Comparison of Cooperative and Isolated Site Binding of T4 Gene 32 Protein to ssDNA by ^{1}H NMR†

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ABSTRACT: Deuteriation of all aromatic protons of gene 32 protein (g32P) from phage T4, followed by selective introduction of specific protons, has allowed the precise identification of the number and magnitude of the chemical shift changes induced in the aromatic protons when g32P binds noncooperatively or cooperatively to nucleotides. Signals from five Tyr residues are shifted by binding of g32P to $d(pA)_8$ or $d(pA)_{40-60}$; however, the change from noncooperative, $d(pA)_8$, to cooperative, $d(pA)_{40-60}$, binding causes significant increases in the magnitudes of the shifts for only two of these Tyr signals. These two Tyr residues may interact directly with the nucleotide bases, while the shifts associated with the other three Tyr may be due to conformational changes in g32P upon ssDNA binding. Similar conclusions can be drawn for two of the six Phe residues whose protons undergo shifts upon nucleotide binding. Observation of selected proton signals allows for the first time detection by ^{1}H NMR of changes in the proton signals from two Trp residues upon nucleotide binding. The side chains of two Tyr, one or two Phe, and one Trp are probably directly involved in nucleotide base–protein interactions. As assayed by the signals from the H2 and H8 protons of adenine, the bases of a bound nucleotide are undergoing a fast chemical exchange in the noncooperative mode of binding, but shift to slow exchange upon assuming the cooperative mode of ssDNA interaction. When bound to a polynucleotide, the A domain of g32P (residues 254–301) becomes more mobile, as reflected in sharpening of the ^{1}H NMR signals from the A domain. This supports the model in which ssDNA binding creates an enhanced potential for interaction of the A domain with auxiliary proteins required for T4 replication in vivo. A comparison of chemical shift changes in protons of g32P induced upon binding to oligo- and polynucleotides reveals no significant structural differences between the sites utilized in the two modes of binding.

Among proteins binding to single-stranded DNA and required for DNA replication, recombination, and repair in most cells, gene 32 protein (g32P)[1] from bacteriophage T4 is the prototype. The protein binds to single-stranded nucleic acids cooperatively without sequence specificity [for a review see Chase and Williams (1986)]. Gene 32 protein has been shown by limited proteolysis to have a distinctive three-domain structure: a C-terminal negatively charged domain (A) of 48 amino acids interacting with T4 DNA polymerase and other proteins of the replisome; a positively charged N-terminal domain (B) of 21 amino acids that modulates protein–protein interactions between cooperatively bound g32P monomers; and a central "core" domain [residues 22–253 (g32P*)] that is highly resistant to proteolysis and contains the nucleotide binding site (Spicer et al., 1979; Prigodich et al., 1984). The core domain contains a single Zn(II) coordinated to three Cys residues which is responsible for the great resistance of the core to further proteolysis by trypsin (Giedroc et al., 1987, 1989; Coleman & Giedroc, 1989). Removal of the A and B domains by trypsin produces g32P* or g32P-(A+B) which shows a large reduction in cooperative binding affinity for an infinite nucleotide lattice, while the intrinsic binding affinity for a single site on a ssDNA lattice (6–8 nucleotides) is unchanged (Spicer et al., 1979).

^{1}H NMR studies of the g32P core and its complexes with oligonucleotides up to 10 bases long are possible, and such studies have shown that five tyrosyl and two phenylalanyl residues appear to participate in ssDNA binding (Prigodich et al., 1984, 1986). Oligomerization of the holoprotein has prevented detailed NMR studies of the unproteolyzed species of g32P (Prigodich et al., 1984). Subsequent site-directed mutagenesis of the Tyr residues has established that at least five Tyr side chains contribute either directly or indirectly to the ssDNA binding affinity (Shamoo et al., 1989). Chemical cross-linking studies also suggest that Phe^{183} is located near the ssDNA binding groove of g32P (Shamoo et al., 1988).

The data from the earlier ^{1}H NMR studies are subject to some ambiguity, however, since the spectra are complex and poorly resolved, and therefore difference spectra were the primary means of detecting spectral changes (Prigodich et al., 1984, 1986). Questions also can be raised as to whether g32P*–oligonucleotide complexes are adequate models for the in vivo protein–ssDNA complexes. We have now simplified the aromatic proton spectra of g32P by placing a single type of aromatic proton in a completely perdeuteriated background. This not only allows us to follow unambiguously the chemical shifts of individual Tyr and Phe residues upon oligonucleotide binding but also allows ^{1}H NMR studies of polynucleotide binding in which the fully cooperative mode of binding is present. Application of selective deuteriation enabled us for

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[1] Abbreviations: g32P, gene 32 protein; g32-(A+B), g32P core protein; g32P*, g32P residues 22–253; NMR, nuclear magnetic resonance; TSP, sodium (trimethylsilyl)tetradeuteriopropionate.

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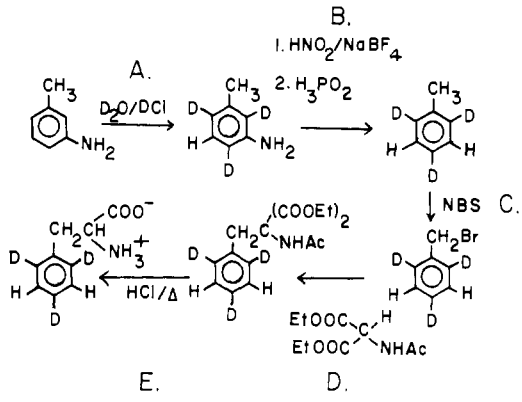
Scheme I



the first time to resolve signals of individual Trp residues and to detect chemical shift changes of Trp signals upon ssDNA binding.

Materials and Methods

Deuteriated Aromatic Amino Acids. Perdeuteriated phenylalanine, tyrosine, and tryptophan were obtained by proton/deuterium exchange in sulfuric acid (Griffith et al., 1976; Matthews et al., 1977). [3,5-^{1}H]Tyr was obtained by exchange of perdeuteriated tyrosine in 20% sulfuric acid at 90 °C for 3.5 days. Greater than 90% of the 3- and 5-positions relative to the β-^{1}H were reexchanged to protons. [2,5-^{1}H]Trp was obtained by exchange of perdeuteriated tryptophan in 1% sulfuric acid at 60 °C for 4.5 days. About 50% of position 2 and 25% of positions 5 were reexchanged for protons.

[3,5-^{1}H]Phe was synthesized as shown in Scheme I. Step A was hydrogen/deuterium exchange of *m*-toluidine in D_2O/DCl under reflux (yield ~100%). Step B was treatment with HBF_4/HNO_2 and H_3PO_2 according to the method of Korzeniowski et al. (1977) (yield ~90%). Step C was a standard NBS reaction catalyzed in the presence of perbenzoic acid (Tuleen & Hess, 1971) (yield ~65%). Step D was treatment with AcHNCH(COOEt)$_2$ according to the method of Ott (1981) (yield ~85%). Step E was treatment with 1% HCl under reflux (Ott, 1981) (yield ~90%). About 60% of the 3- and 5-positions are occupied by protons relative to the β-^{1}H. Preparation of selectively deuteriated g32P is described in Pan et al. (1989). The derivatives of g32P* containing only 3,5-ring protons have been designated [3,5-^{1}H]Tyr-g32P* and [3,5-^{1}H]Phe-g32P*, while the derivative containing Trp with protons at the 2- and 5-positions is designated [2,5-^{1}H]Trp-g32P*.

^{1}H NMR spectra were recorded at 30 °C with a Bruker AM-500 spectrometer. Protein samples were prepared for NMR by exchange with deuteriated buffer on a Sephadex G-25 spun column. Typical experiments were performed on samples 0.20–0.25 mM in g32P* or 0.1–0.4 mM in g32P. Buffers were 50 mM sodium phosphate, pH 7.6, and 50 mM NaCl unless otherwise stated. Chemical shifts are reported relative to TSP as an internal standard.

Results

*^{1}H NMR of [3,5-^{1}H]Tyr-g32P**. The 3,5-^{1}H signals of [3,5-^{1}H]Tyr-g32P* are all singlets, suggesting that all eight Tyr rings flip relatively rapidly (Figure 1A). One of the Tyr residues (8) has a chemical shift of its 3,5-protons that is unusually far upfield (5.6 ppm). This residue may be clustered with other aromatic residues in the core (see the following). Despite the fact that g32P-(A+B) now appears to exist as a well-defined dimer at the concentrations used for the NMR

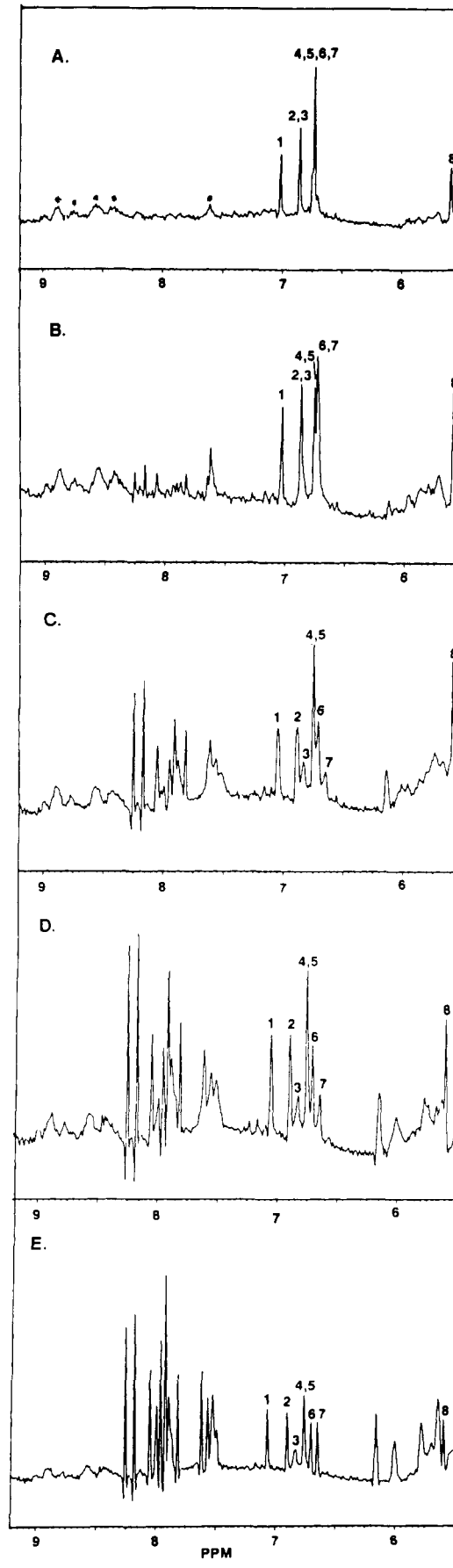


FIGURE 1: Proton signals for the Tyr 3,5-protons during titration of [3,5-^{1}H]Tyr-g32p* with d(pA)$_8$. Nucleotide was added in aliquots to 0.25 mM [3,5-^{1}H]Tyr-g32P* (A) to reach the following nucleotide:protein molar ratios: (B) 1:1; (C) 2:1; (D) 2.5:1; (E) 2.5:1 plus 250 mM NaCl. Signals marked with an asterisk are from nonexchanged amide protons.

Table I: Aromatic Proton Resonances Expected from Gene 32 Protein and Gene 32 Protein-(A+B) (G32P*) and the Shifts in These Proton Resonances Induced by Nucleotide Binding

(A) Aromatic Proton Resonances

	no. of aromatic residues (minimal no. of proton signals expected)		no. of proton signals found in g32P*
	g32p[a]	g32p*	
Tyr	8 (8)	8 (8)	8
Phe	18 (18)	14 (14)	15[b]
Trp	5 (10)	5 (10)	10
His	2 (4)	2 (4)	4[c]

(B) Summary of Chemical Shift Changes upon ssDNA Binding

protein and nucleotide	upfield shifts[d] (ppm)	downfield shifts (ppm)
G32P-d(pA)$_{40-60}$	Tyr^7 (−0.50)	Tyr^1 (+0.10)
	Tyr^6 (−0.25)	Tyr^2 (+0.03)
	Tyr^3 (−0.05?)	
		adenine[e] H2 (+0.39)
		adenine H8 (+0.09)
G32P*-d(pA)$_8$	Tyr^7 (−0.11)	Tyr^1 (+0.04)
	Tyr^6 (−0.04)	Tyr^2 (+0.04)
	Tyr^3 (−0.03)	
G32P*-d(pA)$_{40-60}$	Tyr^7 (−0.29)	Tyr^1 (+0.06)
	Tyr^6 (−0.29)	Tyr^2 (+0.02)
	Tyr^3 (−0.05)	
G32P*-d(pA)$_8$	Phe^2 (−0.20)	Phe^{14} (+0.03)
	Phe^5 (−0.08)	
	Phe^{12} (−0.03)	
	Phe^{10} (−0.02)	
	Phe^9 (−0.02)	
G32P*-d(pA)$_8$	Trp^2 (−0.08)[f]	Trp^5 (+0.04)[g]
	Trp^4 (overlaps 3, but amplitudes of signals from both the 2- and 5-protons change)	
	Trp^3 (broadened beyond detection)	

[a] Positions of the aromatic residues in the primary sequence are as follows: Tyr, 73, 84, 92, 99, 106, 115, 137, 186; Phe, 2, 23, 47, 58, 66, 135, 139, 173, 183, 191, 205, 210, 225, 228, 235, 265, 270, 278; Trp, 31, 72, 116, 144, 168; His, 64, 81. [b] In one case (Phe^3) the Phe side chain is slowly rotating and the 3,5 protons each give rise to a signal. [c] Spectrum shown in Pan et al. (1989). [d] All chemical shift changes are ±0.01 ppm. [e] Bound d(pA)$_{40-60}$. [f] H2 only. At 16:1 nucleotide residues:protein ratio this shift change increases to −0.12 ppm. [g] H5 only.

studies (Pan et al., 1989), the binding of d(pA)$_8$ follows parameters expected for a noncooperative single-site interaction (Prigodich et al., 1984, 1986). The size of the g32P nucleotide binding site is estimated to be 6–8 nucleotide residues in length [reviewed by Chase and Williams (1986)]. The changes in chemical shift of the Tyr 3,5-proton singlets as g32P* is titrated with increasing concentrations of d(pA)$_8$ are shown in Figure 1A–D. Both upfield and downfield shifts are observed which involve five of the eight Tyr residues. A 2:1 nucleotide:protein ratio is required to obtain the completely shifted Tyr ^{1}H spectrum, suggesting that excess nucleotide must be present to saturate the protein at a concentration of 0.25 mM (Figure 1C).

Only one set of signals for the 2- and 8-protons of d(pA)$_8$ are observed at a molar excess of the nucleotide over protein. The chemical shifts of the 2- and 8-protons of adenine are ~0.3 ppm downfield of their position in free nucleotide alone. Thus, the bound and free nucleotide must be a fast exchange, a condition represented by the Tyr ^{1}H resonances as well. If the binding constant for d(pA)$_8$ is assumed to be ~10^6 [reviewed in Chase and Williams (1986)], then under the NMR conditions 1:1 and 2:1 ratios of d(pA)$_8$-g32P* will correspond to 94% and 99.5% complex formation, respectively. The maximum induced upfield shift of the Tyr protons is only 0.10 ppm (Table I) and is not salt sensitive (Figure 1E).

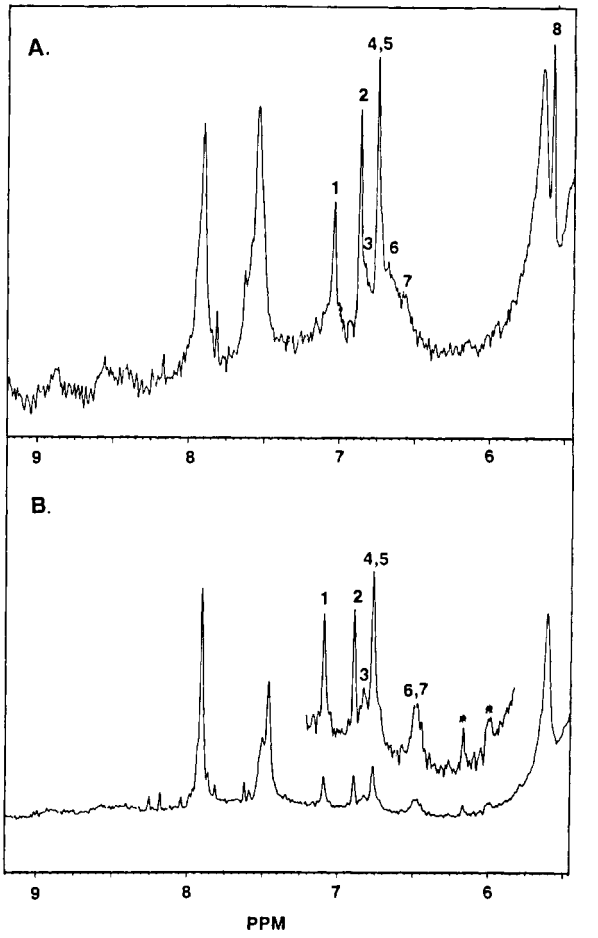


FIGURE 2: Proton signals for the Tyr 3,5-protons during titration of [3,5-^{1}H]Tyr-g32P* with d(pA)$_{40-60}$. d(pA)$_{40-60}$ was added to 0.25 mM [3,5-^{1}H]Tyr-g32P* to a molar ratio of (A) 8 nucleotide residues:1 g32P* monomer or (B) 20 nucleotide residues:1 g32P* monomer in the presence of 150 mM NaCl. Peaks marked with an asterisk are from H1′ of the ribose at the end of d(pA)$_{40-60}$.

^{1}H NMR of Complexes of [3,5-^{1}H]Tyr-g32P with d(pA)$_{40-60}$.* Since g32P* has lost its cooperative mode of binding, the molecular details of its complex formation with a single site on a nucleotide lattice [e.g., d(pA)$_8$] may or may not be similar to complex formation with a multisite lattice. The simple singlet spectrum of [3,5-^{1}H]Tyr-g32P* has enabled us to study its complex with d(pA)$_{40-60}$ which provides 5–7 contiguous sites. Changes in the chemical shifts of the five sets of Tyr 3,5-protons are observed at a lower nucleotide:g32P* ratio than with the octanucleotide, but these changes saturate at a nucleotide residue to protein ratio of 16:1, which corresponds to the 2:1 ratio observed for the d(pA)$_8$ experiment (Figure 2). Since only one set of resonances is again observed for the 2- and 8-protons of the adenine bases, the polynucleotide also appears to be in fast exchange. The total number of shifted resonances are the same as observed for the d(pA)$_8$ complex, but two of the upfield shifts (those for Tyr^6 and Tyr^7) are much larger in magnitude, 0.29 ppm (the two resonances overlap in the complex, Figure 2B).

Holo-[3,5-^{1}H]Tyr-g32P-d(pA)$_{40-60}$ Complex. Truly cooperative binding of g32P to ssDNA can be explored only by studying complexes of holo-g32P with ssDNA. Holo-g32P aggregates in solution as shown by the fact that the resonances of the 3,5-protons of the eight Tyr residues are broadened in the holoprotein (Figure 3A), and the proton spectrum in the aliphatic region consists of a relatively few narrow lines that represent primarily the protons on the more flexible A domain (Prigodich et al., 1984). The resonances from the bulk of the

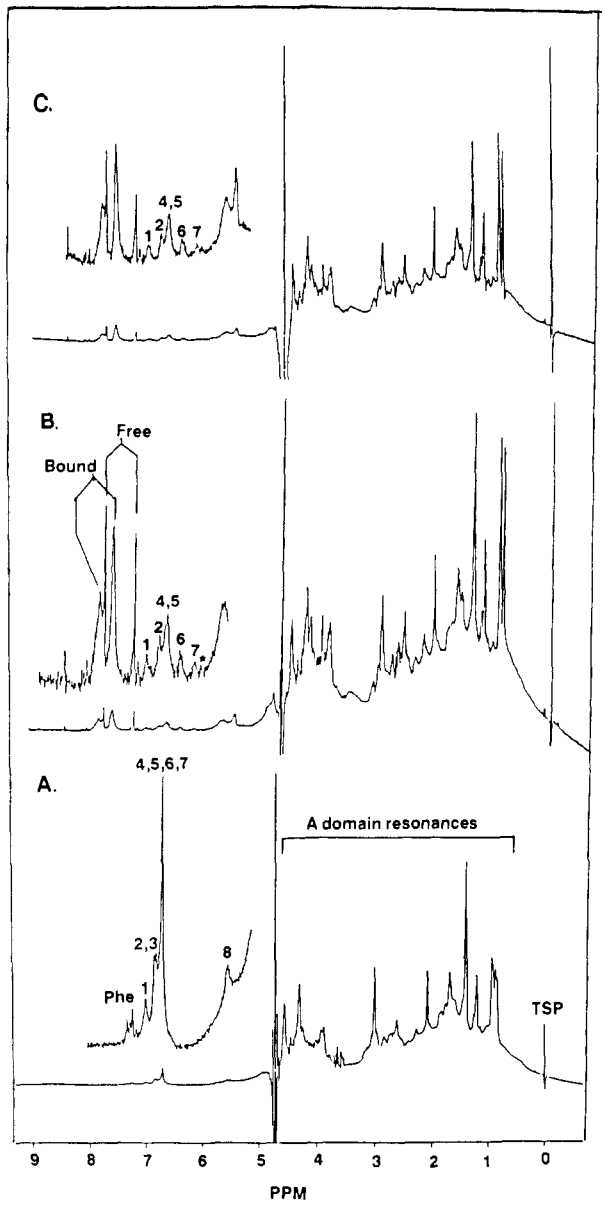


FIGURE 3: Proton signals for the Tyr 3,5-protons during titration of holo-[3,5-^{1}H]Tyr-g32P with $d(pA)_{40-60}$. $d(pA)_{40-60}$ was added to 0.4 mM [3,5-^{1}H]Tyr-g32P (A) to achieve a ratio of 11.5 nucleotide residues:1 g32P monomer (B). Sample C is the same as (B) plus 250 mM NaCl.

protons in the core and B domain are broadened beyond detection. Thus far we have not been able to find conditions that significantly reduce oligomerization of the holoprotein. Nevertheless, in the ^{1}H NMR spectrum of the isolated Tyr 3,5-protons, the signals are broad but still resolved (Figure 3A). The minor peaks, 7.2–7.3 ppm, are from the residual protons of the three Phe residues within the A domain (the phenylalanine is only ~95% deuteriated). The signals of these Phe protons appear so sharp because of the mobility of the A domain relative to the overall tumbling of the protein molecule (Prigodich et al., 1984).

Addition of $d(pA)_{40-60}$ to a final nucleotide residue:g32P ratio of 11.5:1 causes the 3,5-protons of Tyr^6 and Tyr^7 to shift upfield by 0.25 and 0.50 ppm, respectively (Figure 3B). Exchange between the protein–DNA complex and the free nucleotide is now slow judged by the simultaneous presence of signals for the free and bound nucleotide (Figure 3B). Unwinding of stacked adenine rings present in free $d(pA)_{40-60}$ likely accounts for the downfield shifts of the base proton resonances in bound $d(pA)_{40-60}$ (Table I). Increasing the NaCl concentration to 250 mM does not affect the magnitude or the number of nucleotide-induced shifts (Figure 3C). The signals in the aliphatic proton region arising from the A domain of the protein become sharper in the protein–DNA complex (Figure 3B) (see Discussion).

^{1}H NMR of [3,5-^{1}H]Phe-g32P and Its Complex with $d(pA)_8$.* The 18 Phe residues of g32P are relatively evenly distributed in the primary sequence. If 3,5-protonated Phe residues are inserted into the perdeuteriated background, the 3,5-proton resonances from most of the Phe residues are detectable but significantly broadened due to oligomerization (Figure 4A). Here the overlap of the large number of signals remains severe even in the perdeuteriated protein, precluding detailed studies of the holoprotein. The broadening and lack of resolution is removed if the protein is converted to g32P* (Figure 4B). In the spectrum of the holoprotein there is one Phe residue, 15, which consists of two signals, each of which integrates to approximately one proton. Both signals disappear from the spectrum of the core, which identifies this Phe as one of those located in the removed A and B domains. Since the B domain is involved in cooperative interactions between g32P monomers (Spicer et al., 1979) and the A domain has significant motion independent of the core protein, this slowly rotating Phe is most likely Phe^2 in the B domain. All except one of the Phe side chains (3) of g32P* give rise to singlets; thus, the Phe side chains in most environments in g32P* are freely rotating on the NMR time scale.

Binding of the oligonucleotide, $d(pA)_8$, to the 3,5-Phe-g32P* causes only minor changes in the signals of the 3,5-protons (Figure 4C). An expanded difference spectrum, however, reveals that two of the Phe 3,5 singlets (2 and 5) have undergone significant upfield shifts, 0.2 and 0.08 ppm, respectively, and have become slightly broader in the complex (Figure 4D). The resonances from four other Phe side chains undergo even smaller but detectable shifts upon oligonucleide complex formation (Figure 4D).

^{1}H NMR of [2,5-^{1}H]Trp-g32P and Its Complex with $d(pA)_8$.* By employing the 2,5-protonated Trp residues in a background of perdeuteriated aromatic amino acids, we have been able for the first time to isolate the tryptophan ^{1}H resonances from those of other aromatic protons. The 2- and 5-protons of all five Trp residues of g32P* are easily observed (Figure 5A). When the $d(pA)_8$ complex is formed, the signals from both the 2- and 5-protons of Trp^3 broaden beyond detection and there is an upfield shift of 0.08 ppm for the signal from the 2-^{1}H of Trp^2 but no change for the 5-^{1}H of this same residue (Figure 5B). These changes are more graphically illustrated in the expanded difference spectrum between the complex and the unliganded protein shown in Figure 5C. The difference spectrum also reveals a small upfield change in the signal of the 2-proton of Trp^5 but no change in the 5-proton. While we examined the ^{1}H NMR spectra of the $d(pA)_{40-60}$ complexes of both [3,5-^{1}H]Phe-g32P and [2,5-^{1}H]Trp-g32P, the resolution of the spectra, in contrast to the complex with [3,5-^{1}H]Tyr-g32P, does not allow definitive conclusions.

Discussion

Selective Protonation within a Deuteriated Background. Placement of specific protons in an environment where similarly resonating protons are replaced by deuterons greatly improves resolution and enables us to follow isolated signals from one residue type at a time. We have applied this technique to systems such as cooperative g32P–$d(pA)_{40-60}$ assemblies which have M_r of 200000–450000. The g32P–$d(pA)_{40-60}$ complex is of considerable interest since binding is fully cooperative, thus mimicking the in vivo system. There are some

limits to this approach. At present, only aromatic residues are readily adapted to this sort of spectral simplification.[2] If there are large numbers of one type of residue present, then overlapping signals can still limit interpretation of the spectrum.

Gene 32 Protein–Nucleotide Complexes As Studied by NMR of Isolated Protons. Previous ^{1}H NMR studies of fully protonated g32P*–d(pA)$_n$ (n = 2, 4, 6, 8, 10) complexes have led to a model proposing that five Tyr and two Phe are involved in ssDNA binding as inferred from the upfield shifts of their proton resonances induced by nucleotide binding (Prigodich et al., 1984, 1986). By isolating and separating the resonances from the Tyr, Phe, and Trp residues as NMR singlets, the nature of the NMR changes induced by nucleotide binding can be stated with much more precision. The proton signals from two of the five Tyr residues proposed to be involved in nucleotide binding are more dramatically affected by nucleotide binding than the others (Table I). The latter two Tyr residues are also those most affected by the change to the cooperative mode of binding to a long polynucleotide (Figures 2 and 3; Table I). The upfield shifts occurring on polynucleotide binding are from 0.25 to 0.5 ppm. It is possible that the rings of these two Tyr are involved in some sort of stacking interaction with the bases.

Since the original model of tyrosine–base interaction in the base-binding pockets was proposed, seven of the eight Tyr in the molecule have been subjected to site-directed mutagenesis in an attempt to determine the participation of these side chains upon nucleotide binding (Shamoo et al., 1989). One of these mutations, Tyr^{73} → Ser^{73}, is unique. While this mutant g32P protein has no detectable affinity for ssDNA, this change is associated with total loss of Zn(II) binding, yet the protein in general is not unfolded as shown by ^{1}H NMR (King and Coleman, unpublished data). Thus, Tyr^{73} must make a critical structural contribution to the Zn(II) binding domain, and the loss of this side chain must result in localized unfolding. While this result precludes any conclusions about direct interactions between Tyr^{73} and the nucleotides, all the other six Tyr → aliphatic side chain mutations result in significant decreases in the apparent K_a values of these proteins for ssDNA. This change occurs either in the intrinsic binding constant, as measured by the fluorescence quenching method, or in the cooperativity factor, ω. The Tyr^{137} → Ser^{137} mutation is the single exception in which the binding constant as measured by fluorescence quenching is not significantly affected (Shamoo et al., 1989).

The mutations include Tyr 84, 99, 106, 115, 137, and 186. All of these Tyr → aliphatic side chain mutations including that of Tyr^{137} show markedly reduced ability to induce melting of poly[d(AT)]. The melting point depressions for the polymer induced by the mutant g32Ps range from 10 to 35 °C compared to the 43 °C depression shown by the wild-type g32P. These data, however, cannot in every case be translated directly to decreases in nucleotide binding affinity, since several of the mutants show thermal unfolding transitions that occur at significantly lower temperature than the wild-type protein (Keating et al., 1988). There is enough circumstantial data, however, to suggest, as the NMR data does, that Tyr side chains make significant structural contributions to polynucleotide binding to g32P.

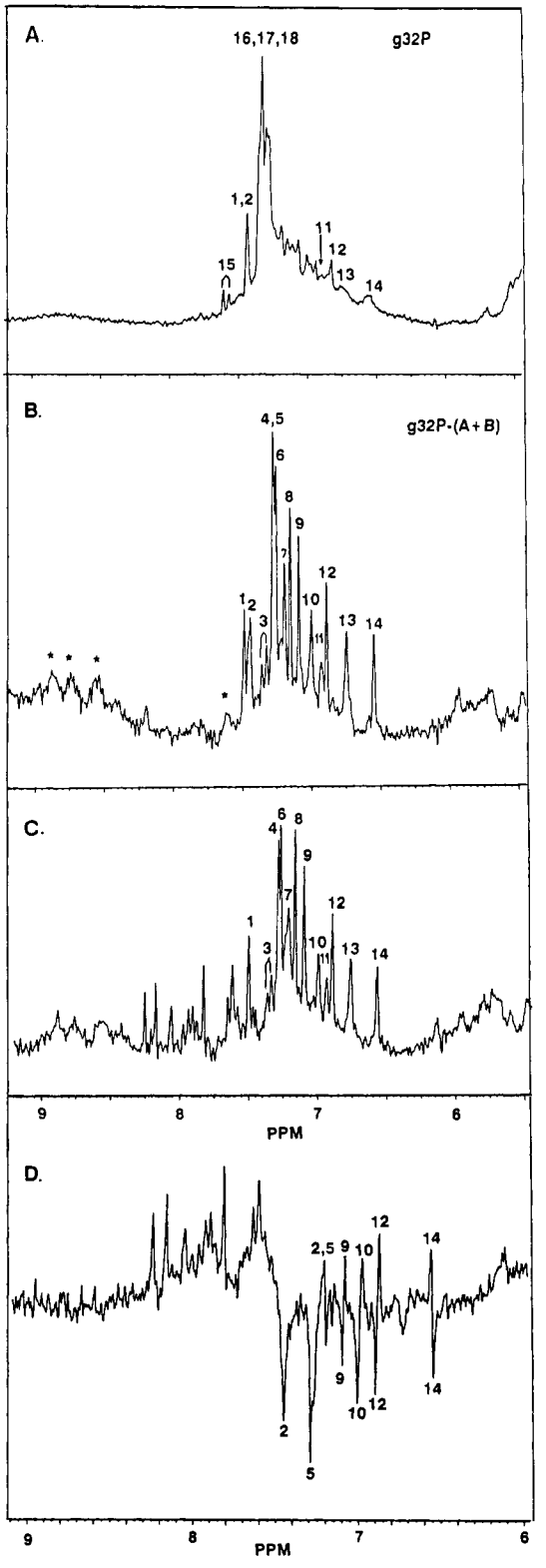


FIGURE 4: Proton signals for the Phe 3,5-protons of 0.25 mM holo-[3,5-^{1}H]Phe-g32P (A) and 0.25 mM [3,5-^{1}H]Phe-g32P* (B). (C) Proton signals of 0.25 mM [3,5-^{1}H]Phe-g32P* in the presence of 1:1 d(pA)$_8$:[3,5-^{1}H]Phe-g32P* monomer. (D) Difference spectrum of spectrum C minus spectrum B.

The present more precise ^{1}H NMR data also confirm that at least two Phe residues are involved in nucleotide binding in some way (Figure 4). The shifted Phe resonance 2 represents the largest upfield shift induced by the g32P*–d(pA)$_8$

[2] The selectively protonated tryptophan obtained by deuterium/hydrogen exchange is only 50% protonated at H2 and 25% at H5. Substantial improvement could be made by taking advantage of the properties of the *aro A* auxotrophic mutant. Preliminary studies show that this auxotrophic host grows by taking up anthranilic acid (Pan and Coleman, unpublished observations). If the *aro A* mutant is grown in the presence of fully deuteriated anthranilic acid, then tryptophans with 100% protonation at the 2-position can be incorporated into proteins of interest without interference of other aromatic protons.

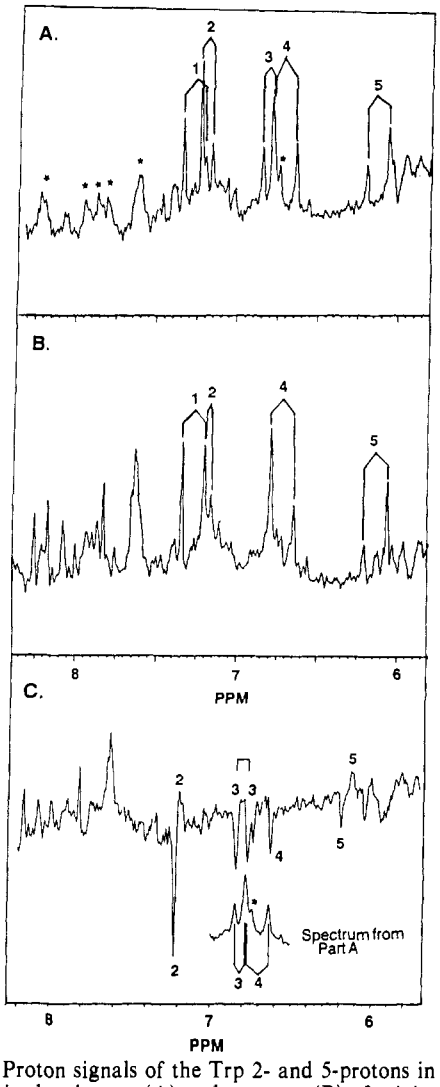


FIGURE 5: Proton signals of the Trp 2- and 5-protons in [2,5-^{1}H]-Trp-g32P* in the absence (A) and presence (B) of a 1:1 molar ratio of $d(pA)_8$:[2,5-^{1}H]Trp-g32P* monomer. (C) Difference spectrum of spectrum B minus spectrum A. The insert in part C shows the original signals for Trp^3 and Trp^4. The Trp 2-position is 50% protonated, while the Trp 5-position is 25% protonated.

complex, 0.20 ppm (Figure 4C). While the Phe proton resonances in g32P show significant chemical shift dispersion, this Phe residue exhibits about the same chemical shift as the Phe 3,5-protons in a random polypeptide and therefore is likely to be a surface residue. It is not unreasonable to suggest that this is Phe^{183} which can be cross-linked to an oligonucleotide by ultraviolet radiation of the complex (Shamoo et al., 1988).

The Trp proton labeling experiments show for the first time that two of the Trp residues are probably close to the nucleotide binding domain. Previous difference spectra have apparently obscured the Trp proton contributions to the shifts because of the complete overlap with Tyr and Phe proton resonances. There are two Trp residues, Trp^{72} and Trp^{116}, that define the ends of the section of primary sequence containing six of the eight Tyr residues as well as the ligands to the Zn(II) ion (Cys^{77}, His^{81}, Cys^{87}, Cys^{90}). The Trp proton signals 2 and 4 were those broadened by Zn(II) removal (Pan et al., 1989) and hence were tentatively assigned to these two residues. Trp signal 2 is shifted by nucleotide binding as well, but Trp signal 4 is only slightly affected (see Figure 5 and Table I). Instead Trp^3 undergoes major broadening upon nucleotide binding, even though its protons were unaffected by Zn(II) removal. Thus, it is possible that three of the five Trp residues may be near the nucleotide binding site.

A series of titrations of g32P* with $d(pA)_8$ have established that the oligonucleotides are in fast exchange on the NMR time scale; the magnitudes of the upfield shifts increase with a higher degree of saturation (Figure 1). It has been shown by independent ^{113}Cd NMR studies that $d(pA)_6$ bound to intact g32P is in fast exchange with unoccupied g32P sites (Giedroc et al., 1989). Thus, rapid exchange may be a reason, in addition to an unfavorable geometry of aromatic rings with respect to the nucleotide bases, for our failure to demonstrate any significant intermolecular NOEs between the aromatic and the base protons of a bound nucleotide.

Cooperative Binding of g32P to ssDNA. Holo-g32P binds ssDNA highly cooperatively ($\omega \sim 1000$) (von Hippel et al., 1982). The method of placing a single type of proton in a deuteriated background allows examination of the Tyr proton chemical shift changes induced by formation of a polynucleotide complex where the cooperative binding mode is present. The resonances of the 3,5-protons of two Tyr residues of g32P, 6 and 7, undergo much larger upfield shifts in the cooperative mode of binding (−0.25 and −0.50 ppm) than in the noncooperative oligonucleotide binding (−0.04 and −0.11 ppm) (Figure 3 and Table I). A similar finding applies to nucleotide-induced upfield shifts of Tyr^{26} in gene 5 protein on shifting from noncooperative to cooperative binding (King & Coleman, 1988). These changes may result from a decrease in aromatic ring–base ring distances in the more "tightly packed" polynucleotide complexes which show $\sim 10^3$ larger K_a values than for binding to a single lattice site.

The aliphatic proton signals assigned to residues from the A domain become sharper upon $d(pA)_{40-60}$ binding (Figure 3). This phenomenon is only observed when the cooperative binding of g32P occurs. This observation agrees with the model in which the A domain can function as an arm or flap to partially occlude the ssDNA binding site (Lonberg et al., 1981; Coleman et al., 1986). Binding of g32P to a polynucleotide is postulated to release the A domain from interaction with core g32P and leaves it to freely rotate and interact more readily with other replisome proteins when present. This release or "activation" coincides with the mobility increase as detected by ^{1}H NMR. The high acidity of the A domain (net charge = −12/58 residues) resembles the activation regions of the eukaryotic transcription factors. Some of these transcription factors may enhance activation also by "freeing" their activation domains when binding to specific DNA sequences.

Summary of the Roles of Aromatic Resonances in ssDNA Binding by Gene 32 Protein. Our results show two general types of upfield shifts to occur upon the binding of g32P to ssDNA. Type I shifts are relatively large (>0.08 ppm) and show significant increases in magnitude upon the transition from noncooperative to cooperative binding to nucleotides. These include the shifts of Tyr^6 and Tyr^7, Phe^2 and Phe^5, and Trp^2 (Table I). Type II shifts are small shifts that do not significantly change between noncooperative and cooperative binding (Table I). These include Tyr^1, Tyr^2, and Tyr^3 (Table I) and Phe^9, Phe^{10}, Phe^{12}, and, Phe^{14} and Trp^5 (Figure 4D). Type I residues may be located in the immediate vicinity of the ssDNA binding surface and interact strongly with the nucleotide bases. Significant protein conformational changes may accompany nucleotide complex formation and could be responsible for the smaller type II shifts.

In the case of gene 5 protein binding to $d(pA)_{40-60}$ there is a −0.85 ppm upfield shift in one of the protons of Tyr^{41} that simply is not present in the noncooperative oligonucleotide binding mode (King & Coleman, 1988). This as well as site-directed mutagenesis suggests that Tyr^{41} participates in

protein–protein interaction. While none of the aromatic protons in g32P shows such a qualitative difference between the two binding modes, the shift of the Tyr^{41} proton in the cooperative g5P complex does show that large upfield shifts in aromatic proton resonances cannot always be associated with ring current shifts induced by the bases of the bound nucleotide. It has also been shown that Leu^{28} of g5P is directly involved in interaction with nucleotide bases (King & Coleman, 1987). Nonaromatic residues of g32P may be candidates for direct interaction with ssDNA, but have not yet been examined.

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A ^{1}H NMR Determination of the Solution Conformation of a Synthetic Peptide Analogue of Calcium-Binding Site III of Rabbit Skeletal Troponin C†

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ABSTRACT: NMR techniques have been used to determine the structure in solution of acetyl (Asp 105) skeletal troponin C (103–115) amide, one of a series of synthetic peptide analogues of calcium-binding site III of rabbit skeletal troponin C [Marsden et al. (1988) *Biochemistry 27*, 4198–4206]. The NMR measurements include ^{1}H–^{1}H nuclear Overhauser enhancements and gadolinium-induced ^{1}H relaxation measurements. The former yield short-range internuclear distances (<4 Å); the latter, once properly corrected for chemical exchange, yield longer range metal to proton distances (5–10 Å). These measurements were then used as pseudo potential energy restraints in energy minimization and molecular dynamics calculations to determine the solution structure. Further information was provided by NMR coupling constants, amide proton exchange rates, and the temperature dependences of amide proton chemical shifts. The solution structure of the peptide analogue is very similar to that of the calcium-binding loop in the protein, the root-mean-square deviation between the backbone atoms being ~1.1 Å.

Calcium plays an important role in many biological systems, often acting as a second messenger (Kretsinger, 1980; Seamon & Kretsinger, 1983), where it effects a structural change in regulatory proteins such as troponin C (Ebashi et al., 1968) and calmodulin (Cheung, 1970; Kakiuchi & Yamazaki, 1970). Comparison of the primary sequence of these proteins reveals that the calcium-binding sites consist of highly homologous regions (Barker et al., 1978; Vogt et al., 1979; Reid & Hodges, 1980; Gariépy & Hodges, 1983). Each calcium-binding site exists in a helix–loop–helix arrangement of 31 residues in length; this arrangement has been designated the "EF hand"[1]

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