The intrinsic fluorescence of the T4 helix-destabilizing protein specified by gene 32 (32P) is not altered by the proteolytic removal of either the 6200-dalton COOH-terminal "A" region (32P*-A) or both the A and the 2300-dalton NH2-terminal "B" region (32P*-(A+B)).

The intrinsic fluorescence of 32P, 32P*-A, and 32P*-(A+B) is decreased 25% by the addition of d(pT)6 and 34% by the addition of poly(dT). Saturation binding curves of the percentage of change in protein fluorescence on a molar basis of nucleic acid can show that the intact 32P as well as the two proteolysis-generated fragments all have association constants of \(10^6\) M\(^{-1}\) for d(pT)6. This demonstrates that the DNA binding site is not contained within either the A or B regions of 32P. Both 32P and 32P*-A bind cooperatively to poly(dT) as evidenced by a 400- to 1000-fold increase in association constant for poly(dT) compared to d(pT)6. Since within the limits of our measurements 32P and 32P*-A bind equally well to poly(dT) (\(K_{assoc} \approx 5 \times 10^8\) M\(^{-1}\)), the enhanced helix-destabilizing properties previously reported for 32P*-A cannot be accounted for by a significant increase in binding affinity of 32P*-A for single-stranded DNA. The binding constant for the 32P*- (A+B):poly(dT) complex is only 3-fold higher than that for the 32P*-(A+B):d(pT), complex, which confirms our proposal that the B region is essential for cooperative 32P:32P protein interactions.

The fluorescence of 32P, 32P*-A, and 32P*-(A+B) in the presence of poly(dT) showed that the B region is essential for cooperative 32P:32P protein interactions.

**Fluorescence Measurement of DNA-Binding Parameters**

Eleanor K. Spicer,† Kenneth R. Williams, and William H. Konigsberg
From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

The purification of gene 32 protein and its partial proteolytic cleavage products is discussed in detail in the preceding paper (1). Protein concentrations were determined by amino acid analysis on a Beckman 121 M amino acid analyzer. Proteins were stored in small aliquots in 10 mM Tris/HCl (pH 8.0), 1 mM \(\beta\)-mercaptoethanol, 50 mM NaCl, and 10% glycerol, at -20°C. Repeated freezing and thawing was found to reduce the DNA binding activity of gene 32 protein and its proteolytic fragments.

Nucleic Acid Ligands—d(pT)6 and poly(dT) were purchased from Collaborative Research, Inc. (Wall, Mass.). The molar extinction coefficient at 260 nm (per mol of nucleoside monophosphate) used in the calculations was 9.1 \(10^4\) cm\(^{-1}\) M\(^{-1}\). For both ligands.

Fluorescence Spectra—Fluorescence spectra were measured on an Aminco-Bowman spectrofluorimeter (American Instrument Company) equipped with a Kipp Zonnen linear chart recorder. A 150-watt xenon lamp (Hanovia, Inc.) was used as a light source. Slits of 5-mm width were placed at the entrance and exit of the sample chamber, and fluorescence was detected at 90° relative to incident excitation light.

Fluorometric Binding Measurements—Protein solutions of 1 to 3 \(10^{-7}\) M concentration (2 to 3 ml volume) were placed in a quartz fluorimetric cuvette containing a Teflon-coated stirrer. The fluorometer sample chamber was equipped with a magnetic stirrer and all measurements were made while the solution was agitated. Five- to 10-\(\mu\)l aliquots of oligo- and polynucleotides were injected into the protein solution using a Hamilton syringe inserted through a small hole in the sample chamber cover. Stepwise titration of fluorescence quenching was monitored at a constant emission wavelength of 348 nm, with constant chart speed. This enabled correction of fluorescence changes for noise introduced by changes in lamp output. All measurements were made at 25 ± 2°C.

When gene 32 protein is diluted to concentrations below 1 \(10^{-7}\) M its DNA binding activity is reduced after standing at 25°C (i.e., a solution is 80% active after 10 min). For this reason, solutions of >1 \(10^{-7}\) M were used, and protein was diluted directly into the fluorimeter cuvette, keeping total elapsed time during fluorescence measurements <5 min.

All experiments were performed in the standard buffer used by Kelly et al. (3) so that our results would be directly comparable. The standard buffer consists of 50 mM Na2HPO4, 1 mM Na2EDTA, and 1 mM \(\beta\)-mercaptoethanol (pH 7.7). 32P has been reported to be mainly in the monomer form at the concentrations used in these studies (5).
Changes in 32P fluorescence were corrected for quenching due to oligonucleotide absorption of incident radiation by subtracting the normalized change in fluorescence of N-acetyltryptophanamide due to oligonucleotide addition. A reference curve of 100 ΔF/F° versus [d(pT)₈] was plotted for N-acetyltryptophanamide for each series of experiments using solutions of total fluorescence equivalent to that of 32P.

**Calculation of Binding Constants**—The association constants for the noncooperative binding of protein to d(pT)₈ were computed from double reciprocal and Scatchard plots (6). The assumption was made that only a single 32P molecule can bind to d(pT)₈, thus the concentration of free d(pT)₈ can be determined by subtracting the concentration of bound protein from the initial d(pT)₈ concentration. The concentration of bound protein was determined from the ratio of the fluorescence change at the particular d(pT)₈ concentration to the maximum fluorescence change (ΔF/ΔFₘₐₓ × initial protein concentration). Double reciprocal plots were constructed in which 1/ΔF was plotted versus 1/(d(pT)₈) (3). The maximum fluorescence change can be determined from the y intercept of these plots, 1/ΔF°. The slope, 1/Kₐₐₐₐ, allows measurement of Kₐₐₐₐ, the association constant. The Scatchard analysis was made in the conventional manner (6) and yielded values which were in good agreement with those computed by reciprocal plot analysis.

Determination of the intrinsic association constant for the cooperative binding of a protein to a multiple site lattice such as poly(dT)₈ is considerably more difficult than the conventional approach required for protein-single site ligand interactions (7). Graphical analysis, which has the advantage of computing Kₐₐₐₐ, values based on all experimental points, becomes difficult to interpret when the interactions involve large cooperativity factors (ω > 10). Thus we have computed an apparent binding constant from the saturation binding curves using the approach described by Kelly et al. (3).

The stoichiometry of binding (the number of nucleotides covered by each protein monomer) for 32P and 32P*-A was estimated from the intersection of the lines approximating the initial and final slopes of the binding curve. The fractional saturation of the protein (6) in the presence of a stoichiometric ratio of nucleotides to protein was determined by monitoring the percentage of maximal change in fluorescence upon nucleotide addition. Kₐₐₐₐ was determined by substituting the computed θ value (representing the ratio of bound to total protein) in the following equilibrium expression

\[ K_{app} = \frac{\theta}{(1 - \theta)[P_s]} \]

where \([P_s]\) is the total concentration of protein.

The association constants we report are averages of three experiments, each varied by a maximum of a factor of two. The error range was largely a function of differences in the activity of the protein preparations.

**RESULTS**

**Proteolytic Removal of the A and B Regions From Gene 32 Protein Does Not Change Its Intrinsic Fluorescence**—Kelly and von Hippel (4) have demonstrated that the intrinsic fluorescence of gene 32 protein is characterized by an excitation maximum at 280 nm and an emission maximum at approximately 350 nm whose intensity and spectral position is a function of the immediate environment of the tryptophan residues. Since all five tryptophan residues are located in the 32P fragment that has had the COOH-terminal A and NH₂-terminal B regions removed (32P*-A+B), it was expected that the fluorescence properties of 32P, 32P*-A, and 32P*-A+B would be similar. This is indeed the case (Fig. 1). The fluorescence emission intensity (at 350 nm) of the two trypsin-generated fragments was the same as that of the intact protein (on a molar basis), and the similarity of peak shape and maxima position for 32P, 32P*-A, and 32P*-A+B suggests that the tryptophan residues are in similar environments in each protein species. Hence, by this criterion, the removal of the A and B regions does not alter the conformation of the core region of gene 32 protein.

**Removal of the A and B Regions Does Not Affect the Affinity of the Proteolytic Fragments for d(pT)₈**—The partial quenching of gene 32 protein fluorescence produced by nucleotide binding (4) was used to measure the affinity of 32P, 32P*-A, and 32P*-A+B for d(pT)₈. The choice of d(pT)₈ as a ligand was dictated by the desire to distinguish the domains of 32P which are involved in DNA binding from those involved in the cooperative protein-protein interactions which lead to a tighter binding to DNA. The nucleotide-binding site size for 32P has been reported to be approximately six nucleotides (3, 8), and the binding of 32P to d(pT)₈, unlike the binding to shorter oligonucleotides, results in a large quenching of protein fluorescence (4).

Fig. 2 shows the saturation binding curves generated by...
measuring the percentage of change in protein fluorescence (100 \( \Delta F/F^0 \)) as a function of \( d(pT)_\text{h} \) concentration. The maximum fluorescence quenching was 21 to 24% for both 32P and the proteolytically derived fragments (Table I), suggesting that \( d(pT)_\text{h} \) interacts with or influences the environments of the tryptophan residues of each protein in the same manner. This is additional evidence that the structural integrity of the core region of 32P remains unchanged after partial proteolysis.

To ensure that the quenching of protein fluorescence which we have measured results solely from the binding of \( d(pT)_\text{h} \) to the proteins, parallel experiments were performed in standard buffer containing 2 M NaCl, conditions which do not allow 32P to bind \( d(pT)_\text{h} \). As anticipated, under these conditions the addition of saturating amounts of \( d(pT)_\text{h} \) to 32P did not result in a decrease in fluorescence.

The association constants for \( d(pT)_\text{h} \) binding to 32P and its proteolytic derivatives were calculated from the fluorescence quenching data by analysis of double reciprocal and Scatchard plots, as detailed under “Materials and Methods.” The double reciprocal plots for 32P, 32P*-A, and 32P*-(A+B) derived from the binding curves are given in Fig. 3. The results of the double reciprocal analysis, summarized in Table II, were in good agreement with the results of the Scatchard analysis (data not shown). The association constant for the (noncooperative) binding of 32P to \( d(pT)_\text{h} \) is \( 6.0 \times 10^9\text{ M}^{-1}\text{ }\text{molecule}^{-1} \), in excellent agreement with the previously reported \( K_{\text{assoc}} \) of \( 3.3 \times 10^9\text{ M}^{-1}\text{ }\text{molecule}^{-1} \) (3), while the association constants for \( d(pT)_\text{h} \) binding to 32P*-A and 32P*-(A+B) are \( 9.8 \times 10^9\text{ M}^{-1}\text{ }\text{molecule}^{-1} \) and \( 1.2 \times 10^9\text{ M}^{-1}\text{ }\text{molecule}^{-1} \), respectively. These data demonstrate that the affinity of 32P for \( d(pT)_\text{h} \) is not reduced by removal of either the A or both the A and B regions.

**Table I**

| Protein | Fluorescence quenching | \( d(pT)_\text{h} \) | Poly(dT) |
|---------|------------------------|----------------|-----------|
| 32P     | 24.5                   | 32.0           |
| 32P*-A  | 21.0                   | 36.5           |
| 32P*-(A+B) | 24.0               | 33.0           |

* Values varied depending upon the binding activity of the protein preparation and are reported for the most active preparation examined. \( \Delta F_{\text{max}} \) is the maximum observed decrease in fluorescence.

**Fig. 3.** Double reciprocal plots of the data in Fig. 2 for the binding of \( d(pT)_\text{h} \) to gene 32 protein and its partial cleavage products. \( \Delta F \) is the change in fluorescence (arbitrary units) at 348 nm.
stantially to the affinity for DNA. In contrast, the association constant for 32P*- (A+B) binding to poly(dT) is $3.4 \times 10^{6}$ M$^{-1}$, two orders of magnitude less than the association constants for the 32P-poly(dT) and 32P*-poly(dT) complexes, with the result that the $\omega$ value for the 32P*- (A+B):poly(dT) complex is only 3. This confirms qualitatively that removal of the B region virtually eliminates the ability of 32P*- (A+B) to bind cooperatively to DNA.

**DISCUSSION**

Moise and Hosoda have previously shown (12) that the interaction of gene 32 protein with DNA is profoundly affected by the proteolytic removal of the NH$_2$-terminal B region or the COOH-terminal A region. Loss of the B region from 32P results in decreased affinity for DNA while loss of the A region from 32P enables the resulting protein to bind dsDNA cellulose (12) and to denature T4 DNA (13), properties not exhibited by the intact 32P.

We have proposed that the B region is essential for cooperative binding of 32P to ssDNA (1), based on the results of differential scanning microcalorimetry of 32P, 32P*-A, and 32P*- (A+B) in the presence of poly(dT). In order to quantitatively estimate the relative contribution of the B region to the 32P:poly(dT) and to 32P:DNA interactions it was necessary to compare the binding constants of 32P*-A and 32P*- (A+B) for d(pT), and for poly(dT). We have shown that 32P*-A and 32P*- (A+B) have similar association constants for d(pT), which confirms that the B region does not contribute to the strength of 32P:DNA interactions. In contrast, 32P*-A binds two orders of magnitude better than 32P*- (A+B) to poly(dT). This 100-fold difference in binding affinity is best accounted for by the contribution of the B region to 32P:poly(dT) interactions.

The exact nature of the involvement of the A region of 32P in DNA binding is not clear. Since 32P*-A but not 32P can denature double-stranded T4 DNA (12), Moise and Hosoda suggested the A region is important for controlling the helix-stabilizing activity of 32P. Presumably, interaction of the A region with other proteins in the replication complex might have the same effect as the proteolytic removal of the A region and thus provide a means for localizing the helix-stabilizing activity of 32P to the region just in front of the replication complex (12). Based on its ability to denature dsDNA and on the calorimetry data in the preceding paper (1), we expected 32P*-A to have a higher affinity than 32P for poly(dT). However, using fluorescence titration no significant difference was found in the association constants for the 32P:poly(dT) and 32P*-Apoly(dT) complexes. The ability of 32P*-A to denature dsDNA is, therefore, either due to an increase in affinity for ssDNA which is too slight to detect using the approximations described by Kelly et al. (3) to determine “apparent” binding constants or it results from some other property of 32P*-A. Two other possible explanations for the enhanced helix-stabilizing activity of 32P*-A compared to 32P are that unlike 32P, 32P*-A binds to double helical DNA and subsequently forces the two strands apart or, more likely, that proteolytic removal of the A region of 32P removes the “kinetic block” that Jensen et al. suggest keeps 32P from denaturing T4 dsDNA in vivo (8). That is, 32P*-A has a faster “on rate” for binding ssDNA than 32P and for this reason 32P*-A can trap transiently “open” single-stranded regions of native DNA which 32P is unable to do for kinetic reasons. We plan to examine the kinetics of 32P and 32P*-A binding to DNA, to test the validity of this hypothesis and its relation to an in vivo mechanism for the control of the helix-stabilizing activity of 32P.

**Acknowledgments**—We are very grateful to Dr. Clifford Slayman for use of the Amino-Bowman spectrofluorimeter. We thank Mary LoPresti for her expert preparation of the T4 gene 32 protein and Dr. Steve Sligar for helpful discussions and critical reading of the manuscript.

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