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Binding properties of T4 gene 32 protein fragments carrying partially cleaved terminal domains

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Analysis of fluorimetric equilibrium-binding isotherms of a proteolytic fragment of bacteriophage T4 gene 32 protein (g32P) lacking residues 1–9 shows that this region contains the site responsible for the function of the NH_2-terminal 'B' domain (residues 1–21). The end codon of the frameshift mutant g32P-PR201 has been identified as TAG at nucleotide position 852. The PR201 gene 32 product ends at Ser^{283} and carries a truncated COOH-terminal 'A' domain (residues 253–301). Fluorimetric titrations of g32P-PR201 with double-stranded DNA show that the functional residues of the A domain are located within the region spanning residues 284–301.

DNA-binding protein, single-stranded; Partial proteolysis; Peptide mapping; Equilibrium-binding isotherm

1. INTRODUCTION

The bacteriophage T4 gene 32 product (g32P) is one of a class of proteins that bind tightly and cooperatively to single-stranded (ss) DNA (for a review, see [1]). In its 301 amino acid residues [2] g32P contains three structural domains; the NH_2- and COOH-terminal domains can be cleaved by a variety of proteolytic enzymes to generate functionally active fragments (see [1], and references therein). Removal of the NH_2-terminal 'B' region (residues 1–21) results in a g32P fragment that binds noncooperatively to ssDNA and no longer forms self-aggregates in solution [1]. Removal of the COOH-terminal 'A' region (residues 253–301) enables g32P to denature naturally occurring double-stranded (ds) DNA in vitro [3,4]. Although the Lys^{253}-Lys^{254} bond is the most sensitive to tryptic cleavage in g32P [4], the region spanning residues 253–275 contains several cleavage sites for trypsin and other proteases; the g32P fragments thus generated appear to exhibit identical properties in vitro [1]. The functional residues of the A peptide must therefore be contained within residues 276–301. To further determine the location of the essential residues, we have studied the frameshift mutant PR201 of g32P, which lacks ~15 residues from the COOH-terminus (see below). A similar study was carried out for the NH_2-terminal domain, since it has been reported [5] that the V8 protease of *Staphylococcus aureus* removes the first nine amino acid residues of g32P to yield a protein fragment (g32P-B′) with no measurable affinity towards g32P-agarose columns. The equilibrium-binding properties of g32P-B′, however, have not been previously investigated.

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Abbreviations: ds, double stranded; g32P, gene 32 product; g32P-A, g32P-B, g32P-B′, cleaved g32P lacking residues 254–301, 1–21 and 1–9, respectively; g32P-PR201, a frameshift mutant of g32P; ss, single stranded

2. MATERIALS AND METHODS

Homogeneous g32P was prepared from T4-infected cells by using standard procedures [6]. g32P-B′ was purified from a limited digest of g32P by the *S. aureus* V8 protease. The digestion was carried out for 7 h at 37°C in a 50 mM $(NH_4)HCO_3$

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buffer with a 1:280 (w/w) ratio of protease. The sample was applied to a ssDNA-cellulose column equilibrated with 50 mM $(NH_4)HCO_3$ and eluted with a linear gradient of 0–1 M NaCl in the same buffer.

T4 phages containing the PR201 mutation were grown in the *E. coli* su$^+$1 strain KL239, and precipitated from cell lysates by sedimentation in the presence of polyethylene glycol [7]. Phage titer was determined by plaque assays. g32P-PR201 was prepared by inoculating the *E. coli* su$^-$ strain BW46 with the mutated phage at a multiplicity of infection of 1.5. The protein was purified by affinity chromatography on ssDNA-cellulose as described [6] with an additional step on a DEAE-cellulose column equilibrated with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% (v/v) glycerol; elution was at a [NaCl] = 0.2 M by a linear gradient of NaCl (0–0.5 M) in the same buffer.

Protein concentration and amino acid compositions were determined using a Beckman 121-M analyzer. For HPLC peptide mapping a total tryptic digest of g32P-PR201, obtained by previously published protocols [6], was injected onto a Waters Associates C-18 μBondapak column equilibrated at a flow rate of 0.7 ml·min^{-1} with 99% buffer A (10 mM potassium phosphate, pH 2.55) and 1% buffer B (0.05% trifluoroacetic acid in acetonitrile). Peptides were eluted by increasing the concentration of buffer B as follows: 0–86 min (1–30%), 86–129 min (30–60%), 129–143 min (60–100%).

Fluorescence measurements were carried out as previously described [8]. Equilibrium-binding isotherms were determined by using standard procedures [9].

3. RESULTS AND DISCUSSION

3.1. *Binding of the g32P-B′ fragment to thymine oligo- and polynucleotides*

Binding of poly(dT) to g32P-B′ results in quenching of its intrinsic tryptophan fluorescence emission (fig.1). Extrapolation of the initial slope to the limiting fluorescence quenching (the plateau region) allows calculation of the occluded binding site size (n). The value found for the g32P-B′/poly(dT) system (n = 7.3) is in good agreement with those reported for g32P and the tryptic fragment g32P-B [1]. However, the binding constant of g32P-B′ for poly(dT) (4.4 × 10^6 M^{-1}) is three orders of magnitude lower than that of g32P [10]. A similar reduction is observed for the tryptic fragment g32P-B [3]. If the intrinsic constant (K_{int}) is similar for the g32P-B and g32P-B′ systems, removal of the first 9 amino acid residues from the N-terminus induces the same effect as the removal of the entire B domain. This has been investigated by binding g32P-B′ to p(dT)$_8$ and p(dT)$_{16}$.

Fluorimetric titrations of g32P-B′ with d(pT)$_8$ (fig.1) show a limiting fluorescence quenching (39%) similar to that observed for g32P with the same oligonucleotide (35%) [11]. The affinity of g32P-B′ for d(pT)$_8$ (1.2 × 10^6 M^{-1}) is also similar to that of g32P (6 × 10^5 M^{-1}) [12] and g32P-B (1 × 10^6 M^{-1}) [3] for this oligonucleotide. Since the binding-site size is ~7 bases, no cooperativity is expected for the binding to d(pT)$_8$ (K_{app} = K_{int}). Binding of g32P-B′ to d(pT)$_{16}$ exhibits a stoichiometry of two protein molecules per oligonucleotide molecule (fig.1) and a K_{app} = 6.9 × 10^6 M^{-1}. The similarity of association constant and limiting fluorescence quenching for the binding of g32P-B′ with d(pT)$_{16}$ and poly(dT) strongly suggest that g32P-B′ exhibits weak cooperativity, if any. Therefore, residues 1–9 contain the functional residues of domain B.

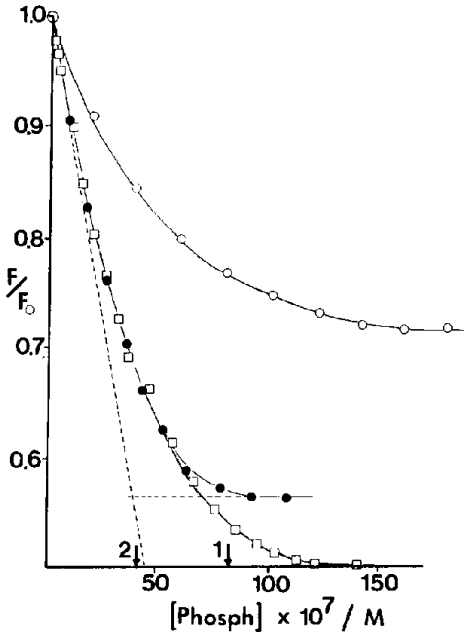

Fig.1. Fluorescence titrations of g32P-B′ (5.3 × 10^{-7} M) with d(pT)$_8$ (○), d(pT)$_{16}$ (□), and poly(dT) (●). Measurements were carried out at 25°C in a 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.2, using an excitation wavelength of 282 nm and an emission wavelength of 340 nm. The stoichiometric point for 1:1 and 2:1 (d(pT)$_{16}$:g32P-B′) complexes are indicated by arrows.

3.2. *Characterization of g32P carrying the frameshift mutation PR201*

Electrophoresis of g32P-PR201 samples in SDS-polyacrylamide gels suggests that the mutated protein is ~2 kDa smaller than the wild-type g32P

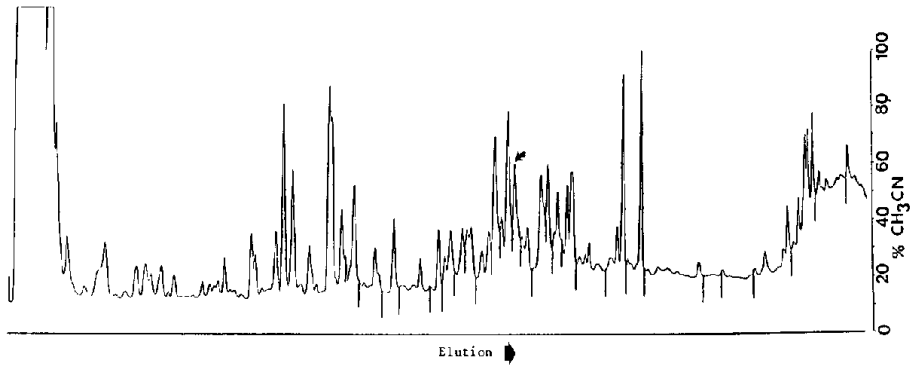

Fig.2. HPLC elution profile of a total tryptic digest of g32P-PR201, using a monitoring wavelength of 210 nm. An arrow indicates the position of the truncated COOH-terminal peptide (T-31′).

(data not shown). Its identification as a frameshift mutant ((Karam, J., personal communication) implies that g32P-PR201 is truncated at the COOH-terminus and lacks about 15 amino acid residues. Total tryptic hydrolysis of g32P-PR201 will yield a fragment of peptide T-31 (residues 274–301). T-31 is a hydrophobic peptide which is eluted in HPLC peptide mapping experiments at high concentration in organic solvent [13] and has the highest content of serine and leucine residues of all tryptic peptides [2]; this allows identification of the fraction containing the truncated peptide T-31′ (fig.2, arrow; table 1). The presence of lysine indicates that there is contamination by another peptide (the

Table 1

Amino acid composition of the chromatographic fraction containing the COOH-terminal peptide of g32P-PR201

Amino acid[a]		Lys = 1	AA residues/ mol peptide	T-31′ (274–283)	T-7 (35–46) + T-6 (33–34)	T-31[b]
Cysteine	–	–	–	–	–	–
Aspartic acid/asparagine	16.05	5.26	5	2	3	9
Threonine	3.51	1.15	1	1	–	2
Serine	12.89	4.23	4	4	–	8
Glutamic acid/glutamine	5.26	1.72	2	1	1	1
Proline	–	–	–	–	–	–
Glycine	6.40	2.10	2	–	2	1
Alanine	5.78	1.90	2	–	2	1
Valine	3.25	1.07	1	–	1	–
Methionine	1.91	0.63	1	1	–	1
Isoleucine	1.59	0.52	1	–	1	–
Leucine	6.24	2.05	2	–	2	4
Tyrosine	0.60	0.20	0	–	–	–
Phenylalanine	3.66	1.20	1	1	–	1
Histidine	0.76	0.25	0	–	–	–
Lysine	3.05	1.00	1	–	1	–
Arginine	–	–	–	–	–	–
Tryptophan	–	–	–	–	–	–
Norleucine	19.11					

[a] Amino acid content (nmol). 20 nmol of norleucine were added as standard
[b] From the sequence in [2]

COOH-terminal tryptic peptide should not contain lysine). The amino acid content was normalized by the lysine content (table 1), therefore assuming that the mixture of the two peptides eluted at this position is equimolar. This is not unlikely, since the recovery yield of different peptides is similar in this zone of the gradient; the nearly even values obtained for several residues also support this assumption. The normalized amino acid composition contains a subset of that expected for the peptide T-31; assignment from the NH_2-terminus of T-31 suggests that T-31′ ends at Ser^{283}. The DNA sequence of g32P [14] shows that there is an amber (TAG) codon out of the normal reading frame near that position, which is read as an end codon due to the deletion of two nucleotide bases. This is the only amber codon present in the DNA sequence encoding the T-31 peptide in the three reading frames. Subtraction of the amino acid composition of T-31′ allows identification of the co-eluting peptide as T-6 + T-7 (a result of incomplete tryptic cleavage). T-6 is the dipeptide Leu^{33}-Lys^{34}; T-7 (residues 35–46) does not contain lysine [2].

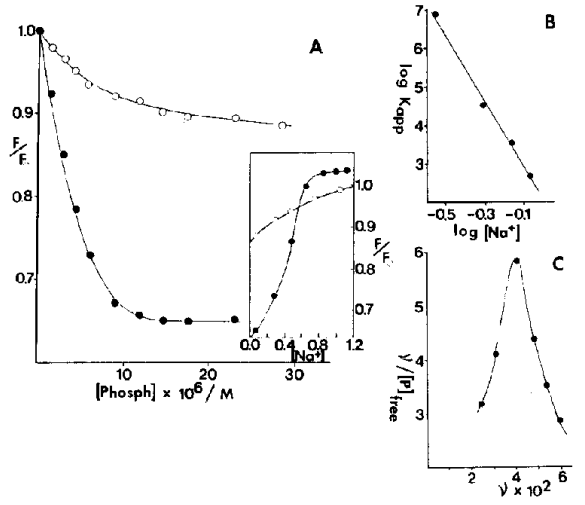

Fig.3. (A) Fluorimetric titration of g32P (2.9×10^{-7} M) (○) and g32P-PR201 (7.5×10^{-7} M) (●) with calf thymus double-stranded DNA. Experimental conditions are as described for fig.1. (Inset) Salt-back titration of the respective complexes. (B) Association constant of g32P-PR201 binding to calf thymus dsDNA as a function of sodium ion concentration. (C) Scatchard plot of the g32P-PR201 equilibrium-binding isotherm of A.

3.3. *Binding of g32P-PR201 to double-stranded DNA*

Fluorimetric titrations of wild-type g32P and g32P-PR201 with calf thymus ds DNA are shown in fig.3A. g32P binds ds DNA with an affinity constant of 7×10^5 M^{-1}, while g32P-PR201 has a $K_{app} = 3.3 \times 10^7$ M^{-1} (at the [g32P-PR201] used in our titrations, this value is a lower limit) and $n \sim 10$ (binding to 6 ± 1 base pairs). The limiting fluorescence quenching of g32P-PR201 bound to ds DNA approaches that induced by ss polynucleotides, as previously reported for the tryptic g32P-A fragment [15]. Analysis of salt-back titrations (fig.3A, inset) indicates a d(log K_{app})/d(log [NaCl]) = −8.5 (fig.3B), comparable to the value of g32P for ss polynucleotides (−6.7 ± 1.4) [16]. Extrapolation to the ionic strength used for our fluorimetric titrations suggests that the affinity of g32P-PR201 for ds DNA exceeds 10^8 M^{-1}. A Scatchard plot of our titration data (fig.3B) is humped upwards, indicating a significant cooperativity [16]. For systems where the cooperativity parameter (ω) is much larger than the binding site size ($\omega > 10n$), the maximum of the Scatchard plot is within the range $\omega/2n < \{(\nu/[P]_{free}) \times (1/K_{int})\}_{max} < \omega/n$. Assuming $K_{int} \sim 10^6$ M^{-1} (as for the noncooperative binding of g32P to ss DNA), g32P-PR201 binds to ds DNA with $\omega \sim 10^2$ ($60 < \omega < 120$).

4. CONCLUSION

Fluorimetric equilibrium-binding isotherms of g32P-B′ exhibit loss of the cooperative binding characteristic of the intact protein. On the other hand, the mutant g32P-PR201 is able to bind and destabilize double-stranded DNA, as shown by the strong cooperativity typical of binding to single-stranded polynucleotides. These results locate the functional residues of domains B and A within the regions spanning residues 1–9 and 284–301, respectively.

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