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 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-\( \beta \)\( ^{B} \), 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, \( \omega \) 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 toselectively remove the carboxyl-terminal “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 mM NaCl, pH 8.1, 20 °C) show that all equilibrium binding isotherms can be fit with \( \omega = 1 \) and \( K_{\text{obs}} = 5.2 \times 10^{4} \text{ M}^{-1} \), with a moderate electrostatic component to the binding free energy, \( \delta \log K_{\text{obs}}/\delta \log [\text{NaCl}] = -3.0 \pm 0.2 \). Under identical solution conditions, g32P-(A + B) derived from g32P-B binds to poly(dT) noncooperatively as expected, but with an \( \approx 80\)-fold higher apparent affinity, \( K_{\text{obs}} = 4.0 \times 10^{7} \text{ M}^{-1} \), and detectably enhanced salt sensitivity, \( \delta \log K_{\text{obs}}/\delta \log [\text{NaCl}] = -3.9 \pm 0.3 \). As the salt concentration is raised, the relative difference in \( K_{\text{obs}} \) between the g32P-(A + B) and g32P-B is gradually reduced such that extrapolation of the log-log plots to 1 mM Na\(^+\) standard state gives similar \( K_{\text{obs}} \) within experimental error. Qualitatively similar observations are also found upon binding to the ribonucleopolymer, 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) specifically relating to the structural disposition of the acidic C-terminal A region relative to the rest of the molecule.

Gene 32 protein (g32P),\(^1\) 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 will 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 cooperative parameter, \( \omega \), reported to range from about 200–2000 (1). The apparent equilibrium association constant (\( K_{\text{app}} \)) of g32P for a polynucleotide lattice is described as \( K_{\text{app}} = K_{\text{obs}} \omega \), where \( K_{\text{obs}} \) 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, \( M_{r} = 33,487 \)) is a multidomain protein of known primary (4) and undefined tertiary structure. Three functional domains become apparent from limited trypsinolysis studies (5, 6). The C-terminal “A” domain (residues 254–301) makes heterogeneous 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

\(^{1}\) The 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-\( \beta \)-thiogalactoside; STD buffer, 10 mM Tris-HCl, pH 8, 1 mM Na\(^{2+}\)EDTA, 1 mM \( \beta \)-mercaptoethanol; STD buffer, 10 mM Tris-HCl pH 8, 0.1 M NaCl, 5% \( \varepsilon \)/\( \gamma \) glycerol; TNGa buffer, 10 mM Tris-HCl, pH 8, 0.1 M NaCl, 5% \( \varepsilon \)/\( \gamma \) glycerol.

Received for publication, January 16, 1990
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 (w = 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 His$^{81}$ on the basis of recent $^3$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 w is essentially independent of nucleic acid base or sugar composition (19, 20). In addition, w 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, g32P$_{22-301}$, 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 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).

MATERIALS AND METHODS

RESULTS

Overexpression of Gene 32 Protein$_{22-301}$ (g32P-B)

The construction of the g32P-B expression plasmid, pT7g32-B-wt, diagrammed in Fig. 1A, is described in the Miniprint. The phage $\lambda$ 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 the T7 RNA polymerase to specifically transcribe its own promoter sequences. Such derepression is conveniently attained by addition of isopropyl-$\beta$-D-thiogalactopyranoside to the exponentially growing culture.

1 Portions of this paper (including "Materials and Methods" and Figs. 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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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 Pl promoter (13). Note that the cells which contain pT7g32P-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) (Fig. 4) 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-Gly3-Phe-Ser-Ser-Glu-Asp-Lys-Gly- the full-length g32P, consistent with the predicted molecular mass some 2500 daltons less than that of the intact protein.

Typical yields of highly purified g32P-B range from 4 to 10 mg/g induced cells. The purification is not especially noteworthy (see “Materials and Methods”). Automated N-terminal sequencing of g32P-B reveals NH2-Gly3-Phe-Ser-Ser-Glu-Asp-Lys-Gly-Glu-ThrP, 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.

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 (Fig. 5). 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.

**Fig. 5.** SDS-polyacrylamide gel electrophoresis analysis of the time course of proteolysis by trypsin of g32P and recombinant g32P-B. The electrophoretic migration of the g32P-(A + B) core fragment is shown in the left-most lane. Each incubation (100 μl) contained 7.16 μM g32P, 0.01 mg/ml trypsin (to 25:1 w/w g32P-trypsin ratio) in 10 mM Tris-HCl, pH 8, 0.1 M NaCl, 5% v/v glycerol, 16 °C. 10-μl aliquots were withdrawn at the indicated times and added to ice-cold SDS-polyacrylamide gel electrophoresis gel loading buffer, mixed, and immediately heated at 75 °C for 5 min and returned to ice until electrophoresis. These are conditions known to quench the proteolysis reaction (12).

**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 reorientation time, as revealed by hydrodynamic measurements (see below). In any case,
N-terminal Deletion of T4 Gene 32 Protein

Fig. 6. 400-MHz $^1$H 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) 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 ($K_w$) of g32P, g32P-B, and g32P-(A + B) obtained at ambient temperature in 10 mM Tris-HCl, 0.1 mM EDTA, 0.1 M NaCl, pH 7.2 as a function of protein concentration as described under "Materials and Methods." In the protein concentration range extending from $2 \times 10^{-5}$ to $5 \times 10^{-6}$ M, we document that g32P-B and the core g32P-(A + B) fragment give the same $K_w$, indicating a constant molecular species. In contrast, as already demonstrated by previous sedimentation experiments (28), the intact protein begins detectable apparent aggregation at $\geq 0.2 \mu M$.

Knowing that at protein concentrations $\leq 10^{-7}$ M, the intact protein is a monomer (28), the observed $K_w$ 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 $f/f_o$ for the monomeric g32P, by assuming an average partial specific volume ($\tilde{\nu} = 0.73 \text{ cm}^3/\text{g}$) as outlined under "Materials and Methods." Since the g32P-B protein also exhibits a $K_w$ 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 $0.05 \text{ M NaCl}$ was not sufficient to eliminate absorption of proteins to the column, whereas extensive exposure of the column to buffers of pH $\leq 8$ 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 Na$_2$EDTA 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].

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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.
Fig. 7. HPLC gel filtration chromatography of g32P, g32P-B, and g32P-(A + B). The elution coefficients ($K_v$) of various g32Ps are plotted as a function of protein concentration determined at $V$, $\Delta$, core g32P-(A + B) fragment; $\bullet$, g32P-B; $\square$, intact g32P. The closed symbols represent runs carried out with a constant sample load volume (20 $\mu$L), whereas open symbols represent all other runs with load volumes varying from 0.5 to 50 $\mu$L as described under "Materials and Methods." The $K_v$ values of bovine serum albumin (BSA; 66,000 Da), ovalbumin (oval; 45,000 Da), and carbonic anhydrase (CA; 29,500 Da) are indicated. Conditions: 10 mM Tris-HCl, 0.1 mM Na$_2$EDTA, 0.1 mM NaCl, pH 7.2, ambient temperature.

**TABLE I**

**Hydrodynamic properties of g32P derivatives derived from analytical gel filtration studies**

<table>
<thead>
<tr>
<th></th>
<th>g32P</th>
<th>g32P-B</th>
<th>g32P-(A + B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M, 33,487)</td>
<td>(M, 30,834)</td>
<td>(M, 29,036)</td>
</tr>
<tr>
<td>Limiting $K_v$</td>
<td>0.420 ± 0.004</td>
<td>0.405 ± 0.005</td>
<td>0.504</td>
</tr>
<tr>
<td>M, $P_j$</td>
<td>50,500</td>
<td>52,500</td>
<td>30,300</td>
</tr>
<tr>
<td>$a/b$</td>
<td>30.2 ± 0.7</td>
<td>31.0 ± 0.4</td>
<td>26.1 ± 1.9</td>
</tr>
<tr>
<td>$f/f_0$</td>
<td>1.42 ± 0.03</td>
<td>1.49 ± 0.02</td>
<td>1.36 ± 0.10</td>
</tr>
<tr>
<td>$a/b^a$</td>
<td>7</td>
<td>6.5</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

*a* Deduced at protein concentration ≤0.1 mM at $V_v$.

*b* 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.

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 and 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 perhaps contributing significantly to the asymmetry of the molecule (compare $f/f_0$ for g32P-B and g32P-(A + B)). We also show that g32P-B remains monomeric over the concentration range tested in contrast to the native protein, indicating that the N-terminal B domain clearly influences g32P self-association. Interestingly, g32P-(A + B) appears to undergo some kind of limited self-association at concentrations ≥10 μM. In any case, at protein concentrations used in the fluorescence 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 mM 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 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 recovered in all regions of 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. 8, followed by monitoring the quenching of the intrinsic tryptophan fluorescence. Since these titrations were done at similar input ligand (protein) concentrations ($L_o$), simple visual inspection clearly shows that under these solution conditions, g32P-B binds with considerably lower

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*G32P-B purified from E. coli BL21(DE3) cells transformed with or without pLysS (a plasmid which provides a source of T7 lysozyme (cf. Ref. 38)) give indistinguishable $K_{obs}$ for the homopolymer poly(dT) under identical solution conditions.
acid binding equilibria provides molecular information on the nucleic acid lattice resulting from electrostatic interactions upon release of cations thermodynamically associated with the nucleic acid, whereas the second term incorporates preferential anion effects as the net number of anions displaced from the nucleic acid, whereas the second term incorporates preferential anion effects as the net number of anions displaced from the protein (31). With such an approach, we can ascertain if the lower \( K_{\text{obs}} \) of g32P-B for poly(dT) at low salt relative to the core protein is due to a differential net release of ions concomitant with complex formation. We have obtained the [NaCl] dependence of the binding equilibria, i.e. \( \partial \log K_{\text{obs}}/\partial \log[\text{NaCl}] \), for both g32P derivatives via analysis of individual “salt-back” titrations where the protein-nucleic acid complex is formed at low salt and is gradually dissociated upon incremental increases in solution [NaCl], monitored by an increase in protein fluorescence (32). At each NaCl concentration, \( L_p \) and \( L_o \) can be calculated from Equations 1–3 under “Materials and Methods,” permitting calculation of \( K_{\text{obs}} \) at each NaCl concentration, according to Equation 4. This salt-back analysis also requires that \( Q_{\text{max}} \), \( n \), and the cooperativity parameter \( \omega = 1 \) not change as a function of solution NaCl concentration (32). Analysis of reverse titrations carried out with g32P-B and g32P-(A + B) at various [NaCl] reveal that all titrations can be fit satisfactorily with an \( \omega = 1 \) and \( n \) within the range indicated, whereas best-fit \( Q_{\text{max}} \) values do not vary systematically by ±5% in either case (data not shown).

Fig. 8. Reverse fluorescence titrations of recombinant g32P-B (2.98 x 10^{-7} M) (C) and g32P-(A + B) derived from g32P-B (3.23 x 10^{-7} M) with poly(dT) in 10 mm Tris-HCl, 0.1 mm Na2EDTA, pH 8.1, 0.05 M NaCl at 20 °C. The solid lines drawn through the experimental points represent the best-fit theoretical binding isotherms for each titration predicted by the McGhee-von Hippel cooperative overlap binding model (30) with the binding parameters obtained as follows: g32P-B, \( K_{\text{obs}} = 5.15 \times 10^{-4} \) M^{-1}, \( \omega = 1, n = 7 \); g32P-(A + B), \( K_{\text{obs}} = 3.20 \times 10^{-4} \) M^{-1}, \( \omega = 1, n = 7 \). We assume that \( L_p/Q_{\text{obs}} = 0.1 \) and the binding density obtained from degree of quenching, \( v_{\text{obs}} = (L_p/Q_{\text{obs}}) \), directly reflects the true binding density, \( v = 0.435 \) for the g32P-(A + B) core fragment and 0.445 for the g32P-B under these conditions.

net number of ions (cation and anion) released concomitant with formation of the complex according to \( \partial \log K_{\text{obs}}/\partial \log[\text{NaCl}] = m \mu + a \) (31). The first term encompasses cation release and screening effects primarily associated with the nuclear acid, whereas the second term incorporates preferential anion effects as the net number of anions displaced from the protein (31). With such an approach, we can ascertain if the lower \( K_{\text{obs}} \) of g32P-B for poly(dT) at low salt relative to the core protein is due to a differential net release of ions concomitant with complex formation. We have obtained the [NaCl] dependence of the binding equilibria, i.e. \( \partial \log K_{\text{obs}}/\partial \log[\text{NaCl}] \), for both g32P derivatives via analysis of individual “salt-back” titrations where the protein-nucleic acid complex is formed at low salt and is gradually dissociated upon incremental increases in solution [NaCl], monitored by an increase in protein fluorescence (32). At each NaCl concentration, \( L_p \) and \( L_o \) can be calculated from Equations 1–3 under “Materials and Methods,” permitting calculation of \( K_{\text{obs}} \) at each NaCl concentration, according to Equation 4. This salt-back analysis also requires that \( Q_{\text{max}} \), \( n \), and the cooperativity parameter \( \omega = 1 \) not change as a function of solution NaCl concentration (32). Analysis of reverse titrations carried out with g32P-B and g32P-(A + B) at various [NaCl] reveal that all titrations can be fit satisfactorily with an \( \omega = 1 \) and \( n \) within the range indicated, whereas best-fit \( Q_{\text{max}} \) 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 \( L_p/L_o \) ratio versus total [NaCl] obtained for g32P-B and g32P-(A + B), respectively. The uncertainty shown in the experimental determinations of \( L_p/L_o \geq 0.85 \) and ±0.15 incorporate the experimentally observed uncertainty in \( Q_{\text{max}} \) (which primarily affects the low salt region) in addition to the actual measurement of \( Q_{\text{max}} \) values (difficult to obtain in the high salt region). Note that the middle portion of the curve, i.e. where \( 0.15 \leq L_p/L_o \leq 0.85 \), is not appreciably affected by either uncertainty. We thus use this portion of the curve to estimate the [NaCl] dependence plotted as \( \log K_{\text{obs}} \) versus log [NaCl], shown for both proteins as indicated in Fig. 9C. The linear least squares analysis of these data yield the slope \( \partial \log K_{\text{obs}}/\partial \log[\text{NaCl}] \) and the extrapolated log \( K_{\text{obs}} \) (1 M Na+ ) value compiled in the legend to Fig. 8. The solid lines drawn through the experimental points in A and B

<table>
<thead>
<tr>
<th>Poly(dT)</th>
<th>Poly(dT)</th>
</tr>
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<tbody>
<tr>
<td>( K_{\text{obs}} ) M^{-1}</td>
<td>( K_{\text{obs}} ) M^{-1}</td>
</tr>
<tr>
<td>( \omega )</td>
<td>( n )</td>
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<tr>
<td>( \delta \log K_{\text{obs}}/\delta \log[\text{NaCl}] )</td>
<td>( \log K_{\text{obs}} ) (1 M Na+ )</td>
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<tr>
<td>( \delta \log K_{\text{obs}}/\delta \log[\text{NaCl}] )</td>
<td>( \log K_{\text{obs}} ) (1 M Na+ )</td>
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* 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.

* \( Q_{\text{max}} = 0.46, n = 7 \) and \( \omega = 1 \) were used to calculate the indicated parameters.

* \( Q_{\text{max}} = 0.44, n = 7 \) and \( \omega = 1 \) were used to calculate the indicated parameters.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( K_{\text{obs}} ) M^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>g32P-B</td>
<td>5.2 x 10^6 (±1.6)</td>
</tr>
<tr>
<td>g32P-(A + B)</td>
<td>4.0 x 10^7 (±2.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L_p/Q_{\text{obs}} )</td>
<td>0.1</td>
</tr>
<tr>
<td>( v_{\text{obs}} )</td>
<td>0.435 for g32P-(A + B)</td>
</tr>
<tr>
<td>( v )</td>
<td>0.445 for g32P-B</td>
</tr>
</tbody>
</table>

For g32P-B, the site size \( n \) for g32P-B and g32P-(A + B) derived from g32P-B (3.23 x 10^{-7} M) (C) with poly(dT) in 10 mm Tris-HCl, 0.1 mm Na2EDTA, pH 8.1, 0.05 M NaCl at 20 °C. The solid lines drawn through the experimental points represent the best-fit theoretical binding isotherms for each titration predicted by the McGhee-von Hippel cooperative overlap binding model (30) with the binding parameters obtained as follows: g32P-B, \( K_{\text{obs}} = 5.15 \times 10^{-4} \) M^{-1}, \( \omega = 1, n = 7 \); g32P-(A + B), \( K_{\text{obs}} = 3.20 \times 10^{-4} \) M^{-1}, \( \omega = 1, n = 7 \). We assume that \( L_p/Q_{\text{obs}} = 0.1 \) and the binding density obtained from degree of quenching, \( v_{\text{obs}} = (L_p/Q_{\text{obs}}) \), directly reflects the true binding density, \( v = 0.435 \) for the g32P-(A + B) core fragment and 0.445 for the g32P-B under these conditions.

Table II. We found no evidence that \( n \) for the g32P-(A + B) core fragment is as small as 5.0 ± 0.5 as reported previously (11). We are presently exploring the question of site size for both g32P derivatives with short single-site oligonucleotides of varying length (l) by directly determining stoichiometries according to Bujalski and Lohman (39).
represent the theoretical curve obtained when those parameters derived from an analysis of the data cast in the linear format of Fig. 9C are recast, indicating an acceptable fit to the non-transformed experimental data, given the indicated uncertainty. Table II gives the average values for $\partial \log K_{obs}/\partial \log[NaCl]$ and $\log K_{obs}$ (1 M Na$^+$) and incorporates data from additional salt-back experiments carried out at differing initial binding densities ($v_0$, $[L_T]$) and initial $[NaCl]$, as well as analysis of standard reverse titrations performed at various $[NaCl]$.

The $[NaCl]$ dependence of the binding of g32P-(A + B) to poly(dT) of $-3.9 \pm 0.3$ is identical to that determined previously by Lonberg et al. (11), $-3.8 \pm 0.4$. The new data for the g32P-B protein indicate a reduced salt dependence and thus a net release of fewer ions upon complex formation by this molecule. We see, however, that the different $[NaCl]$ dependences for both species reduce the relative difference in $K_{obs}$ for both proteins as the $[NaCl]$ is increased such that the extrapolated $\log K_{obs}$ (1 M Na$^+$) values are quite similar to one another within experimental error. In other words, the unfavorable effect of the A domain on core domain nucleic acid binding in g32P-B at low salt is clearly relieved at higher monovalent ion concentrations.

In Table II, we also show preliminary data on the binding of g32P-B and g32P-(A + B) to the ribohomopolymer, 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 ($-100$-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 thus 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-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$_{32-301}$ or g32P-B. The purified protein has the expected and desired primary structure in the non-transformed experimental data, given the indicated uncertainty. Table II gives the average values for $\partial \log K_{obs}/\partial \log[NaCl]$ and $\log K_{obs}$ (1 M Na$^+$) and incorporates data from additional salt-back experiments carried out at differing initial binding densities ($v_0$, $[L_T]$) and initial $[NaCl]$, as well as analysis of standard reverse titrations performed at various $[NaCl]$. The $[NaCl]$ dependence of the binding of g32P-(A + B) to poly(dT) of $-3.9 \pm 0.3$ is identical to that determined previously by Lonberg et al. (11), $-3.8 \pm 0.4$. The new data for the g32P-B protein indicate a reduced salt dependence and thus a net release of fewer ions upon complex formation by this molecule. We see, however, that the different $[NaCl]$ dependences for both species reduce the relative difference in $K_{obs}$ for both proteins as the $[NaCl]$ is increased such that the extrapolated $\log K_{obs}$ (1 M Na$^+$) values are quite similar to one another within experimental error. In other words, the unfavorable effect of the A domain on core domain nucleic acid binding in g32P-B at low salt is clearly relieved at higher monovalent ion concentrations.

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and specific proteolysis by trypsin of only the N-terminal B domain after Lys \( ^{21} \) with the linkage connecting the core domain and the C-terminal A domain (after Lys \( ^{28} \)) remaining intact. In fact, this particular molecule, g32P22-301, 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 either trace amounts of intact g32P or the respective core fragment, all of which exhibit wide disparity 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 non-cooperatively to polynucleotides. However, the most striking result from the current studies is that the equilibrium binding affinity of g32P-B for poly(dT) \( ^{l} \) and poly(U) \( ^{l} \) 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 B domain is exclusively of electrostatic origin.

Our results with g32P \( ^{2} \) (with or without the A domain) which lack the B domain and therefore bind non-cooperatively 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 \( (e.g. d(pT)_{8}) \) (11, 19). With oligonucleotides of length, \( l < 6 \) nucleotides, both g32P and g32P-A exhibited similar affinities and salt dependences (11). However, with \( l \geq 6 \), g32P-A showed a detectably greater affinity at low salt concentration and enhanced salt dependence relative to the native protein (11). For example, g32P-A binds to d(pA)\( _{6} \) at 0.1 M NaCl with a \( K_{\text{obs}} \approx 5.0 \times 10^{6} \) M\(^{-1} \) and a \( \delta \log K_{\text{obs}}/\delta \log [\text{NaCl}] \approx -1.3 \pm 0.1 \), whereas the same values for the intact g32P are \( \approx 1.6 \times 10^{7} \) M\(^{-1} \) and \( \approx -0.3 \pm 0.1 \), respectively.\(^{7} \) Evaluation of \( K_{\text{obs}} \) 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 nonelectrostatic contribution to the binding free energy for both proteins. Since the anion component to the binding free energy appeared negligible in this system, Lomerberg et al. (11) suggest that the effect of removing the B domain is to increase the binding affinity for these oligonucleotides manifest entirely through electrostatic interactions. This would further imply a greater number of cations \( \approx 2 \) displaced from the oligonucleotide in g32P-A, relative to the native protein \( (\approx 1) \). Our preliminary results with the single-site oligonucleotide, d[T(pT)\( _{8} \)] \( (l = 8) \), 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 \( K_{\text{obs}} \) becomes 2–3-fold higher upon proteolysis of the A domain (11) with no detectable change in the cooperativity parameter (11); however, \( \delta \log K_{\text{obs}}/\delta \log [\text{NaCl}] \) values are indistinguishable for both polynucleotides and significantly more negative (\( \approx -6.5 \pm 1.0 \)) than those obtained with the B domain deletions (Table II).

From these studies, a structural model was developed in which the negatively charged A domain forms all or part of an arm or “flap” that forms electrostatic interactions with a cluster of positively charged side chains on the core domain \( (1–3, 19) \), which prevents g32P from adopting a highly salt-dependent polynucleotide-type binding mode with oligonucleotides \( l \approx 8 \). Some or all of this cluster of cationic side chains, previously occluded by the A domain, are destined to interact electrostatically with the acidic phosphodiester backbone when bound to polynucleotide. Thus, a structural transition in g32P from the so-called oligonucleotide to polynucleotide conformation can be conceptually visualized as movement of all or part of the A domain such that an electrostatic sub-site and thus the entire nucleic acid binding groove becomes uncovered. In this model, the binding affinity of A domain containing g32Ps is simply weaker because fewer electrostatic interactions are formed in the complex and/or energy must be expended to “move” the flap to one or more environments. Structural evidence for such a conformational change is the observation that the A domain becomes more susceptible to proteolytic cleavage upon binding cooperatively to polynucleotides (6); recent \(^{1} \)H NMR experiments are also consistent with a faster average mobility of A domain resonances in the cooperative binding conformation (26).

As pointed out by Burke et al. (8), this modular feature of the A domain with respect to the rest of the bound g32P molecule would appear to be well suited to hold the helixdestabilizing activity of g32P in check by providing a kinetic block to uncontrolled DNA duplex melting (9). Such inhibition could be selectively relieved only when needed, e.g. near the point of duplex unwinding in an active assembled replication complex carrying out fully coordinate DNA synthesis. Here, the energy required to move the A domain on a small subset of g32P molecules might come from heterologous protein-protein interactions with accessory proteins, e.g. the gene 41/61 primsome complex and the DNA polymerase holoenzyme on the lagging and leading strands, respectively (47, 48).

Since the binding of g32P to homopolynucleotide lattices is expected to model many of the fundamental features of the productive binding conformation in the replicative complex, our data would indicate that g32P which selectively lack the B domain experience considerable difficulty in adopting the conceptualized “polynucleotide binding conformation,” in clear contrast to native g32P. We thus provide evidence consistent with the scenario that some of the additional binding free energy derived from B domain-dependent cooperative interactions on polynucleotides be used to drive or facilitate an A domain conformational change such that the respective structural transitions or roles of the A and B domains are energetically coupled. Again, this makes good biological sense from the standpoint that as a result of high affinity, cooperative binding by g32P monomers (which requires the B domain), the A domain becomes liberated to appropriately enable or disable any number of different activities. As to why the marked unfavorable influence of the A domain at equilibrium was not fully appreciated in previous studies, it could simply be that by removing only the N-terminal B domain, we are able to quantify polynucleotide binding of g32P at salt concentrations low enough such the effect is quite dramatic and easily measured. At these low concentrations of salt, both g32P and g32P-A simply bind too

\(^{7} \) \( K_{\text{obs}} \) 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 \( K_{\text{obs}} \) cannot be measured accurately with fluorescence techniques. For example, at more moderate \([\text{NaCl}](e.g. 0.4 \text{ M})\), the reported difference in \( K_{\text{obs}} \) 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 \( \approx 2\)-fold) as the solution \([\text{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 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 equilibria. 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 \(^1\text{H} \text{NMR}\) experiments which carefully examined the isolated site (mimicked by g32P-(A + B) binding to d(pA)\(_{40-60}\)) and the cooperative binding modes (intact g32P complexed with d(pA)\(_{40-60}\)) 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 \( K_{\text{obs}} \) 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 \( \delta \log K_{\text{obs}}/\delta \log [\text{NaCl}] \) 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. Youssif 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\(_x\), expression plasmid pTLOW, and S. Silber for help in acquiring the NMR spectra.

REFERENCES

N-terminal Deletion of T4 Gene 32 Protein

41. Portno, J. (1963) Pure Appl. Chem. 6, 233

Material and Methods

Materials

Animals were purchased from 11 animal colonies maintained in our laboratory (Materials at 4). The enzyme of the gene 32 protein coding sequences was the replicative form of an in-line replication in which the T4 gp22 structural gene is found in the 5' end with the E. coli plasmid and the 3' end with a lambda plasmid (kindly provided by Y. Saenko). The gp22 open reading frame was inserted into the plasmid pBR322 digested with the same enzymes. The gp22 coding sequences were then inserted into an ampicillin mini-culture line containing pBR322 (3.9 kb) which contains the T4 gp22 coding sequences in the opposite orientation relative to the lac promoter (1). This form of the protein was used as a template for oligonucleotide-directed mutagenesis according to Taylor et al. (2) in a set purificaton from expression vectors. 15.1, with the gp22 fusion protein

Methods

Subcloning of gp22 coding sequences into the T4 DNA polymerase expression vector

Materials. All buffers were prepared with double distilled and deionized Mill-Q water. T4 DNA polymerase was released according to Alberts et al. (39) using 0.6 M HCl denatured calf thymus DNA (Sigma) per 100 g of calf thymus (300 mg). T4 gp22 coding sequences were obtained from Sigma. Poly(dG) - poly(dG) and poly(dC) - poly(dC) were obtained from Sigma. Poly(dG) - poly(dG) and poly(dC) - poly(dC) were purchased from Millipore Cellfree Reactant (Oldendorf, CA). The following reactions were performed from either Nebraska Biolabs (Broken Arrow, OK) or BioMakor Chemicals (Khoury, Israel).

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N-terminal Deletion of T4 Gene 32 Protein

cytochrome c reductase activity against 50 mM sodium phosphate, pH 8, 30 mM NaCl and native microinjection (14.00000) to remove debris. The sample was then loaded onto two successive Sephadex G-25 (Pharmacia) spin columns (5x0.8 mL) equilibrated in 0.2M NaCl, 200 mM NaCl buffer. 

- NMR spectra were recorded with a JEOL JNM-GSX 400 spectrometer equipped with a T250 mm probe. Temperature was controlled to within ±0.1°C.

- Chemical shifts are reported relative to the internal standard, sodium3-(trimethylsilyl) 2,2,2-trifluoroacetamide (TFA). Changes in the residual water peak was accompanied by a 2.5 s relaxation delay prior to acquisition. A 1.5 s acquisition time and a 3.8 s pulse angle were used; 2000 transients were collected.

- Gel filtration of g232p. Analytical gel filtration was carried out with a Waters PowerLine High Pressure Liquid Chromatography system operating at ambient temperature (22.0°C) with flow-rate and solvent composition controlled by a Waters gradient system controller. The column employed was a diethylaminoethyl (DEAE)-Cellulose, 100x2.6 cm. The sample was loaded in 50 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.1% (v/v) 2-mercaptoethanol, 0.005% (v/v) sodium dodecyl sulfate, 0.04% (v/v) Triton X-100, and the elution was monitored at 280 nm. The column was calibrated with ribonuclease A, 5.28 mg; alpha-chymotrypsin, 4.8 mg; trypsin, 1.0 mg; 2-mercaptoethanol, 0.5 mg; and 2-mercaptoethanol, 0.1 mg.

- Protein characterization. Protein concentration was determined using the method of Lowry et al. [11] or by optical density measurement at 280 nm in a Beckman DU-70 spectrophotometer.

- Fluorescence measurements. Fluorescence spectra of the g232p derivative was measured on a Shimadzu RF-5301 spectrophotometer, equipped with a thermally controlled cell holder. The samples were excited at 280 nm. The excitation and emission slits were 5 and 10 nm, respectively. The excitation wavelength was fixed at 280 nm, and the emission was scanned from 300 to 500 nm. The fluorescence intensity was corrected for the scattering of the sample and the path length of the cuvette.

- Data analysis. The data were analyzed using the Origin software package (OriginLab Corporation, Northampton, MA). The fluorescence spectra were fitted to a single exponential function using a nonlinear least-squares fitting routine. The fitted parameters included the slope, the maximum fluorescence intensity, and the lifetime of the excited state.

Figure 2. DE-52 anion exchange chromatography of low-speed supernatant of BL21(DE3)/pET30a (L365). Lane A, absorbance at 260 nm (A260) and 280 nm (A280) of fractions collected following application of the linear (10% to 100%) gradient. Lane B, rechromatography of peak 1 on the ion-exchange column. Lane C, rechromatography of peak 2 on the ion-exchange column. Lane D, rechromatography of peak 3 on the ion-exchange column. Lane E, high-speed supernatant of BL21(DE3)/pET30a (L365). Lane F, rechromatography of peak 1 on the ion-exchange column. Lane G, rechromatography of peak 2 on the ion-exchange column. Lane H, rechromatography of peak 3 on the ion-exchange column. Lane I, rechromatography of peak 4 on the ion-exchange column. Lane J, rechromatography of peak 5 on the ion-exchange column. Lane K, rechromatography of peak 6 on the ion-exchange column. Lane L, rechromatography of peak 7 on the ion-exchange column. Lane M, rechromatography of peak 8 on the ion-exchange column. Lane N, rechromatography of peak 9 on the ion-exchange column. Lane O, rechromatography of peak 10 on the ion-exchange column. Lane P, rechromatography of peak 11 on the ion-exchange column. Lane Q, rechromatography of peak 12 on the ion-exchange column. Lane R, rechromatography of peak 13 on the ion-exchange column. Lane S, rechromatography of peak 14 on the ion-exchange column. Lane T, rechromatography of peak 15 on the ion-exchange column. Lane U, rechromatography of peak 16 on the ion-exchange column. Lane V, rechromatography of peak 17 on the ion-exchange column. Lane W, rechromatography of peak 18 on the ion-exchange column. Lane X, rechromatography of peak 19 on the ion-exchange column. Lane Y, rechromatography of peak 20 on the ion-exchange column. Lane Z, rechromatography of peak 21 on the ion-exchange column.
Figure 3. SDS-PAGE analysis of fractions resulting from chromatography of a DE-52 pool on a ssDNA-cellulose column. A. Elution profile of a DE-52 pool on ssDNA-cellulose plotted as absorbance at 280 nm vs. fraction number. B. The left gel shows the time-course of induction of g32P-B expression pertaining to this experiment. Cells harvested at 120 min were used to make the low-speed supernatant (cf. "Materials and Methods"), applied to the DE-52 column and pooled as indicated in Fig. 2A, resulting in the protein fraction designated DE-52 Pool (Load). This material was applied to the column and representative fractions from panel A are shown on the right gel. Flow-through, fractions 25, 27 and 30; 0.5M NaCl Elute, fractions 102, 104, 105, 106 and 108; 2M NaCl Elute, fraction 155. The fractions designated POOL were combined, dialyzed and applied to the phenyl-sepharose column described in Fig. 4.

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), (S) 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).
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