Overexpression, Purification, and Characterization of Recombinant T4 Gene 32 Protein22–301 (g32P-B)*

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Gene 32 protein (g32P), the replication accessory protein from bacteriophage T4, is a zinc metalloprotein which binds with high cooperativity to single-stranded (ss) nucleic acids. The basic N-terminal 21 amino acids (termed the “B” domain) is required for highly cooperative (\(\omega \approx 500\)) binding of g32P monomers to ss nucleic acids. As part of our studies to systematically evaluate the structural features of the B domain important for cooperative binding, a homogeneous source of g32P-B which binds noncooperatively to nucleic acids (\(\omega = 1\)) and is devoid of contamination by native g32P is needed. Herein, we describe large-scale overexpression and purification of recombinant g32P-B lacking the tryptic N-terminal B domain (residues 1–21), designated g32P-B*, as well as its physicochemical and nucleic acid binding properties. G32P-B is readily purified from the soluble fraction of Escherichia coli BL21(DE3) transformed with the plasmid pT7g32-B, which contains the g32P-B coding sequences under inducible transcriptional control of T7 RNA polymerase. Anion exchange, ssDNA-cellulose and phenyl-Sepharose chromatographies give rise to highly homogeneous g32P-B, free of contaminating nucleic acid. Recombinant g32P-B has the expected N-terminal primary structure and contains stoichiometric Zn(II). It also has the expected globular structure as shown by \(^1\)H NMR spectroscopy, hydrodynamic measurements, and the ability to selectively remove the carboxyterminal “A” domain to form the trypsin-resistant g32P-(A + B) DNA-binding core fragment.

Quantitative ss nucleic acid binding experiments of g32P-B to poly(dT) (0.05 M NaCl, pH 8.1, 20 °C) show that all equilibrium binding isotherms can be fit with \(\omega = 1\) and \(K_{obs} = 5.2 \pm 1.6 \times 10^5 \text{M}^{-1}\), with a moderate electrostatic component to the binding free energy, \(\delta \log K_{obs}/\delta \log [\text{NaCl}] = -3.0 \pm 0.2\). Under identical solution conditions, g32P-(A + B) derived from g32P-B binds to poly(dT) noncooperatively as expected, with an \(\approx 80\)-fold higher apparent affinity, \(K_{obs} = 4.0 \pm 2.0 \times 10^7 \text{M}^{-1}\), and detectably enhanced salt sensitivity, \(\delta \log K_{obs}/\delta \log [\text{NaCl}] = -3.9 \pm 0.3\). As the salt concentration is raised, the relative difference in \(K_{obs}\) between the g32P-(A + B) and g32P-B is gradually reduced such that extrapolation of the log-log plots to 1 M NaCl standard state gives similar \(K_{obs}\) within experimental error. Qualitatively similar observations are also found upon binding to the ribohomopolymer, poly(U). Elimination of cooperativity from g32P binding has allowed us to investigate the role of the A domain directly; our data demonstrate that the A domain provides an unfavorable energetic component to the formation of the protein-polynucleotide complex. We discuss these data in the context of a previous model of the “polynucleotide binding conformation” of g32P (Kowalezykowski, S. C., Lonberg, N., Newport, J. W., and von Hippel, P. H. (1981) J. Mol. Biol. 145, 75–104) particularly relating to the structural disposition of the acidic C-terminal A region relative to the rest of the molecule.

Gene 32 protein (g32P), encoded by gene 32 of bacteriophage T4, is a single-stranded (ss) nucleic acid binding protein which binds to regions of ssDNA formed transiently during replication and repair processes (1, 2). At intermediate binding densities and nucleic acid excess, gene 32 protein monomers tend to cluster on both naturally occurring and synthetic homopolymeric ssDNA and RNA lattices, a binding mode thought to be important for preparing the ssDNA in a conformation suitable for the enzymatic machinery in vivo, e.g. DNA replication, as well as afford protection of the single strand against degradation by intracellular nucleases. Such cluster formation at equilibrium is a consequence of the high cooperativity of binding, and is contained within the cooperativity parameter, \(\omega\), reported to range from about 200–2000 (1). The apparent equilibrium association constant \((K_{app})\) of g32P for a polynucleotide lattice is described as \(K_{app} = K_{obs} \omega\), where \(K_{obs}\) is the intrinsic association constant of a g32P monomer for an isolated lattice binding site of \(n\) (where \(n = 7–10\) nucleotides (3)).

G32P (301 amino acids, \(M_s = 33,487\)) is a multidomain protein of known primary (4) and undefined tertiary structure. Three functional domains become apparent from limited tryptic-sinolysis studies (5, 6). The C-terminal “A” domain (residues 254–301) makes heterologous contacts with other proteins in an active replication complex, including the DNA polymerase and accessory proteins associated with both leading strand and lagging strand DNA synthesis (7, 8). Tryptic cleavage of this domain from g32P to form g32P-A also removes a kinetic

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1The abbreviations used are: g32P, gene 32 protein, 301 amino acids; g32P-B, gene 32 protein lacking only the B domain (residues 1–21); g32P-A, gene 32 protein lacking only the A domain (residues 254–301), also referred to as g32P-E; g32P-(A + B), gene 32 protein lacking both the A and B domains, also referred to as g32P DNA-binding core fragment and g32P-III; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; IPTG, isopropyl-\(\beta\)-D-thiogalactopyranoside; STD buffer, 10 mM Tris-HCl, pH 8, 1 mM Na2EDTA, 1 mM \(\beta\)-mercaptoethanol; STDG buffer, STD buffer plus 5% v/v glycerol; TNGa buffer, 10 mM Tris-HCl, pH 8, 0.1 M NaCl, 5% v/v glycerol.

11444
block in the ability of g32P to destabilize natural double-stranded DNAs; equilibrium (thermodynamic) binding parameters remain relatively unchanged (9). The N-terminal basic or "B" domain (residues 1-21) is required for g32P to bind with high cooperativity (10). This conclusion has been reached on studies carried out with g32P lacking both the A and B domains, termed the trypsin-resistant DNA-binding core fragment or g32P-(A + B); g32P-(A + B) has been shown to bind noncooperatively (ω = 1) to poly(dT) (11). The core fragment coordinates an intrinsic Zn(II) ion, which a variety of spectroscopic studies indicate is coordinated by the side chain S- atoms of three Cys (residues 77, 87, and 90) and a fourth non-sulfur liganding donor atom, proposed to be His61 on the basis of recent 'H NMR studies (12-14). The Zn(II) ion provides structural stabilization to the core domain of the molecule, as shown by the reduced thermal stability (15) and greatly enhanced susceptibility of the core domain to proteolysis (12, 16).

The molecular mechanism of cooperative binding by this prototype ss nucleic acid binding protein remains obscure, aside from the fact that the N-terminal 21 amino acids (and probably as few as residues 1-9) (17, 18) are phenomenologically required. Cooperative binding would appear to derive exclusively from protein-protein interactions between contiguous g32P monomers bound to a lattice, since the value of ω is essentially independent of nucleic acid base or sugar composition (19, 20). In addition, ω appears to be minimally, if at all, influenced by the monovalent salt concentration, with the entire salt dependence contained within $K_{obs}$ (19-21). To initiate experiments designed to identify the amino acid side chains important for molecular cooperativity, we require a well characterized g32P species completely lacking this region of the molecule and devoid of even trace amounts of native intact g32P. In this paper, we describe an overexpression and purification strategy which provides a rich source of homogeneous tryptic N-terminally deleted g32P, g32P22-301, or g32P-B, heretofore unavailable. We verify that the recombinant protein has the expected tertiary structure on the basis of recent 'H NMR studies (12-14). The Zn(II) ion introduces a unique NdeI (..CATATG..) restriction site at nucleotides +58-63 which correspond to codons 20 and 21. Upon subcloning the so-generated 1-kilobase pair NdeI/BamHI fragment into vector PET-3b (4.5 kilobase pair), the ATG specifies the new initiation codon of the N-terminally deleted g32P-B gene product. g32P-B is under transcriptional control of the T7 RNA polymerase promoter. B, SDS-polyacrylamide gel electrophoresis analysis of the time course profiles of total cellular protein taken from liquid cultures at the indicated times following induction (0 min) of the following gene products: g32P, E. coli HB101 transformed with the plasmid pT7g32.N20H.K21M, which expresses the double mutant full-length g32P obtained after introduction of the described (A) NdeI site. In this construct, the 1.1-kilobase pair NdeI/BamHI fragment is cloned into the same sites of the expression vector, pET3b (48). Expression is from the phage λ P1 promoter induced by temperature jump from 30 to 40 °C exactly as described previously (13); g32P-B, E. coli BL21(DE3) transformed with pT7g32-B.wt; pET3b. E. coli BL21(DE3) transformed with the parent vector, pET3b. The electrophoretic migration of purified g32P is indicated in the left-most lane.

MATERIALS AND METHODS

RESULTS

Overexpression of Gene 32 Protein22-301 (g32P-B)

The overexpression of the g32P-B expression plasmid, pT7g32-B.wt, diagrammed in Fig. 1A, is described in the Miniprint. The phage λ lysogen, BL21(DE3) contains a single copy of the gene for T7 RNA polymerase stably integrated into the bacterial chromosome and under the inducible transcriptional control of the lacUV5 promoter (22, 23). High level expression of a target gene positioned downstream of a T7 RNA polymerase promoter can be initiated in BL21(DE3) simply by derepressing the lacUV5 promoter. This provides sufficient amounts of T7 RNA polymerase to specifically transcribe its own promoter sequences. Such derepression is conveniently attained by addition of isopropyl-β-D-thiogalactopyranoside to the exponentially growing culture. Fig. 1B, lanes 7-10, shows the profile of cellular proteins from BL21(DE3) transformed with pT7g32-B.wt obtained prior to (0 min) and 30, 60, and 120 min following addition of isopro-
N-terminal Deletion of T4 Gene 32 Protein

Pyl-β-D-thiogalactopyranoside. This is compared with the same cells which harbor the parent vector, PET-3b (23) (lanes 11-14). Also shown for comparison (lanes 2-6) are the induction profiles of Escherichia coli HB101 transformed with a plasmid which inducibly expresses a full-length g32P from the phage λ P2 promoter (13). Note that the cells which contain pT7g32-B.wt specifically express a protein with a relative molecular mass some 2500 daltons less than that of the full-length g32P, consistent with the predicted molecular mass of 30,834 daltons for authentic g32P-B. We note the level of induction can be significantly improved upon this experiment, with some experiments approaching g32P-B levels upwards of 30% of the cellular protein and 50-60% of the soluble protein by weight (cf. “Materials and Methods” and Fig. 2B).

As described in the Miniprint, recombinant g32P-B is highly soluble in cells which express it. Upon lysis of cells by the resident T7 lysozyme after a single freeze-thaw cycle, the protein is readily purified to homogeneity from the low-speed supernatant by a combination of anion exchange (Fig. 2), ssDNA-cellulose (Fig. 3), and hydrophobic interaction (e.g. phenyl-Sepharose) chromatographies. All g32P-B protein preparations following the phenyl-Sepharose step are ≥99% homogeneous as shown by gel electrophoresis of large quantities of protein (~25 μg) and are free of detectable single-stranded DNA endonucleases (see “Materials and Methods”). The only visible contaminant in g32P-B preparations, if present at all, comigrates with the g32P-(A + B) core fragment, likely obtained by in situ proteolysis of g32P-B during purification (see below). Automated N-terminal sequencing of g32P-B reveals NH2-Gly-Phe-Ser-Ser-Glu-Asp-Lys-Gly-Trp3, the expected primary structure (4) provided the initiator Met from the cloning vector is cleaved upon/after biosynthesis. All g32P-B preparations contain stoichiometric Zn(II) by atomic absorption analysis.

Typical yields of highly purified g32P-B range from 4 to 10 mg/g induced cells. The purification is not especially noteworthy (see “Miniprint”), as we and others utilize the precise sequence of chromatographic steps to purify intact g32P (24). The ssDNA-cellulose column step, however, requires some comment (see below).

Recombinant g32P-B Possesses a Native Globular Structure

Limited Proteolysis—The N terminus of any given protein may potentially influence the folding pathway from the nascent polypeptide chain in vivo, especially if the reaction involves the concerted folding of distinct structural domains in a multidomain protein. Although nothing is known of the folding pathway for g32P, we do know that the native structure contains distinct structural domains, the B domain being one. Considerable effort was therefore expended to substantiate that the recombinant N-terminally deleted g32P-B possesses a wild-type structure, both with regard to specific features of the DNA-binding core and the A domain, as well as the physical relationship of these two domains to one another.

A simple, albeit low resolution, indication of the expected tertiary globular structure in g32P-B takes advantage of the resistance to proteolysis of the core domain of the molecule (5, 6). Fig. 5 shows that upon treatment of the recombinant g32P-B with trypsin under conditions known to result in quantitative conversion of the wild-type g32P to the g32P-(A + B) fragment (cf. Ref. 12), the g32P-B is also converted to the g32P-(A + B), with no other proteolytic fragments of lower molecular weight apparent even after 90 min of trypsin incubation.

1H NMR Spectroscopy—Further evidence for a native structure is provided by 1H NMR spectroscopy of g32P-B. The 1H NMR spectrum of wild-type g32P is dominated by resonances of the C-terminal A domain (25, 26). This is due to the fact that g32P aggregates extensively which severely broadens most of the resonances; the A domain on the other hand experiences faster average motion than that described by the aggregate, resulting in an NMR spectrum comprised of some rather sharp resonances superimposed on a broad featureless spectrum. In contrast to this situation with the intact protein, the g32P-B derivative should show reduced if not eliminated aggregation (see below) which should enhance the overall resolution of the 1H NMR spectrum.

The 400 MHz 1H NMR spectrum of g32P-B (Fig. 6A) is compared with that of g32P-(A + B) (Fig. 6B). Even with g32P-B, there exists a subset of narrow resonance lines superimposed on less well resolved resonances. This is clearly seen, for example, in the aromatic region at 7.2-7.3 ppm where a set of sharp overlapping resonances assignable to protons of the three A domain Phe ring systems, and therefore specific to g32P-B, are found. Similar groups of A domain resonances, e.g. at ∼4.45, ∼3.85, ∼2.90, ∼2.60, and ∼0.85 ppm, can easily be seen throughout the aliphatic region as well. This spectrum provides compelling evidence that in g32P-B, the A domain is conformationally more mobile than the rest of the molecule, giving rise to a relatively small set of narrow lines, whose chemical shifts exactly correspond to those found with the A domain of the intact protein (26), albeit better resolved here. In addition, careful inspection of the remaining spectral features associated with the core domain in both g32P-B and g32P-(A + B) spectra indicate common single resonance or groups of overlapping resonances on the chemical shift axis. For example, the single peak at 5.52 ppm, which likely corresponds to the 3.5 protons of Tyr6 in Pan et al. (26), is clearly present in both spectra. However, this and all other common core domain resonances generally appear broader in the g32P-B sample (cf. the aromatic envelope, from 6 to 8 ppm), consistent with its 23% greater mass, but also perhaps reflecting a greater overall globular asymmetry, and thus larger effective reorientaiton time, as revealed by hydrodynamic measurements (see below). In any case,
FIG. 6. 400-MHz ¹H NMR spectra of g32P-B (A) and g32P-(A + B) (B) core fragment. Both spectra were recorded at a protein concentration of 0.4 mM in 50 mM NaPi, pH 8, 30 mM NaCl at 22 °C. The sharp peak at ~4.8 ppm is an artifact from pre-irradiation of the residual HOD peak in the D₂O-exchanged samples. The other extremely sharp resonances at 3.5-3.7 ppm are residual buffer resonances not completely eliminated during the exchange procedure (cf. "Materials and Methods").

these spectra implicate a globular structure for the recombinant g32P-B totally consistent with previous studies of both the tryptic core fragment and intact proteins (25-27).

**Analytical Gel Filtration of g32P-B**—It has been reported previously that both g32P-B' (obtained by limited proteolysis by Staphylococcal protease following amino acid Glu' in the R domain)³ and g32P-(A + B) obtained by limited proteolysis of g32P migrate on gel filtration columns (e.g. Sephadex G-100) as monomers at low salt and near neutral pH (17). However, there are no reports on the effects of protein concentration or salt concentration, data essential to correctly interpret the equilibrium binding studies described below. To quantitatively assess the solution aggregation properties of the recombinant g32P-B protein, we performed analytical HPLC gel filtration studies with a Waters Protein Pak 300sw column. In Fig. 7, we plot the observed elution coefficients (Kₐw) of g32P, g32P-B, and g32P-(A + B) obtained at ambient temperature in 10 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM NaCl, pH 7.2 as a function of protein concentration as described under "Materials and Methods." In the protein concentration range extending from 2 × 10⁻⁶ to 5 × 10⁻⁴ M, we document that g32P-B and the core g32P-(A + B) fragment give the same Kₐw, indicating a constant molecular species. In contrast, as already demonstrated by previous sedimentation experiments (28), the intact protein begins detectable apparent aggregation at ≥0.2 μM.

Knowing that at protein concentrations ≤10⁻⁷ M, the intact protein is a monomer (28), the observed Kₐw value (0.420 ± 0.004) must represent a limiting value, i.e. that of a monomer. Since the monomer molecular weight is known precisely (4), one can estimate the apparent Stokes radius a, and frictional coefficient f/f₀ for the monomeric g32P, by assuming an average partial specific volume (v = 0.73 cm³/g) as outlined under "Materials and Methods." Since the g32P-B protein also exhibits a Kₐw similar to the intact protein at very dilute concentrations, we assume that this value also incorporates the hydrodynamic properties of monomeric g32P-B. We adopt 0.05 mM NaCl was not sufficient to eliminate absorption of proteins to the column, whereas extensive exposure of the column to buffers of pH ≥ 8 proved deleterious to column performance. Thus, a pH value closer to neutrality and a [NaCl] of 0.1 M was employed with the Tris-HCl and Na₂EDTA concentrations the same in both experiments. Identical gel filtration behavior of g32P-B and g32P-(A + B) was observed at 0.2 and 0.4 M NaCl, revealing no change in aggregation state in either case as a function of [NaCl].

³ Conditions can be found which utilize staphylococcal protease to preferentially cleave after Glu' to give the previously described g32P-B'. (17, 18). Even so, this reaction readily proceeds to the limit digest core fragment.

⁴ It proved impossible to perform HPLC gel filtration under precisely the same solution conditions as the fluorescence measurements.
 Porcine retrovirus experiments, both g32P-B and g32P-(A + B) are clearly monomers. Finally, at very dilute concentrations, the g32P-B, although smaller than the intact protein by some 2500 daltons, behaves consistently as a comparably slightly larger (more asymmetric) particle than the intact protein. Identical trends are observed at 0.2 and 0.4 M NaCl (data not shown). Although interesting, further experiments are required to interpret such behavior in molecular terms.

G32P-B Possesses a Significantly Reduced Equilibrium Binding Affinity for ssDNA Than Does g32P-(A + B)

This became apparent during the ssDNA-cellulose chromatography step early in our attempts to purify large quantities of g32P-B. One would have expected, based on published methods to purify the noncooperatively binding ssDNA-binding core fragment g32P-(A + B) (13), that g32P-B would bind tightly to the column and be quantitatively eluted with a 0.5 M NaCl elution step (cf. Fig. 3A); intact wild-type g32P is eluted only at much higher NaCl concentrations (1–2 M [NaCl]). However, we noticed that under conditions where similar loads of g32P-(A + B) core fragment are quantitatively bound by the column at low salt and eluted specifically and predictably upon addition of 0.5 M NaCl to the elution buffer, g32P-B is recovered in all regions of the elution profile, including the flow-through, low-salt washes and the 0.5 M NaCl elution step (data not shown). The 0.5 M NaCl fraction appears as a recognizable shoulder on a very broad g32P-B peak. By UV spectroscopy, the weakly bound g32P-B fraction does not differ from the 0.5 M NaCl eluted fraction in the amount of contamination by nucleic acid (both of which are trace), which could affect its elution properties on ssDNA-cellulose. Replication of the flow-through fraction to the same column under the same conditions results in essentially the same elution profile, showing that the loosely bound protein is functional, and the column under these conditions appears as if it is simply being overloaded (data not shown). In fact, if the amount of the g32P-B load is significantly reduced, all of the protein binds to the column and is eluted only at 0.5 M NaCl, as shown in Fig. 3. Remarkably, if this ssDNA-cellulose flow-through pool of g32P-B is first treated with trypsin (which converts the g32P-B to the g32P-(A + B), (cf. Fig. 5) and then applied to the same ssDNA-cellulose column under identical conditions, the resulting g32P-(A + B) is totally absorbed by the column under low-salt conditions and eluted only and specifically upon application of the 0.5 M NaCl elution buffer (data not shown).

Although these are qualitative and nonequilibrium experiments, these observations would tend to indicate that g32P-B binds with detectably lower affinity to ss nucleic acids than does the g32P-(A + B) core fragment. In order to substantiate this proposal and obtain quantitative determinations of $K_{obs}$ and $\omega$, we carried out a series of binding measurements with the homopolymer poly(dT) for recombinant g32P-B, purified from the 0.5 M NaCl elution pool of two separate runs of the ssDNA-cellulose column, and the g32P-(A + B) core protein, derived from g32P-B by proteolysis and re-purified via ssDNA-cellulose as described above.

Representative reverse titrations of g32P-B and g32P-(A + B) with poly(dT) at low salt (0.05 M NaCl), pH 8.1 and 20 °C are shown in Fig. 5, followed by monitoring the quenching of the intrinsic tryptophan fluorescence. Since these titrations were done at similar input ligand (protein) concentrations ($L_0$), simple visual inspection clearly shows that under these solution conditions, g32P-B binds with considerably lower

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# Table I

|            | g32P     | g32P-B   | g32P-(A + B) |
|------------|---------|---------|-------------|
|            | (M, 54,447) | (M, 50,834) | (M, 25,006) |
| Limiting $K_m$ | 0.420 ± 0.004 | 0.405 ± 0.005 | 0.504 |
| M, $\mu$M | 50,500 | 59,800 | 39,300 |
| $a/A$, $\Delta$ | 30.2 ± 0.7 | 31.0 ± 0.4 | 26.1 ± 1.9 |
| $K_{obs}$, $\mu$M | 1.42 ± 0.03 | 1.49 ± 0.02 | 1.35 ± 0.10 |
| $a/b$ | 7 | 8 | 6 ± 1 |

* Deduced at protein concentration ≤0.1 μM at Vn.

* Calculated (42) according to Laurent and Killander (40), Porath (41), and Ackers (39) (see "Materials and Methods"). The $a$ values obtained by each method were averaged with the mean and standard errors shown. The standard error in the frictional coefficient incorporates the uncertainty in $a$.

* Assuming a prolate ellipsoidal globular shape.
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Similar reasoning for the core g32P-(A + B) fragment. Compiled in Table I are estimates of the apparent molecular weights, Stokes radii, frictional coefficients, and calculated axial ratios for g32P derivatives, the latter assuming a prolate ellipsoidal globular structure as was done previously for the intact protein (29).

These data show that recombinant g32P-B migrates on gel filtration with an apparent particle size and shape characteristics similar to those of native g32P under conditions where both proteins are monomeric. All g32P derivatives are considerably asymmetric (Table I), with the C-terminal A domain considerably more asymmetric (Table I), with the C-terminal A domain less symmetric (Table I), with the C-terminal A domain this proposal and obtain quantitative determinations of $K_{obs}$ and $\omega$, we carried out a series of binding measurements with the homopolymer poly(dT) for recombinant g32P-B, purified from the 0.5 M NaCl elution pool of two separate runs of the ssDNA-cellulose column, and the g32P-(A + B) core protein, derived from g32P-B by proteolysis and re-purified via ssDNA-cellulose as described above.

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* Assuming a prolate ellipsoidal globular shape.
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affinity than does the core fragment. Superimposed on the experimental data are the visual best-fit theoretical binding isotherms predicted by the large ligand cooperative overlap binding model of McGhee and von Hippel (30). Such an analysis is strictly justified since we have shown that the protein is monomeric under these conditions. The binding parameters which describe these best fit isotherms (K_m, n, and w) are indicated in the legend to Fig. 8, with the compiled results from several experiments summarized in Table II.

As found previously for the g32P-(A + B) core fragment (11), recombinant g32P-B can be quantitatively shown to bind noncooperatively (w = 1) to poly(dT). Furthermore, K_m for the g32P-B binding affinity is approximately 80-fold lower than that found for the core fragment. We point out that the g32P-B binding affinity is equivalent to that reported by other recombinant g32P-B can be quantitatively shown to bind noncooperatively (w = 1) to poly(dT). Moreover, K_m for the g32P-(A + B) core fragment is as small as 5.0 2 0.5 as reported previously (11). We are presently exploring the question of site size for both g32P-B and g32P-(A + B) derivatives according to Bujalowski and Lohman (33).

Equilibrium binding parameters for g32P-B and g32P-(A + B)

Conditions for all experiments: 10 mM Tris-Cl, 0.1 mM Na_2EDTA, pH 8.1, 0.05 M NaCl at 20 °C. Protein concentration ranged from 2.0 10^{-7} to 1.6 10^{-7} M in the case of g32P-B and 2.21 10^{-7} to 3.23 10^{-7} M for g32P-(A + B).

| Poly(dT) | g32P-B | g32P-(A + B) |
|----------|--------|-------------|
| K_{obs} M^{-1} | 5.2 10^{6} (1.6) | 4.0 10^{5} (2.0) |
| ω | 1 | 1 |
| n | 8 (1) | 7 (1) |
| δ log K_{obs}/δ log[NaCl] | -3.00 (0.18) | -3.91 (0.28) |
| log K_{obs} (1 M Na^{+})^w | 3.50 (0.22) | 2.33 (0.36) |
| Poly(dT) | g32P-B | g32P-(A + B) |
| K_{obs} M^{-1} | 5.2 10^{6} | 5.9 10^{6} |
| δ log K_{obs}/δ log[NaCl] | -2.2 | -3.5 |
| log K_{obs} (1 M Na^{+})^w | 1.4 | 1.0 |

* The indicated range incorporates uncertainty from multiple titrations as well as within individual titrations, the latter due primarily to the range of acceptable values of n.
* Extrapolated.
* Extrapolated.
* Extrapolated.
* Extrapolated.

The net number of ions (cation and anion) released concomitant with formation of the complex according to "salt-back" titrations where the protein-nucleic acid complex is formed at low salt and is gradually dissociated upon increment increases in solution [NaCl], monitored by an increase in protein fluorescence (32). At each NaCl concentration, L_0 and L_P can be calculated from Equations 1–3 under "Materials and Methods," permitting calculation of K_{obs} at each NaCl concentration, according to Equation 4. This salt-back analysis also requires that Q_{max}, n, and the cooperativity parameter ω = 1 not change as a function of solution NaCl concentration (32). Analysis of reverse titrations carried out with g32P-B and g32P-(A + B) and poly(dT) at various [NaCl] reveal that all titrations can be fit satisfactorily with an ω = 1 and n within the range indicated, whereas best-fit Q_{max} values do not vary systematically by ±5% in either case (data not shown).

Fig. 9, A and B, show representative salt-back titrations presented as L_0/L_P ratio versus total [NaCl] obtained for g32P-B and g32P-(A + B), respectively. The uncertainty shown in the experimental determinations of L_0/L_P ≥ 0.85 and ≤0.15 incorporate the experimentally observed uncertainty in Q_{max} (which primarily affects the low salt region) in addition to the actual measurement of Q_{max} (difficult to obtain in the high salt region). Note that the middle portion of the curve, i.e., where 0.15 ≤ L_0/L_P ≤ 0.85, is not appreciably affected by either uncertainty. We thus use this portion of the curve to estimate the [NaCl] dependence plotted as log K_{obs} versus log [NaCl], shown for both proteins as indicated in Fig. 9C. The linear least squares analysis of these data yield the slope (δ log K_{obs}/δ log[NaCl]) and the extrapolated log K_{obs} (1 M Na^{+}) values compiled in the legend to Fig. 8. The solid lines drawn through the experimental points in A and B

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**Table II**

Equilibrium binding parameters for g32P-B and g32P-(A + B)

| Condition | g32P-B | g32P-(A + B) |
|-----------|--------|-------------|
| K_{obs} M^{-1} | 5.2 10^{6} | 4.0 10^{5} |
| ω | 1 | 1 |
| n | 8 (1) | 7 (1) |
| δ log K_{obs}/δ log[NaCl] | -3.00 (0.18) | -3.91 (0.28) |
| log K_{obs} (1 M Na^{+})^w | 3.50 (0.22) | 2.33 (0.36) |

The indicated range incorporates uncertainty from multiple titrations as well as within individual titrations, the latter due primarily to the range of acceptable values of n.

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**Fig. 8. Reverse fluorescence titrations of recombinant g32P-B (2.98 10^{-7} M) and g32P-(A + B) derived from g32P-B (3.23 10^{-7} M) with poly(dT) in 10 mM Tris-Cl, 0.1 mM Na_2EDTA, pH 8.1, 0.05 M NaCl at 20 °C. The solid lines drawn through the experimental points represent the best-fit theoretical isotherms for each titration predicted by the McGhee-von Hippel cooperative overlap binding model (30) with the binding parameters obtained as follows: g32P-B, K_{obs} = 5.15 10^{6} M^{-1}, ω = 1, n = 7; g32P-(A + B), K_{obs} = 3.20 10^{5} M^{-1}, ω = 1, n = 7. We assume that L_0/L_P = Q_{obs}/Q_{max} and the density obtained from degree of quenching, v_{Q} = (L_0/L_P), directly reflects the true binding density, v_{Q} = 0.435 for the g32P-(A + B) core fragment and 0.445 for the g32P-B under these conditions.**
The [NaCl] dependence of the binding of g32P-(A + B) to poly(dT) varies from that observed in g32P-B, which incorporates data from additional salt-back experiments carried out at differing initial binding densities ($u_0$, $L_T$), and initial [NaCl], as well as analysis of standard reverse titrations performed at various [NaCl].

In Table II, we also show preliminary data on the binding of g32P-B and g32P-(A + B) to the ribonucleopolymer, poly(U), to which native g32P binds with much less affinity (20). The trends for poly(U) are qualitatively as outlined for poly(dT) binding. Although both molecules bind less tightly to poly(U) than poly(dT) (Table II), g32P-B is characterized by a greatly reduced binding affinity ($\approx 100$-fold) at low salt and a salt dependence considerably less than g32P-(A + B) core fragment under the same conditions of pH and temperature. Thus, the qualitative trends we see with poly(dT) appear independent of the base and sugar composition of the nucleic acid molecule and must reflect generic features of the noncooperative linear lattice binding mode of g32P.

**DISCUSSION**

Cooperative binding by g32P to ss nucleic acids requires the N-terminal B domain as evidenced by quantitative equilibrium binding experiments carried out with the tryptic core g32P-(A + B) fragment on the polynucleotide, poly(dT), i.e. $\omega = 1$ or noncooperative binding (11). Analogous quantitative measurements of equilibrium binding parameters for g32P-(A + B)' remain unreported, although qualitatively the binding affinity of g32P-(A + B)' for poly(dT) (17, 18) and poly(d[A-T]) (16) is of lower affinity and generally nonstoichiometric under low salt conditions, relative to the intact protein. As the mechanism of cooperative binding by this prototype ss nucleic acid binding protein remains obscure, we intend to elucidate the functional importance of individual amino acids in the B domain, by systematically creating a large library of B domain point mutations to be functionally characterized in the context of the entire molecule. In order to initiate these experiments, we require a predictable and large-scale source of a g32P molecule completely lacking only this domain and one devoid of contamination by the intact protein.

In this report, we describe overexpression, purification, and characterization of the N-terminal tryptic deletion fragment of T4 gene 32 protein, g32P$_{2-301}$ or g32P-B. The purified protein has the expected and desired primary structure in the N-terminal region, NH$_2$-Gly$_2$-etc. and contains stoichiometric Zn(I1). We provide evidence from $^1$H NMR and hydrodynamic experiments that the recombinant protein has tertiary and quaternary structural properties indistinguishable from the proteolytic fragments obtained in the usual way via partial proteolysis of intact g32P. The described means of obtaining homogeneous g32P-B represent a considerable improvement over the techniques of protein chemistry which rely on partial
and specific proteolysis by trypsin of only the N-terminal B domain after Lys21 with the linkage connecting the core domain and the C-terminal A domain (after Lys22) remaining intact. In fact, this particular molecule, g32Pb22-301, is impossible to make with trypsin, as the A domain is preferentially removed prior to the B domain. In all cases, the possibility persists that these preparations can be contaminated with other trypsin, as the A domain is preferentially removed prior to the B domain.4 In all cases, the possibility persists that these preparations can be contaminated with either or both of the respective core fragment, all of which exhibit widely disparate binding affinities (Ref. 11; this work). Reflecting these limitations, at least in part, the physical and nucleic acid binding properties of g32P lacking only all or part of amino acids 1–21 are only qualitatively described (17, 18).

We demonstrate quantitatively that g32P-R binds noncooperatively to polynucleotides. However, the most striking result from the current studies is that the equilibrium binding affinity of g32P-B for poly(dT) and poly(U) is significantly reduced relative to core fragment derived from it via proteolytic cleavage of the C-terminal A domain. Thus, the A domain contributes unfavorably to the binding free energy of the core nucleic acid binding domain to an isolated lattice site. We also show that this unfavorable contribution is diminished as the solution salt concentration is raised, such that the non-electrostatic component of the binding free energy for both g32P derivatives would appear to be similar (Table II). However, since the anion component to the salt dependences thus far remain undefined for both proteins, we cannot as yet conclude that the unfavorable influence of the C-terminal A domain is exclusively of electrostatic origin.

Our results with g32Ps (with or without the A domain) which lack the B domain and therefore bind noncooperatively to polynucleotides (Table II), are qualitatively consistent with previous studies which examined the noncooperative binding of intact g32P versus g32P-A to short oligonucleotides of maximum length (l) nucleotides less than that able to span more than one protein binding site (e.g., d(pT)l2) (11, 19). With oligonucleotides of length, l < 6 nucleotides, both g32P and g32P-A exhibited similar affinities and salt dependences (11). However, with l ≥ 6, g32P-A showed a detectably greater affinity at low salt concentration and enhanced salt dependences relative to the native protein (11). For example, g32P-A binds to d(pA)l2 at 0.1 M NaCl with a Kobs ≈ 5.0 × 106 M–1 and a a log Kobs/θ log [NaCl] ≈ –1.3 ± 0.1, whereas the same values for the intact g32P are 1.6 × 106 M–1 and = –0.3 ± 0.1, respectively.7 Evaluation of Kobs at various higher NaCl concentrations permitted construction of a log log plot which upon tenuous extrapolation to 1 M Na+ standard state revealed a similar non-electrostatic contribution to the binding free energy for both proteins. Since the anion component to the binding free energy appeared negligible in this system, Lonberg et al. (11) suggest that the effect of removing the A domain is to increase the binding affinity for these oligonucleotides manifest entirely through electrostatic interactions. This would further imply a greater number of cations (=2) displaced from the oligonucleotide in g32P-A, relative to the native protein (=1). Our preliminary results with the single-site oligonucleotide, d[T(pT)]l (l = 8), also indicate similar disparities in equilibrium affinities of g32P-B and g32P-(A + B), assuming a 1:1 oligonucleotide:protein stoichiometry.8

Quantitative determinations of equilibrium binding parameters of g32P and g32P-A, on polynucleotides mirror the results with oligonucleotides in that Kobs becomes 2-3-fold higher upon proteolysis of the A domain (11) with no detect-

7 Kobs values were obtained assuming an oligonucleotide:protein monomer stoichiometry of 1:1.
8 D. Giedroc and R. Khan, unpublished observation.
tightly (or stoichiometrically) to poly(dT) and poly(U) such that $K_{obs}$ cannot be measured accurately with fluorescence techniques. For example, at more moderate [NaCl] (e.g. 0.4 M), the reported difference in $K_{obs}$ for g32P and g32P-A bound in the cooperative binding mode on polynucleotides (11) would appear to be comparable to the difference in affinities which we measure for g32P-D and g32P-(A + D) bound noncooperatively under similar solution conditions.

Watanabe (21) has recently suggested that A domain function may, in fact, be intimately incorporated into the cooperative binding mode of g32P. Data were presented which showed that the cooperativity parameter exhibits a weak salt dependence, becoming slightly larger (by ~2-fold) as the solution [NaCl] is raised. Titrations carried out with NaF gave indistinguishable values for $\omega$, whereas MgCl$_2$ seemed to facilitate cooperative interactions. He suggested that the negative charge of the A domain must be effectively "screened" by cations in order to g32P to bind tightly (cooperatively) to polynucleotides. Thus, although not physically associated in the thermodynamic sense, solution cations may, in fact, be intimately incorporated into the cooperative interactions. He suggested that the negative charge of the A domain must be effectively "screened" by cations in order for g32P to bind tightly (cooperatively) to polynucleotides. Thus, although not physically associated in the thermodynamic sense, solution cations may enhance the equilibrium binding affinity of g32P by reducing the repulsion of negatively charged A domains on contiguously bound g32P monomers through a simple ionic strength effect (21). In this line of thinking, a trivial interpretation of our binding data thus far might be that the binding energetics of g32P-B simply represent the sum of such a screening effect (or weak cation uptake component) specific to the A domain and the particular energetics which characterize the core fragment-polynucleotide complex.

Clearly, additional experiments beyond the scope of the current work are required to further elucidate the role of the A domain in molecular terms with regard to linear lattice binding by g32P. Such experiments are facilitated by the B domain deletion molecules since cooperativity is eliminated from the binding equilibrium. As we show, this permits direct determination of affinities and salt-dependences of the isolated site binding mode under a wide variety of conditions, vastly reducing the complexity of the system. We point out that very recent high resolution $^1$H NMR experiments which carefully examined the isolated site (mimicked by g32P-(A + B) binding to d(pA)$_n$-d) and the cooperative binding modes (intact g32P complexed with d(pA)$_n$-d) reveal that the protein-nucleic acid interfacial region must be very similar in both conformations (26). Studies are underway which systematically alter the nature of the cation and anion species of the dissociating salt, providing molecular information as to whether the observed differences in $K_{obs}$ between g32P-B and g32P-(A + B) are manifest as a result of anion or cation effects contributed by the C-terminal A domain. Initial studies by Lonberg et al. (11) with g32P-(A + B) suggest a considerable anion component of the salt dependence for this interaction with $\delta \log K_{obs}/\delta \log [NaCl]$ obtained in NaF and MgCl$_2$ only marginally reduced relative to that obtained in NaCl (11). Our experiments with g32P-B as well as g32P-(A + B) should extend these observations as well as provide additional detail concerning the generic mechanisms of high affinity linear lattice binding by this prototype ss nucleic acid binding protein.

Acknowledgments—We thank Dr. T. M. Lohman and members of his laboratory for assistance in analyzing the fluorescence experiments in the early stages of this work as well as their ongoing interest in these studies. We also thank Drs. Yousif Shamoo and Kenneth R. Williams for providing the original g32P construct in M13, Dr. F. William Studier for providing plasmids and host strains of the T7 RNA polymerase overexpression system, Dr. T.-C. Lin for the gift of AP$_2$, expression plasmid pTLOW, and S. Silber for help in acquiring the NMR spectra.

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N-terminal Deletion of T4 Gene 32 Protein

Materials and Methods

Materials

All buffers were prepared with doubly distilled and deionized Mill-Q water. The enzyme stock solution was prepared according to Alberts et al. (1979) by using 0.6 g denatured calf thymus DNA (Sigma) per 100 ml of buffer. The buffer used for the T4 gene 32 protein was 50 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4. The polymerase used was obtained from Sigma. Poly(dT)12-18 and poly(U)15 were purchased from Midland (Clemson, SC). All radioactive tracers were obtained from either New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL).

Methods

Subcloning of gII-2 coding sequences into the T7 RNA polymerase expression vector. All molecular biological methods were carried out essentially as described in Maniatis et al. (1982). The source of the gene 32 protein coding sequences was the recombinant form of an M13mp18 derivative in which the gII-2 structural gene is on the 59 side with an E.coli promoter and the 39 side with a bacterial gIII gene (kindly provided by G. Snell). The gII-2 insert lacks the autogenous transcriptional repression determinant (38) but contains a consensus ribosome binding site upstream of the natural gII gene. This plasmid was digested with EcoRI and BamHI and the resulting 1.1 kb EcoRI/BamHI fragment was subcloned into pBR322 (B. Hansen, New Haven, CT) digested with the same enzymes. The recombinant vector was then converted to a plasmid site with synthetic linkers containing pMD2.27 (9.9 kb) which contains the gII-2 coding sequences in the opposite orientation relative to the lac promoter (Fig. 1). The 5.9 kb of this vector was used as a template for oligonucleotide-directed mutagenesis according to Taylor et al. (1981) in a kit purchased from amersham (Arlington Heights, IL), with the oligonucleotides 5'GTGAGATCCATGTTTAAATTTTTTTCTGATACCAGATTAAGCTTTT>C (Fig. 1A) for the introduction of a unique restriction site at nucleotides 65-67 (36) which changes the gII-2 34440-A sequence to 34440-TG. With this construct, the lac operator was deleted and the gII-2 34500-A fragment cloned into pBR322 (generously provided by F. W. Studier) similarly digested, creating pT7GII-2-Bst (Fig. 1A). Expression of gII-2 protein. The E. coli host (BL21DE3) was transformed to ampicillin (100 µg/ml) resistance with pT7GII-2-Bst. For analytical induction experiments (as shown in Fig. 1B), a sterile transformed culture of cells was inoculated to 5 ml in Luria broth (LB) containing 100 µg/ml ampicillin and 10 µg/ml chloramphenicol. After 3 h of growth at 37°C, chloramphenicol was supplied to a final concentration of 1 µg/ml. Protein samples were prepared from the induced cultures according to the method of Clontech Laboratories (Palo Alto, CA).

Results

This is the section where the results of the experiments are described. The results are presented in a logical order, with the most significant findings highlighted. The data are supported by figures, tables, and other visual aids as necessary.

Discussion

The discussion section is where the results are interpreted and compared to previous findings. It is also where the implications of the results are discussed and the potential applications of the findings are considered.

Conclusion

The conclusion section is where the main findings are summarized and their significance is highlighted. The conclusion should also include suggestions for future research.

Supplementary Material

A supplementary material section is where additional data, methods, or results that do not fit into the main text are included. This section should be clearly marked as supplementary and should be referenced in the main text.

References

A list of references is provided at the end of the document. The references should be numbered and cited in the appropriate places in the text.

Tables

Tables are used to organize and present data in a clear and concise manner. Each table should have a clear title and be self-explanatory.

Figures

Figures are used to illustrate data and concepts. Each figure should have a clear title and be labeled with numbers and legends.

Appendices

Appendices are used to provide additional information that is not essential for the main text. They should be clearly marked and referenced in the main text.
N-terminal Deletion of T4 Gene 32 Protein

dyes against 50 mM sodium phosphate, pH 8, 30 mM NaCl and trypsin microradiography (14,000000) to remove debris. The sample was then loaded onto two successive Sepharose 2B (Pharmacia) spin columns (0.4 x 30 cm). Following washing with 2 mL of 0.1 M NaCl and 20 mM Na3EDTA, the sample was eluted with a linear gradient (0-200 mM NaCl) in 0.1 M Na3EDTA, pH 8, and 0.2% (v/v) Triton X-100. The elution profile was checked by SDS-PAGE.

Chemical shifts are reported relative to the standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (TSP). In the presence of detergent, the resonances of T4 protein 32 were shifted downfield by 0.1 ppm. The resonance of T4 protein 32 was monitored in the presence of T4 protein 32 and T4 protein 32, and the resonance of T4 protein 32 was shifted upfield by 0.1 ppm.

We have determined the secondary structure of T4 protein 32 using CD spectroscopy and NMR spectroscopy. The CD spectrum of T4 protein 32 in 0.1 M NaCl, pH 8, is consistent with a helical structure. The NMR spectrum of T4 protein 32 in 0.1 M NaCl, pH 8, shows two distinct sets of resonances, indicating a mixture of structures.

Figure 2. DE-52 anion exchange chromatography of low-speed supernatant of BL21(DE3)/pT7-30.

A. Absorbance at 260 nm (A260) and 280 nm (A280) of fractions collected following application of the low-speed supernatant to the column. The absorbance at 260 nm is indicated by the lower curve. The absorbance at 280 nm is indicated by the upper curve.

B. The elution profile shows the time course of elution of a DE-52 anion exchange column. The column was washed with 10 mM sodium phosphate, pH 8, and then eluted with a linear gradient of 0-1 M NaCl.

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Figure 4. SDS-PAGE analysis of fractions resulting from phenyl-Sepharose chromatography of the ssDNA-cellulose pool from Fig. 3: LOAD, ssDNA-cellulose pool, fractions 21-34, non-bound protein fractions; fractions 114-125, protein bound to the column and eluted near the low salt end of the reverse (IM-NaCl) gradient (cf., "Materials and Methods"). The indicated inclusive fractions were pooled as indicated (POOL) and used for initial characterization experiments.
Overexpression, purification, and characterization of recombinant T4 gene 32 protein22-301 (g32P-B).
D P Giedroc, R Khan and K Barnhart

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