °C. This translates into binding affinities of 7.3×10^8 and $4.5 \times 10^8\ M^{-1}$ for the wild-type and R4K mutant, respectively (Table III), or a difference of almost 2-fold. This difference in affinity for poly[d(A-T)] agrees well with that determined for poly(A) (Table I). In the temperature scans for the wild-type and R4K gp32s at about 56 °C, there is a negative absorption of the same magnitude, followed finally by a positive absorption transition at or near the transition temperature of the uncomplexed polynucleotide. The negative transition corresponds to thermal unfolding of the protein with the simultaneous renaturation of the copolymer, which then melts at

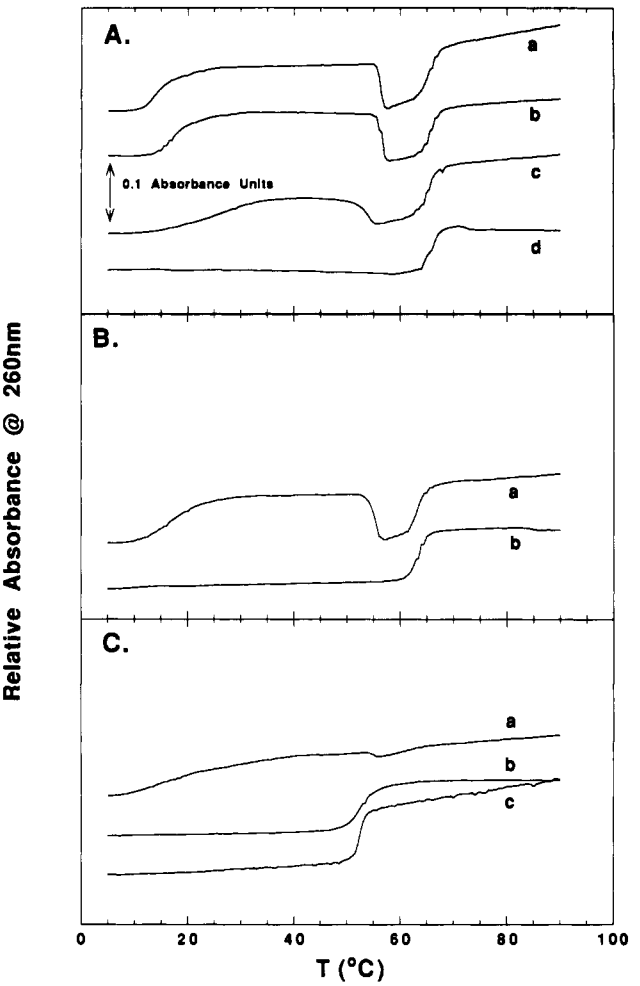

FIGURE 5: Thermal denaturation of poly[d(A-T)] monitored at 260 nm as a function of temperature in the presence of various gp32 derivatives. The concentrations of gp32 (6.0×10^{-6} M) and polynucleotide (2.5×10^{-5} M nucleotide) are the same in all experiments. The temperature ramp rate was 0.5 °C/min. (A) T/0.15 M NaCl, curve a, wild-type gp32; curve b, R4K gp32; curve c, R4Q gp32; curve d, no added gp32; (B) T/0.125 M NaCl, curve a, R4Q gp32; curve b, no added gp32; (C) T/0.025 M NaCl, curve a, R4T gp32; curve b, R4G gp32; curve c, no added gp32.

Table III: Association Constants (M^{-1}) of Wild-Type and Arg⁴ Gene 32 Protein Mutants Estimated from Poly[d(A-T)] T_m Depression Experiments[a]

	K_{app} (M^{-1}) at [NaCl] (M)			
protein	0.150	0.125	0.050	0.025
wild-type	7.3×10^8			
R4K	4.5×10^8			
R4Q	$1.0\ (\pm 0.5) \times 10^8$ [b]	3.0×10^8		
R4T	ND[c]	ND	*d*	$1.2\ (\pm 0.4) \times 10^7$
R4G			ND	ND

[a] Determined in T buffer with the indicated total [NaCl] at a scan rate of 0.5 °C/min with 2.5×10^{-5} M poly[d(A-T)] and 6.0×10^{-6} M gp32 (see Figure 5). K_{app} calculated as described under Materials and Methods. The enthalpy of complex formation was assumed to be relatively similar for all gp32 proteins and in any case small in magnitude compared to the enthalpy of formation of a base pair [≤25% of this value; see Kowalczykowski et al. (1981b)]. [b] Determined from an average of extrapolation of 0.125 M NaCl data assuming $\partial \log K_{app}/\partial \log$ [NaCl] of −2.2 ($1.9 \times 10^8\ M^{-1}$) and estimation from replicate melts performed at 0.15 M NaCl where the transition is about 75% complete prior to onset of protein denaturation ($K_{app} \approx 5 \times 10^7\ M^{-1}$) (see Figure 5A). [c] ND, no T_m depression detected or less than 10% of transition observed. [d] About 40% poly[d(A-T)] melted prior to the onset of protein denaturation.

its characteristic denaturation temperature. This provides direct evidence that the R4K substitution has essentially no effect on the denaturation temperature of the free gp32.

Under these same solution conditions, as was the case in the poly(A) titrations, R4Q gp32 is less effective at destabilizing poly[d(A-T)], with only about 70% of the polymer melted in a broad transition, prior to the onset of protein denaturation. An upper estimate of the T_m under these conditions is 28 °C, giving a $K_{app} \leq 6 \times 10^7$ M^{-1}, or about 12-fold lower than the wild-type protein (Table III). In Figure 5B, the melting activity of R4Q gp32 is given at 0.125 M NaCl, conditions under which the copolymer becomes more destabilized due to lowering the [NaCl]. Here, the T_m = 16.5 °C in the presence of saturating protein with a total T_m depression of 46.4 °C, corresponding to $K_{app} = 3.0 \times 10^8$ M^{-1}. Again, the denaturation temperature of the Q protein is ≈55 °C, similar to that of the wild-type protein.

Both R4T and R4G gp32s are unable to depress the T_m of poly[d(A-T)] appreciably at [NaCl] greater than 0.05 M. As shown in Figure 5C, however, R4T gp32 is able to completely perturb the helix-to-coil transition at 0.025 M NaCl. The T_m is estimated to be 27 ± 2 °C in the presence of saturating R4T gp32, while it is 52.5 °C in its absence, giving a K_{app} of 1.2 (±0.4) × 10^7 M^{-1}. Note that at about 52–55 °C, the protein melts and the polynucleotide denatures approximately simultaneously, obscuring the helix-to-coil transition of uncomplexed poly[d(A-T)] in protein-containing solutions. Under the same conditions, R4G gp32 is essentially inactive in this activity (Figure 5C), requiring that $K_{app} \leq 10^6$ M^{-1} (Table III) for this mutant. The relative efficacy of helix destabilization of poly[d(A-T)] by the Arg^4 gp32 mutants parallels their relative affinity for poly(A).

DISCUSSION

The phage T4 gene 32 protein system represents an ideal system to address and define the molecular details of cooperative binding by this class of single-strand-specific binding proteins. Regions of the molecule previously shown to be important for ssDNA binding by the monomer and monomer–monomer cooperativity appear physically separated along the gp32 primary structure. A large body of evidence reveals that the N-terminal "B" domain (residues 1–21) is required for highly cooperative binding by gp32 on single-stranded nucleic acids (Giedroc et al., 1990; Lonberg et al., 1981; Spicer et al., 1979; Kowalczykowski et al., 1981a; Karpel, 1990), although the mechanistic details are currently unclear. Its removal gives rise to a protein which binds nonspecifically to single-stranded nucleic acids but without cooperativity (Giedroc et al., 1990). Comparative ^1H NMR studies of the noncooperative gp32-(A+B)–oligonucleotide complex and the cooperatively bound gp32–polynucleotide complex suggest that many protein–ssDNA contacts are common to both complexes (Pan et al., 1989). In addition, the "B" domain promotes ssDNA-independent monomer–monomer self-association (Giedroc et al., 1990), but it is not known whether these contacts are similar to or different from those which occur on the ssDNA lattice (Kowalczykowski et al., 1981a).

A complementary but indirect approach to understanding cooperativity in gp32 was recently taken by Casas-Finet et al. (1992). This approach rests on the model that cooperativity in gp32 is accomplished only by direct interactions of the N-terminal domain with another region within the core domain of an adjacently bound gp32 monomer (Williams & Konigsberg, 1978; Kowalczykowski et al., 1981b). These experiments assessed the relative affinity that peptides based on sequences of various overlapping regions of the N-terminal "B" domain have for intact gp32. It was found that peptides containing the positively-charged Lys^3-Arg^4-Lys^5-Ser^6-Thr^7 region possessed measurable affinity for intact gp32, formed a 1:1 complex, and also appeared to inhibit the binding of gp32 to polynucleotides. This led Casas-Finet et al. (1992) to propose that this sequence formed the core of the cooperative binding domain in gp32 and that the as yet unmapped peptide interactive site serves as the primary determinant for "B"-domain–core domain interactions in the cooperative binding conformation. Interestingly, this $(Lys/Arg)_3(Ser/Thr)_2$ or LAST motif is found in many other nucleic acid binding proteins and is also repeated at amino acids 110–114 in the core domain of gp32.

In this report, we present the first direct data which underscores the importance of any one particular amino acid in this domain, namely, Arg^4. Although we do not present an exhaustive substitution survey of this or other residues outside of Arg^4, detailed characterization of four Arg^4 gp32 mutants clearly shows that this residue itself is critical. Point mutations deposited here can reduce the K_{app} and helix-destabilizing activity by over 3 orders of magnitude in a manner which clearly depends on the nature of the substitution (Table I). Second, although resolution of K_{int} and ω is experimentally difficult in this system for the reasons discussed, even a qualitative interpretation of our findings points toward significant perturbations in the magnitudes and energetics of both the K_{int} and ω terms (Table II).[3] This contrasts sharply from simple models of cooperative binding by gp32 where the N-terminal domain would determine only the magnitude of ω. Thus, the mutations deposited here appear to have a global effect on the binding equilibria, and appear inconsistent with the loss of one or two interactions in which Arg^4 must participate during cooperative binding. Binding experiments carried out at extremely low binding densities, in which the degree of binding is determined only by K_{int} (Kowalczykowski et al., 1986) and by electron microscopy studies of mutant gp32–ss nucleic acid complexes, which would provide an independent estimation of cooperativity [cf. Bear et al. (1988)], are currently in progress to provide additional support for the findings reported here.

The hierarchy of binding affinities that we observe for Arg^4 substitution mutants deserves some comment, although without a full range of mutations it is not possible to draw molecular conclusions. Further, since we have not attempted in this paper to address the contributions that entropy and enthalpy changes make to the free energy changes upon formation of the wild-type and mutant protein–poly(A) complexes, any discussion of molecular interactions must be made cautiously. A preliminary investigation of the temperature dependence of the binding of the wild-type and R4T gp32s to poly(A), however, reveals that K_{app} for both proteins varies inversely with temperature over the range of 10–37 °C, and is indicative of an enthalpy-driven [large negative $\Delta H°$ and small negative $\Delta S°$; see also Kowalczykowski et al. (1981b)] reaction at 20 °C in both cases (J. L. Villemain and D. P. Giedroc, unpublished observations). It is clear that Lys^4 is an excellent substitute for Arg^4 but at the same time is easily distinguished from the wild-type protein (Tables I and II). Arg is a better hydrogen bond donor than is Lys, which suggests that while the positive charge of residue 4 appears critical, additional interactions may be important for cooperative binding. The characteristics of the Gln^4 mutant support this proposal, since Gln, although neutral, is an excellent hydrogen bond donor which also maintains a high cooperativity of binding not readily

distinguished from the wild-type and R4K gp32s (Table II). This mutation appears to primarily reduce the magnitude of K_{int}. Thr[4], while preserving some hydrogen bond donating capacity of Gln and Arg, binds significantly weaker (by about 10-fold) to poly(A) than does Gln[4] gp32. Both the intrinsic association constant and the cooperativity parameter appear affected to relatively similar extents relative to the wild-type protein (Table II).

Thr[4] is a significantly better substitution than is Gly[4]. This could either be due to configurational entropy effects and/or be due to a specific loss of the side chain at this position. Finally, residual cooperativity characterizes R4G gp32 relative to the deletion mutant which lacks the first 21 amino acids, gp32-B (Table II), revealing that the other amino acids in this domain contribute in some way to cooperative binding. Although the energetic contributions that these other residues make in the cooperative binding conformation are unknown, they are unlikely to be simply additive. In other words, equally debilitating effects can probably be found for other point mutations deposited elsewhere in this domain. Physical characterization of other "B"-domain mutants is required to test this proposal.

The [NaCl] dependence of the cooperative binding of gp32 to polynucleotides has been shown to reside exclusively in the K_{int} term, and appears to contain major contributions from anion displacement from the protein as well as cation displacement from the polynucleotide upon formation of the complex (Kowalczykowski et al., 1981b; Lohman, 1984). The reduced [NaCl] dependence of the binding of R4T, R4Q, and R4G gp32s, relative to R4K and wild-type gp32s, suggests that a fewer net number of ions are released in the mutant protein complexes. However, since it is not possible to determine the [NaCl] dependence of K_{app} for the wild-type vs Q, T, or G mutants over overlapping ranges of [NaCl], a direct comparison requires that the [NaCl] dependence observed be linear outside of the physically accessible range (Ha et al., 1992). Support for this with wild-type gp32 comes from the constancy of the log–log slopes obtained on different polynucleotide lattices over the range of 0.15–0.8 M NaCl (Newport et al., 1981). With this consideration, most or all of this change in $\partial \log K_{app}/\partial \log$ [NaCl] in R4T gp32 appears to reside in the K_{int} term as ω for R4T gp32 appears largely [NaCl] independent (Figure 3). This does not necessitate a large-scale change in the nature and number of ion pairs which are resident at the gp32 monomer–ssDNA interface. A net change in the nature and/or number of strongly bound anions released from gp32 may lie at the molecular basis for the reduced [NaCl] dependence of the K_{int} term (Figure 3, Table II). Preliminary experiments suggest that this may indeed be the case.[4] It seems unlikely that the Lys[3]-Arg[4]-Lys[5] sequence itself forms a tight anion binding site, since ω shows no appreciable net [NaCl] dependence.

As expected, as the "B"-domain substitution becomes incrementally debilitating (Arg[4] to Gln→Thr→Gly) and altogether removed (in gp32-B), the resultant changes in the energetics begin to resemble more closely a gp32–ssDNA complex which adopts the *non*cooperative rather than the cooperative polynucleotide conformation (e.g., $\partial \log K_{app}/\partial \log$ [NaCl] is significantly reduced; Table II) (Kowalczykowski et al., 1981b; Giedroc et al., 1990). Arg[4] in the "B" domain must participate in some way to drive this conformational change which is linked to formation of cooperative clusters of gp32 at equilibrium. The extreme case would have the "B" domain as required only to create a conformation of bound gp32 which is *competent* to interact with other bound monomers, but where the domain itself does not directly participate in the interaction. This behavior, in fact, appears to describe the R4Q mutant rather well since K_{int} appears affected to a much greater extent than ω (Tables I and II). Indeed, our data do not even rule out simple models in which the N-terminal B domain functions directly in forming contacts with the ssDNA specific to the cooperatively bound conformation. This is potentially consistent with the reduced [NaCl] dependence and significantly perturbed K_{int} which seem to characterize the binding by the Arg[4] mutants. It is the case that the gp32 N-terminal peptides bind weakly to nucleic acids (Casas-Finet et al., 1992) and that other LAST motifs in fact function in DNA binding.

These studies also suggest that the metal-free apoprotein binds with significant cooperativity, but about 5-fold less cooperatively than does the wild-type protein. This is consistent with an overall ≈6-fold decrease in K_{app} (Tables I and II). This is in contrast to initial conclusions drawn largely from oligonucleotide binding experiments that the cooperativity in apo-gp32 was largely eliminated upon metal depletion (Giedroc et al., 1987). The $\partial \log K_{app}/\partial \log$ [NaCl] value obtained for apo-gp32 is either comparable to or slightly greater than that of the wild-type protein (Table II and Figure 4). These findings are consistent with those of Nadler et al. (1990) as well as electron microscopy studies of metal-free apo-gp32–ssDNA complexes. These latter studies showed that apo-gp32 gave rise to an average smaller protein cluster size distribution on M13 ssDNA at equilibrium than the wild-type protein (Keating et al., 1987). Overall, this perturbation in K_{app} is relatively small, about midway in magnitude between the R4K and R4Q mutants. The proposal has been made that the apoprotein defect may lie elsewhere, e.g., in nucleation of cooperative binding of gp32 to a *gene* 32 mRNA structural element in the autoregulatory process (Shamoo et al., 1991). A more general kind of *kinetic* deficiency in helix melting or ssDNA complex lifetime (Giedroc et al., 1987) is also a possibility. However, it is important to point out that mutagenesis of the Zn(II)-coordinating ligand in gp32 (Cys[87]) results in a metal-free protein which shows little affinity for ssDNA and is destabilized to an extent far greater than the apo-gp32 prepared *in vitro* by chemical expulsion of the Zn(II) from the wild-type protein (Giedroc et al., 1992). Why this is the case is currently under investigation. However, in the cell, the Zn(II) complex in gp32 may function primarily as an important folding element in a way which may be unrelated to the observed small defect in the cooperativity of linear lattice binding noted here.

Although it is always conjectured, it is thus far unknown whether or to what extent gp32 must bind cooperatively to ssDNA substrates to assist replication and recombination processes. The cassette mutagenesis approach which we outline here will enable us to generate a large number of single- and multiple-substitution mutants in a way in which their biological activity *in vivo* can be readily established. These data should provide an unbiased and comprehensive survey of the amino acid functional group requirements at each amino acid residue within this short domain (Clarke et al., 1991;

[4] Analysis of the binding equilibria of R4T gp32 as a function of [NaF] (from 0.1 to 0.25 M) reveals $\partial \log K_{app}/\partial \log$ [NaF] = −1.5 (±0.3), similar in magnitude to the [NaCl] dependence (Table II). This suggests that the anion component to the binding equilibria is minimized in this mutant. This is in contrast with the published behavior of the wild-type protein (Kowalczykowski et al., 1981b) as well as our findings where $\partial \log K_{app}/\partial \log$ [NaF] ≈ −3.0 ([NaF] ranging from 0.4 to 0.5 M) for wild-type gp32 (J. L. Villemain and D. P. Giedroc, unpublished observations).

Knight & Sauer, 1989). When coupled with *in vitro* reconstitution of gp32-requiring replication and recombination (Kodadek, 1990) assays, the functional ramifications of a particular kind of thermodynamic defect in cooperative gp32 binding can be readily investigated.

ACKNOWLEDGMENT

We thank Dr. Michael L. Johnson of the University of Virginia for the NONLIN program and Fortran code which we used in the analysis of gp32–poly(A) binding isotherms, Mr. Raza Khan for the collection of the apo-gp32 binding data, and Mr. Huawei Qiu for kindly providing the wild-type gp32 used in these experiments. We also gratefully acknowledge the constructive comments of the two anonymous reviewers of the work presented here.

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