Circular Dichroism Spectroscopy of Three Tyrosine-to-Phenylalanine Substitutions of fd Gene 5 Protein†

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ABSTRACT: Circular dichroism spectroscopy was used to study mutants of phage fd gene 5 protein (Y26F, Y34F, and Y41F) in which three of the five tyrosines, Tyr-26, Tyr-34, and Tyr-41, were individually substituted with phenylalanine. The tyrosine 229 nm CD bands of the wild type, Y26F, and Y41F gene 5 proteins decreased in magnitude during complex formation with either fd ssDNA or poly[d(A)]. However, the Y34F gene 5 protein showed no decrease in the 229 nm band during titrations of these nucleic acids. This suggested that Tyr-34 of the wild type gene 5 protein dominated the 229 nm CD changes upon binding to single-stranded DNA. Titrations of poly[d(A)] or fd ssDNA with wild type, Y26F, Y34F, or Y41F gene 5 proteins resulted in perturbations of the nucleic acid near-UV CD bands, specific for the particular nucleic acid, but similar for all four proteins (in 2 mM sodium phosphate buffer, pH 7.0). For both nucleic acids, the addition of protein beyond a certain [protein monomer]/[nucleotide] ratio (0.25 for poly[d(A)] or 0.33 for fd ssDNA) resulted in a partial reversal of the CD change of the nucleic acid. These data are interpreted to mean that, in addition to the two well-known $n = 4$ and $n = 3$ stoichiometric modes of binding, there is a third mode of binding in which the nucleic acid is in limited contact with the protein. As shown by salt dissociation studies of complexes with poly[d(A)], the binding affinites, $K\omega$, of the proteins were in the order: wild type > Y26F ≫ Y34F ≥ Y41F (for the $n = 4$ binding mode in 0.1−0.2 M NaCl). Our data indicate that Tyr-34 plays a more important role in forming a complex with ssDNA than is apparent in current models of the g5p•ssDNA complex. We suggest that the hydroxyl moieties of Tyr-34 and Tyr-41 are both somehow involved in stabilizing the interface of bound protein dimers.

Essential proteins that bind nonspecifically, cooperatively, and preferentially to ssDNA[1] are required in the life cycles of bacteriophage (Alberts & Frey, 1970) and prokaryotes (Sigal *et al.*, 1972) and are also found in eukaryotes from yeast (Heyer *et al.*, 1989) to humans (Fairman & Stillman, 1988; Wold & Kelly, 1988). The gene 5 protein encoded by filamentous Ff bacteriophages is a well-studied model for proteins that bind to ssDNA (Alberts *et al.*, 1972; Kowalczykowski *et al.*, 1981; Ray, 1978; Bulsink *et al.*, 1985).

Several functions for Ff gene 5 protein (g5p) have been elucidated. First, it facilitates the switch from the production of replicative form dsDNA to the production of ssDNA viral genomes by sequestering the nascent ssDNA genomes into helical complexes as they are synthesized from a rolling circle replicative intermediate (Gilbert & Dressler, 1969). Second, the g5p•ssDNA complex contacts the *Escherichia coli* inner membrane, where the genome is released from g5p and becomes complexed with the coat protein (g8p) (Rasched & Oberer, 1986). The g8p•ssDNA complex, along with some minor coat proteins, is then extruded from the cell as a mature virus particle. Third, g5p binds to certain mRNA sequences and translationally represses the mRNA of g2p (Model *et al.*, 1982; Yen & Webster, 1982) and other viral mRNAs (Zaman *et al.*, 1991). However, deletion of the major protein binding site from gene 2 mRNA shows that this function of g5p is nonessential (Zaman *et al.*, 1992).

The g5p has a $M_r = 9690$ and exists as a dimer in solution (Oey & Knippers, 1972). One monomer of the g5p dimer covers 3−4 nucleotides (see Kansy *et al.*, 1986, Table 1; Bulsink *et al.*, 1986, 1988). The superhelical complex formed by cooperative binding to the ssDNA genome in the $n = 4$ mode is a left-handed helix with 103 ± 3 helical turns, a diameter of 80 ± 10 Å , and a variable pitch of 60−120 Å (C. W. Gray *et al.*, 1982; Gray, 1989; Olah *et al.*, 1995).

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[1] Abbreviations: CD, circular dichroism; dsDNA, double-stranded DNA; *E. coli, Escherichia coli*; Ff, filamentous phage (M13, f1, fd); g2p, fd gene 2 protein (similar definitions for g5p and g8p); IPTG, isopropyl thio-β-D-galactoside; $K\omega$, the product of the intrinsic binding constant K and a cooperativity factor ω; mdeg, millidegrees; n, the number of nucleotides covered by the fd g5p monomer; NMR, nuclear magnetic resonance; OB-fold, (oligonucleotide/oligosaccharide binding) fold; P/N, molar ratio of [gene 5 protein monomer]/[nucleotide]; ssDBP, single-stranded DNA binding protein; ssDNA, single-stranded DNA; SSC, standard saline citrate buffer containing 0.195 Na^+, pH 8; UV, ultraviolet; WT, wild type.

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The DNA is located in the interior of the helix (D. M. Gray *et al.*, 1982; Olah *et al.*, 1995).

The most recent crystal structure of g5p has been described as a five-stranded antiparallel β-sheet, with two of the β-strands being interrupted by a 3_{10} helix (Skinner *et al.*, 1994), all forming a distorted β-barrel. Since g5p has not been cocrystallized with ssDNA, information about specific protein−DNA interactions and dimer−dimer interactions in the complex has come from solution and modeling studies. The architecture of g5p falls within the OB-fold category of proteins, the OB-fold being a structural motif found in a variety of five-stranded antiparallel β-sheet proteins (Murzin, 1993; Schindelin *et al.*, 1993). The ligands for OB-fold proteins are generally located where three loops of the β-barrel are in close proximity. Two of these loops in fd g5p contain Tyr-26 and Phe-73, aromatic residues implicated by NMR measurements to be directly involved in binding to DNA (see below).

Gene 5 protein has a total of three phenylalanines, five tyrosines, and no tryptophans (Cuypers *et al.*, 1974; Nakashima *et al.*, 1974a,b) among its 87 amino acids. The five tyrosines (Tyr-26, Tyr-34, Tyr-41, Tyr-56, and Tyr-61) are conserved in the g5p of another class I phage, IKe (Peeters *et al.*, 1983; de Jong *et al.*, 1989), and the spacing of tyrosines is conserved among different ssDNA binding proteins, including the ssDBP from phage Pf3, the phage T4 gene 32 protein, and the *E. coli* proteins RecA, ssDNA binding protein, and F sex factor (Prasad & Chiu, 1987). Additionally, the ssDNA binding proteins of bacteriophages Pf1 and Pf3 probably form DNA binding wings similar to that of fd g5p, with Tyr-30 of the Pf1 protein and Tyr-22 of the Pf3 protein being equivalent to Tyr-26 of fd g5p (Plyte & Kneale, 1993; Powell & Gray, 1995). In four known structures of single-strand nucleic acid binding proteins, aromatic side chains help to form pockets for the recognition of bases, as recently shown for T4 g32p (Shamoo *et al.*, 1995). Figure 1A shows the positions of the five tyrosines in the g5p dimer (Skinner *et al.*, 1994). The symmetrically related DNA-binding wings contain Tyr-26 and Tyr-26′.

^{1}H-NMR measurements by King and Coleman (1988) using gene 5 proteins with selectively deuterated tyrosines showed Tyr-26 to be the only tyrosine that directly interacts with ssDNA. The authors suggest that Tyr-26 may be hydrogen-bonded to the sugar−phosphate backbone, in addition to being involved in base stacking, since intermolecular NOEs show the ring protons of Tyr-26 to be close to sugar groups. In general, ^{1}H-NMR data have lead to a concurrence that, among the aromatics of g5p, only Tyr-26 and Phe-73 interact directly with the ssDNA (King & Coleman, 1987, 1988; Folkers *et al.*, 1991a,b). Both of these amino acids may make important stacking interactions with DNA, as indicated by the site-saturation mutagenesis work of O'Donohue *et al.* (1993). In addition, from studies of WT and mutant Y26F proteins, Turner and Kneale (1995) determined that Tyr-26 of the WT protein is 50% quenched when the protein binds to poly[d(T)].

There is little published information regarding the function of Tyr-34 in g5p. NMR experiments by Folkers *et al.* (1991a) suggest that Tyr-34 may possibly have a role in protein−protein interactions, since ^{1}H resonances for Tyr-34 are slightly shifted upon a change of solution conditions that affect protein solubility. *In vivo* experiments by

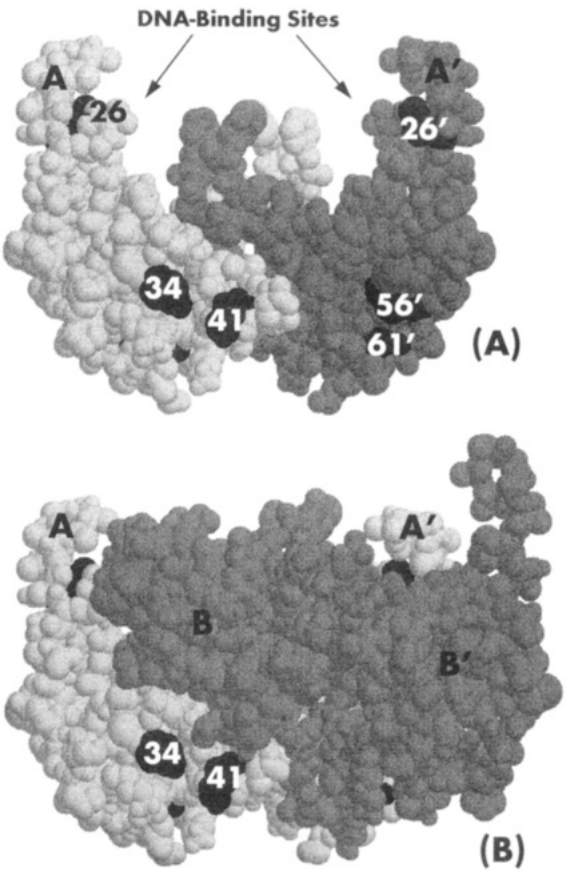

FIGURE 1: (A) Gene 5 protein dimer from the crystal structure of Skinner *et al.* (1994). In this view perpendicular to the 2-fold rotational axis of the dimer, Tyr-26, Tyr-34, and Tyr-41 are visible in monomer A, while Tyr-26′, Tyr-56′, and Tyr-61′ are visible in monomer A′. The DNA-binding wings, where Tyr-26 is located, have a relatively conserved sequence of residues among ssDNA-binding proteins. (B) Two gene 5 protein dimers. The dimer in the background is the same as in (A). The dimers are as proposed by Skinner *et al.* (1994) for a left-handed helical structure. Tyr-34 and Tyr-41 are not quite within the dimer−dimer contact area.

Terwilliger *et al.* (1994) showed that Tyr-34 is sensitive to mutation, since Y34S and Y34P were inactive in the inhibition of *E. coli* growth (Y41F was active) and Y34S was inactive in the support of phage propagation. We now have evidence from *in vitro* CD experiments that, indeed, Tyr-34 plays an important role in complexation.

The g5p, along with a few other proteins (Woody, 1978), has a unique positive CD band at 229 nm. For Ff g5p, this large CD band is due primarily to the L_a bands of the five tyrosyl residues (Murrell, 1963; Day, 1973). Typically, the 229 nm CD band is decreased upon binding to nucleic acids. Day (1973) suggested that this reduction is the result of a change in the environment or in the orientation of the tyrosyl chromophores. Kansy *et al.* (1986) found that the CD of the 229 nm band was decreased by only 4−5% when short oligomers [e.g., $d(pT)_7$ and $d(pA)_7$] were titrated with the WT protein, compared with a 26−43% decrease when fd ssDNA or long ssDNA polymers were titrated. One possible explanation for this difference is that one or more tyrosines that are perturbed by cooperatively-interacting proteins are responsible for the change in the 229 nm band. Consistent with this explanation, the results of our present work identify Tyr-34, a tyrosine near the dimer−dimer interface in models of the superhelical complex (Skinner *et al.*, 1994; Guan *et al.*, 1994; Folmer *et al.*, 1994; Olah *et al.*, 1995), as the main

tyrosine responsible for the change in optical activity upon binding to ssDNA.

^{1}H-NMR studies have strongly implicated Tyr-41 as being important in dimer−dimer interactions. Tyr-41 proton resonances are insignificantly perturbed when g5p binds noncooperatively to oligonucleotides (King & Coleman, 1987). However, when the protein binds cooperatively to long oligonucleotides (i.e., d[pA]$_{40-60}$), the Tyr-41 proton resonances undergo significant upfield shifts (King & Coleman, 1988). In addition, a change in salt concentration or protein concentration causes g5p to aggregate concomitantly with an upfield shift of the Tyr-41 proton resonances (Folkers *et al.*, 1991a; de Jong *et al.*, 1987).

Folkers *et al.* (1991a) observed that a Y41H mutation makes the protein significantly more soluble, while maintaining its ability to bind to ssDNA. Stassen *et al.* (1992a) found that Y41H and Y41F, as well as Y26H, were inactive in repressing the translation of a reporter protein whose promoter and translational initiation sequence was that of the g2p. In other work, Y41H was inactive in the inhibition of *E. coli* growth (Terwilliger *et al.*, 1994).

The crystal structures of Y41F and Y41H gene 5 proteins have now been solved (Guan *et al.*, 1994). Guan *et al.* (1994) suggest that when gene 5 protein forms a superhelical complex with ssDNA in solution, the protein dimer−dimer interactions approximate the crystal packing characteristics of the Y41H protein. Figure 1B is a view of two g5p dimers from the Skinner *et al.* (1994) model of the superhelix, which shows that Tyr-34 and Tyr-41 are adjacent to the dimer−dimer interface. This superhelix model is similar to that derived by Guan *et al.* (1994).

Tyr-56 and Tyr-61 of g5p are located in β-strand 4 and are part of the core barrel. We did not give preference to the study of Tyr-56 and Tyr-61, as they are not thought to participate in protein−protein or protein−DNA interactions. Skinner *et al.* (1994) found that Tyr-61 forms two hydrogen bonds to the peptide nitrogen and oxygen of Ala-57 and thereby helps to stabilize one of the β-loops. Tyr-61 may also participate in structure stabilization with Tyr-56 in an aromatic−aromatic interaction as described more generally by Burley and Petsko (1985).

Depending on buffer conditions, WT g5p has been found to bind to nucleic acids in more than one mode (see Kansy *et al.*, 1986). To determine if the substitution of Phe for Tyr-26, Tyr-34, or Tyr-41 affects the binding stoichiometry, number of binding modes, or binding affinity, we performed CD titrations and salt dissociation experiments with each of the four proteins, WT, Y26F, Y34F, and Y41F, in a buffer of 2 mM Na^+ (phosphate buffer, pH 7). All of the proteins exhibited the expected $n = 3$ mode of binding to fd ssDNA, and all but the Y41F protein showed a previously undetected $n \approx 2$ mode of binding. For poly[d(A)], two modes of binding, $n = 4$ and $n \approx 2.5$, were found for all four proteins. We suggest that when g5p binds to nucleic acids in the $n \approx 2-2.5$ mode, there is a less stringent association of the nucleic acid with the protein than is currently thought to be the case for the $n = 4$ and $n = 3$ binding modes.

MATERIALS AND METHODS

Isolation of Protein. Competent *E. coli* K561 cells were transformed with plasmids (Terwilliger, 1988) containing the WT and mutant g5p genes. The cells were grown in a New Brunswick Scientific Co. 10-L fermenter in a broth containing 5 g of NaCl, 1 g of Tris, 32 g of tryptone, and 20 g of yeast extract per liter, plus 150 μg/mL of ampicillin (Sigma). The culture was induced with 1 mM IPTG (Fisher Biotech) at an OD(600) of 0.5. The cells were harvested by centrifuging at 4090*g* for 10 min at 4 °C and were frozen at −70 °C. WT and mutant proteins were isolated essentially as described by Day (1973). Fractions were eluted from a DNA affinity column by a step gradient of 0.2, 0.3, 0.4, and 0.6 M NaCl in a buffer of 0.02 M Tris, 5 mM EDTA, 0.01 M NaCl, 1 mM β-mercaptoethanol, and 10% glycerol at pH 7.0. Pooled samples were then concentrated and applied to a Sephadex G-75 column (Pharmacia) to separate any high molecular weight contaminants. Purity of the protein samples was checked by 18% SDS−PAGE. After staining with Coomassie Blue, the gel was digitized using a Model 2000i Gel Print System (BioPhonics). Quantitation with ImageQuant Ver. 3.3 (Molecular Dynamics) showed that each of the proteins was >98% pure. The proteins were dialyzed into 2 mM Na^+ (phosphate buffer, pH 7.0) and stored at −20 °C.

Protein concentrations were determined from absorption measurements using a molar extinction coefficient $\epsilon(276)$ of 7074 $M^{-1}\cdot cm^{-1}$ (Day, 1973) for the WT protein monomer and a calculated $\epsilon(276)$ of 5660 $M^{-1}\cdot cm^{-1}$ for the mutant proteins. Using an $\epsilon(276)$ for tyrosine of 1386.8 $M^{-1}\cdot cm^{-1}$ and an $\epsilon(276)$ for phenylalanine of 2.2 $M^{-1}\cdot cm^{-1}$ (Fasman, 1989), we calculated the $\epsilon(276)$ for the mutant proteins (which contain four Tyr and four Phe) as follows: $(4 \times 1386.8) + (4 \times 2.2) = 5556$ $M^{-1}\cdot cm^{-1}$. We used a value of $\epsilon(276) = 5660$ $M^{-1}\cdot cm^{-1}$, 2% higher than calculated, for determining concentrations of the mutant proteins, since for WT g5p the experimentally determined extinction coefficient was 2% larger than the similarly calculated value. The value of 5660 $M^{-1}\cdot cm^{-1}$ was in close agreement with the value of 5680 $M^{-1}\cdot cm^{-1}$ determined by the Bio-Rad protein assay for the Y41F protein (Folkers *et al.*, 1991a).

Nucleic Acids. Poly[d(A)] was purchased from Sigma Chemical Co. Poly[d(A)] was thoroughly dialyzed first against 1.0 mM Na_2HPO_4, 0.5 M NaCl, and 0.01 EDTA, pH 7.0, next against 1.0 mM Na_2HPO_4 and 0.5 M NaCl, pH 7.0, and finally into 2 mM Na^+ (phosphate buffer, pH 7.0). The poly[d(A)] concentration was obtained using an $\epsilon(260)$ of 9650 $M^{-1}\cdot cm^{-1}$ (Bollum, 1966). The fd ssDNA was isolated from phage as previously described (D. M. Gray *et al.*, 1982). The fd ssDNA concentration was obtained using an $\epsilon(260)$ of 8730 $M^{-1}\cdot cm^{-1}$ for the ssDNA in 2 mM Na^+ (phosphate buffer, pH 7.0). The extinction coefficient for ssDNA in this buffer was determined by the relative absorption of the DNA when diluted into this buffer and into SSC buffer, for which the extinction coefficient is known (Berkowitz & Day, 1974).

UV Absorption and CD Measurements. UV absorption spectra were measured using a Cary Model 118 spectrophotometer. CD spectra were measured using either a Jasco Model J500A or a J710 spectropolarimeter. The spectropolarimeters were calibrated as described by Gray *et al.* (1995). CD data were plotted at nanometer intervals as $\epsilon_L - \epsilon_R$ in units of $M^{-1}\cdot cm^{-1}$ per mole of nucleotide or per mole of protein monomer. Most CD data were taken with the Jasco J500. Far-UV CD spectra of the individual proteins were taken either in a 0.2 mm cell or in a 1 mm cell at a scan speed of 1 nm/min, with a sensitivity range of 1 mdeg/cm,

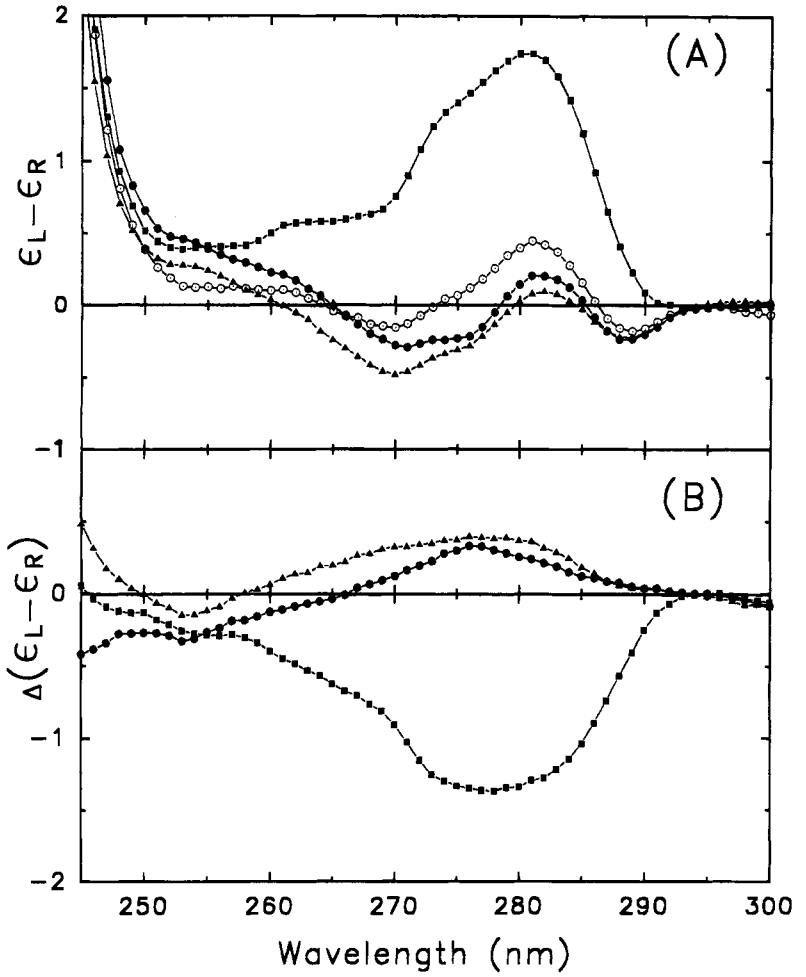

FIGURE 2: (A) Near-UV CD spectra of g5p. Spectra are for free WT (○), Y26F (●), Y34F (■), and Y41F (▲) gene 5 proteins, taken in a 1.0 cm cell. (B) Difference spectra of above. CD(WT) − CD(Y26F) (●), CD(WT) − CD(Y34F) (■), and CD(WT) − CD(Y41F) (▲). The average of five spectra is plotted for each protein. The standard deviation was within the size of the symbols. $\epsilon_L - \epsilon_R$ is in units of $M^{-1}\cdot cm^{-1}$, per mole of protein monomer, in this figure and Figure 3. For the spectra in this figure and Figures 3−7, the buffer was 2 mM Na^+ (phosphate, pH 7.0) unless otherwise indicated.

using a time constant of 4 s. Near-UV CD spectra were generally taken in a 10 mm cell at 10 nm/min, 2 mdeg/cm, and a time constant of 1 s. The Jasco J710 was used to acquire salt dissociation data at different protein concentrations; three repetitive scans were taken at 50 nm/min with a response time of 0.25 s. All spectral data were taken at 20 ± 0.5 °C.

Titrations. Titrations of nucleic acids with the WT and mutant proteins were performed as described by Kansy *et al.* (1986). Starting concentrations of fd ssDNA were (4.6−6.6) × 10^{-5} M. Starting concentrations of poly[d(A)] were (4.2−5.9) × 10^{-5} M. The buffer used was 2 mM Na^+ (phosphate buffer, pH 7.0), except for one titration in the presence of added 0.1 M NaCl. Light scattering during the titrations was minimal, with the absorption at 320 nm being at most 4.5% of the absorption at 260 nm for samples at the highest P/N ratios.

Dissociations. Salt dissociations for the complexes in 2 mM Na^+ (phosphate buffer, pH 7.0) were performed as described by Kansy *et al.* (1986) and Sang and Gray (1989), by increasing the salt concentration with aliquots of 4 M NaCl in 2 mM Na^+ (phosphate buffer, pH 7.0). The sample solution was weighed after each addition. The density of the added NaCl solution was taken into account in calculating the dilution of the sample.

The percentage of maximum CD change of the complexes at different salt concentrations was derived from the percentage of the maximum change in molar CD of the nucleic acid at 270 nm found upon forming a saturated complex: % maximum CD change = [(CD(isolated components) − CD(complex plus NaCl))/(CD(isolated components) − CD(complex with max negative CD))] × 100. That is, % maximum CD change = ($\Delta CD_{meas}/\Delta CD_{max}$) × 100.

The titration of free fd ssDNA or poly[d(A)] with NaCl resulted in minor changes in the CD at 270 nm, amounting to less than 4% (at 0.5 M NaCl) or less than 3% (at 0.3 M NaCl), respectively, of the total CD changes found upon forming complexes with the WT g5p. Therefore, CD values at 270 nm for fd ssDNA and poly[d(A)], obtained at 0.5 and 0.3 M Na^+, respectively, were used for the isolated nucleic acid components when calculating the percentage of maximum CD change at all salt concentrations using the above equation. Titration of the free WT g5p from 2 to 400 mM Na^+ produced an insignificant change of CD in the near-UV region, relative to the CD changes of the nucleic acid in the complex. For example, the small negative band at 270 nm (−0.15 $M^{-1}\cdot cm^{-1}$) of the protein became slightly more negative (by 0.25 $M^{-1}\cdot cm^{-1}$) as salt was added. Thus, the CD values of the proteins at 270 nm in 2 mM Na^+ were used for the isolated protein component when calculating

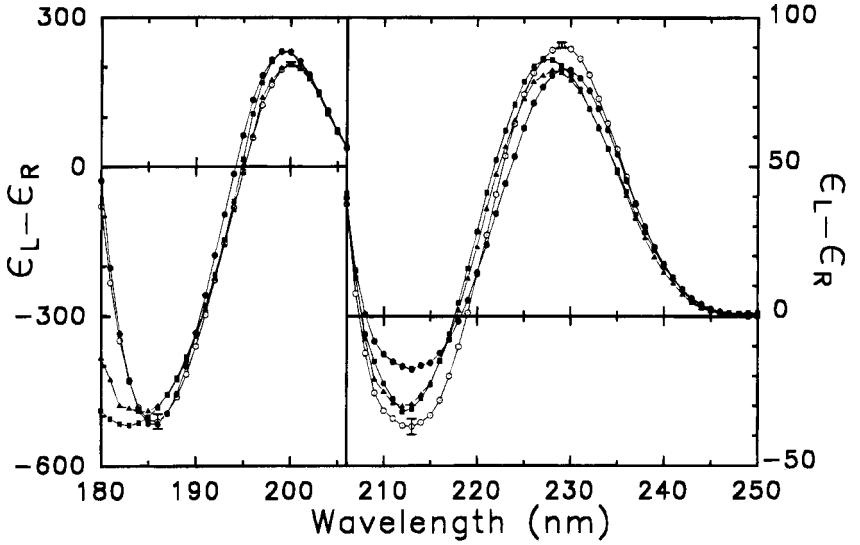

FIGURE 3: Far-UV CD spectra of g5p. Spectra are for free WT (○), Y26F (●), Y34F (■) and Y41F (▲) gene 5 proteins, taken in a 0.2 mm cell. Spectra were normalized to the value of the 229 nm band value obtained in a 1 mm cell. Error bars at the positive and negative peaks show the range of spectra of three samples of WT g5p.

the percentage of maximum CD change at all salt concentrations using the above equation.

RESULTS

CD Spectra of Free Proteins. Each protein had a characteristic spectrum in the near-UV region at wavelengths above 250 nm (Figure 2A). Y34F had the most distinctive CD spectrum, with a positive band at 280 nm. Since phenylalanine CD bands are small above 270 nm, this suggested that Tyr-34 in the WT protein contributes a negative CD in the near-UV. See Figure 2B, which shows the CD contributions of the individual tyrosines minus those of the substituted phenylalanines.

The far-UV CD spectra were qualitatively similar for all four proteins as shown in Figure 3. The proteins all had a significant L_a band at about 229 nm, but differed in that there was a slight blue shift of this band for the Y34F and Y41F mutant proteins. As can be seen in Figure 3, the CD magnitude at 229 nm was less by 9%, 8%, and 10% for Y26F, Y34F, and Y41F, respectively, compared with the WT g5p. The overall similarity in the far-UV CD spectra of the mutant and WT proteins indicated that the mutant proteins maintained a native secondary structure. For comparison, WT g5p that has been denatured by 4 M guanidine hydrochloride shows a complete loss of the tyrosyl 229 nm band (Liang & Terwilliger, 1991). That the mutant proteins had essentially native structures was evident in that they all similarly perturbed the CD of fd ssDNA and poly[d(A)] as shown below. Furthermore, the C_α atoms in the crystal structure of Y41F g5p have a root mean square deviation from the C_α atoms in the crystal structure of the WT g5p of only 0.30 Å (Guan *et al.*, 1994), showing that the tertiary structure of Y41F is well-maintained.

CD Changes of Nucleic Acids upon Titration. CD changes were essentially the same upon titration of fd ssDNA with WT g5p (Figure 4A) and Y34F g5p (Figure 4B). The free nucleic acid had a positive CD signal at 270 nm. As the DNA was titrated, the CD band above 260 nm decreased and became negative. The crossover shifted to longer wavelengths, until a [g5p protein monomer]/[nucleotide], or P/N, ratio of about 0.33 was attained (solid symbols). As

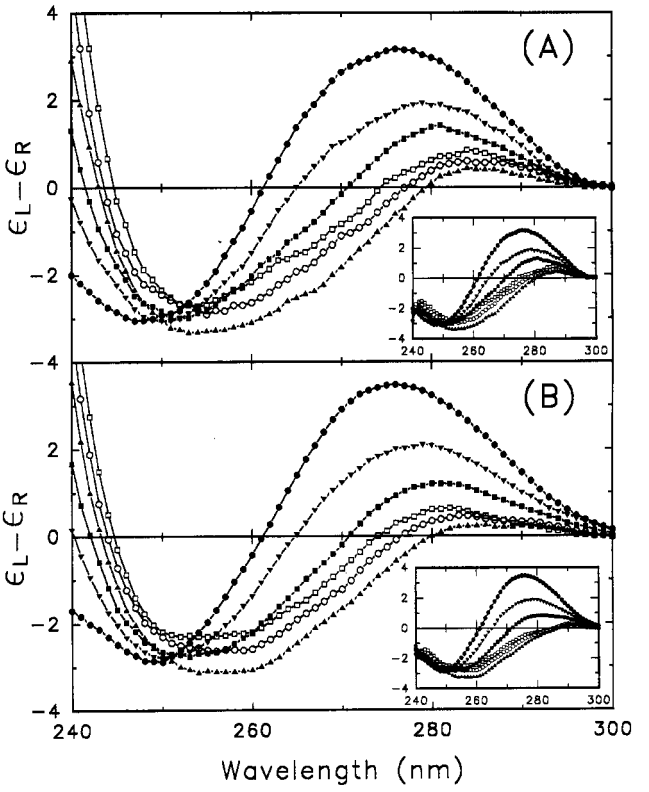

FIGURE 4: A comparison of the fd ssDNA CD changes upon binding WT g5p (A) and Y•34F g5p (B). Representative spectra are shown from example titrations. (A) Spectra are for free fd ssDNA (●) and WT protein•fd-ssDNA complexes at P/N ratios of 0.12 (▼), 0.21 (■), 0.33 (▲), 0.40 (○), and 0.56 (□). (B) Spectra are for free fd ssDNA (●) and Y34F protein•fd-ssDNA complexes at P/N ratios of 0.12 (▼), 0.21 (■), 0.33 (▲), 0.41 (○), and 0.50 (□). Insets show the same spectra, but with the CD of free protein subtracted. $\epsilon_L - \epsilon_R$ is in units of $M^{-1}\cdot cm^{-1}$, per mole of nucleotide, in this figure and Figures 5−7.

additional protein was added to give larger P/N ratios, the CD change reversed (i.e., the CD above 260 nm became less negative) until a P/N ratio of 0.45−0.5 was reached (open symbols). In previous work (Kansy *et al.*, 1986), a reversal in the CD change was not found during titrations of fd ssDNA with WT g5p in 50 mM Tris-HCl, pH 7.8, due to

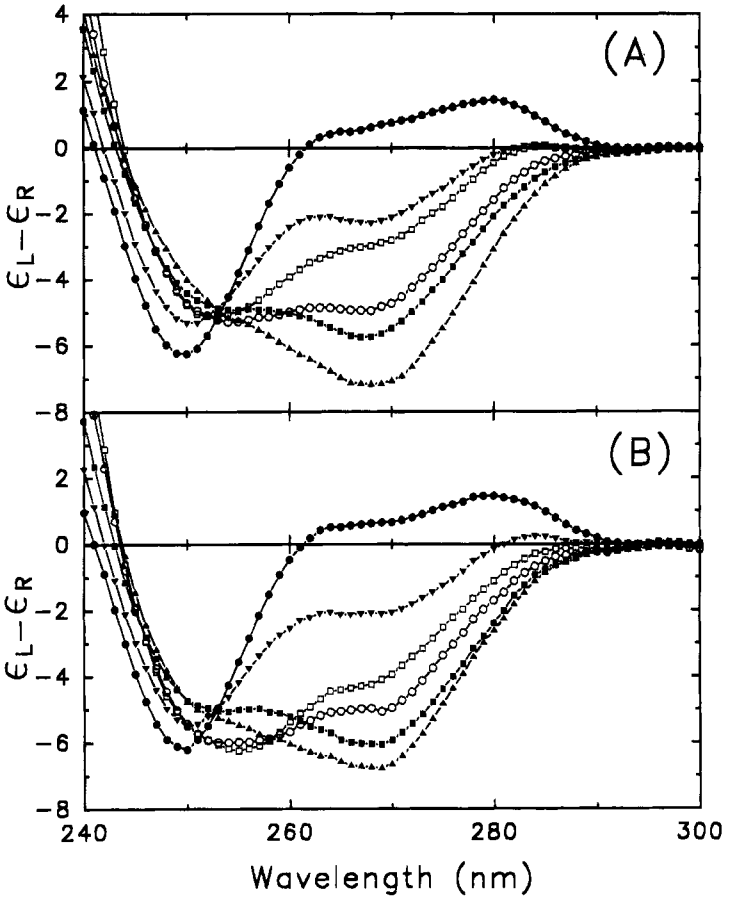

FIGURE 5: A comparison of the poly[d(A)] CD changes upon binding WT g5p (A) and Y34F g5p (B). Representative spectra are shown from example titrations. Spectra in (A) are for free poly[d(A)] (●) and WT protein•poly[d(A)] complexes at P/N ratios of 0.10 (▼), 0.20 (■), 0.26 (▲), 0.34 (○), and 0.48 (□). Spectra in (B) are for free poly[d(A)] (●) and Y34F protein•poly[d(A)] complexes at P/N ratios of 0.09 (▼), 0.18 (■), 0.27 (▲), 0.36 (○), and 0.45 (□).

the salt sensitivity of the binding at higher P/N ratios (see below).

Per mole of protein, WT g5p had small CD bands above 250 nm, as shown in Figure 2. The inset to Figure 4A shows the same spectra as in Figure 4A but after subtraction of the CD contributions of appropriate submolar amounts of WT protein. An assumption in making this subtraction was that the bound protein had the same CD spectrum as the free protein. The subtraction made an insignificant difference at 270 nm. The Y34F mutant protein had larger molar CD bands in the near-UV than did the WT g5p (Figure 2). The inset to Figure 4B shows spectra of the mixtures after subtraction of the appropriate submolar CD contributions of the added Y34F protein. The effect at 270 nm was minimal, but at the higher P/N ratios there was a small but detectable difference at 280 nm where the CD spectrum of Y34F had a maximum. In general, however, the proteins made a minimal contribution to the near-UV CD of the complexes, and it was reasonable to attribute the markedly similar near-UV CD titration changes in panels A and B of Figure 4 to similar conformational changes of the nucleic acid. Equivalent results were obtained with the Y41F and Y26F mutant proteins (not shown).

Titrations of poly[d(A)] with all four proteins also showed two stages of conformational changes of the titrated polymer. Example titrations with the WT g5p and Y34F g5p are shown in Figure 5. While free poly[d(A)] had a positive CD at 270 nm, titration of the polymer with the gene 5 proteins induced large negative CD bands above 250 nm until P/N ratios of about 0.25 were reached (solid symbols). As the titration was continued to give larger P/N ratios, the CD became less negative (open symbols). Kansy *et al.* (1986) previously reported this effect for titrations of poly[d(A)] with the WT protein in 5 mM Tris, pH 7.0−7.8. These authors also reported that there was only one titration breakpoint, and that the CD did not reverse, when the titration was done in the presence of 0.1 M NaCl.

Binding Modes. Example CD titration curves at 270 nm for fd ssDNA during titrations with WT and Y34F gene 5 proteins in 2 mM Na^+ (phosphate buffer, pH 7.0) are plotted in panels A and B, respectively, of Figure 6. CD titrations were also performed with the Y26F and Y41F proteins (not shown). For fd ssDNA at low ionic strength, the addition of any of the four proteins up to a P/N ratio of about 0.33 resulted in proportional CD changes in the ssDNA spectrum, indicating that the binding was stoichiometric. Breakpoints at P/N $\cong$ 0.33 showed that all four proteins bound fd ssDNA in an $n = 3$ mode. As protein was added to give P/N ratios >0.33, the CD at 270 nm reversed its direction of change and appeared to level off only after ratios of 0.45−0.5 were reached. For simplicity, we will refer to the second breakpoint near a P/N ratio of 0.5 as representing an $n \approx 2$ binding mode. In the case of Y41F (data not shown), there was only a slight reversal in the direction of the titration curve between the first and second breakpoints, indicating that this mutant protein could not readily form a complex

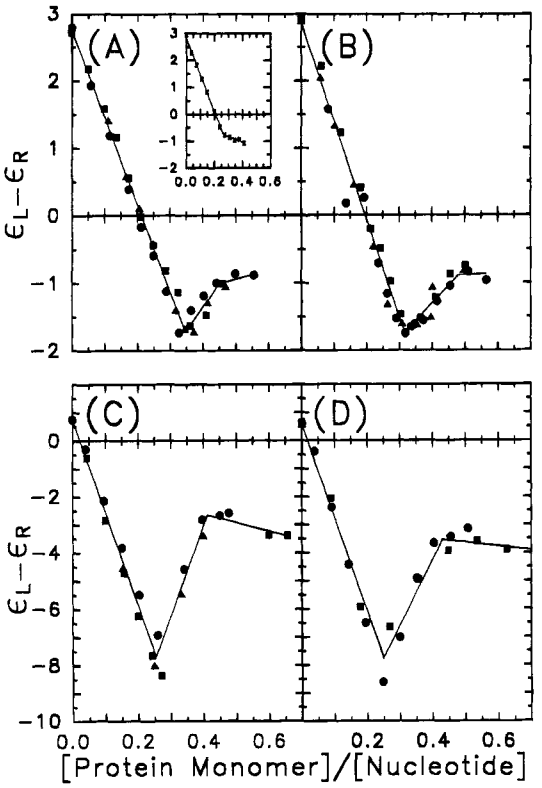

FIGURE 6: CD titration plots at 270 nm of fd ssDNA with (A) WT and (B) Y34F gene 5 proteins and of poly[d(A)] with (C) WT and (D) Y34F gene 5 proteins. The inset in (A) shows data from a titration of fd ssDNA with WT protein in 0.1 M NaCl and 2 mM Na^+ (phosphate, pH 7.0). All other titrations were done in the same buffer without 0.1 M NaCl. In this figure and Figure 7, different symbols represent data from different titrations.

with ssDNA in the $n \approx 2$ binding mode. The hydrogen bonding ability of Tyr-41 may be important in stabilizing the $n \approx 2$ mode with fd ssDNA.

CD titration curves at 270 nm for poly[d(A)] during titrations with WT and Y34F gene 5 proteins in 2 mM Na^+ (phosphate buffer, pH 7.0) are shown in panels C and D, respectively, of Figure 6. The CD titration curves reached their most negative values at P/N ratios of about 0.25, indicating an $n = 4$ mode of binding. Further titration of poly[d(A)] with any of the proteins resulted in a dramatic reversal of the CD change by up to 50%. Titration end points were eventually reached at P/N ratios of about 0.4, with about 2.5 nucleotides per protein monomer. We will term this latter mode of binding an $n \approx 2.5$ mode. The titration breakpoints from all the titrations are summarized in Table 1A.

An $n = 4$ mode of binding to fd ssDNA has been described as the end point found by fluorescence and other techniques (Kansy *et al.*, 1986; Bulsink *et al.*, 1988) in the presence of higher salt concentrations. A titration is included as an inset to Figure 6A to show that this mode of binding, with saturation at a P/N ratio of 0.25, is also evident by CD measurements when 0.1 M NaCl is present. Therefore, depending on the conditions, either the $n = 4$ or the $n = 3$ mode of binding to fd ssDNA can be detected by CD measurements, and the reversal of the long-wavelength CD changes at higher P/N ratios under low salt conditions represents a third mode of binding that is available to g5p. In this mode, each protein monomer accommodates only about 2 nucleotides of fd ssDNA.

Changes in the Tyrosyl 229 nm CD band. Figure 7 is a plot of the tyrosyl 229 nm CD band during titrations of fd ssDNA with the WT and mutant gene 5 proteins. The lines without data points represent the expected CD of free protein if no changes were to occur upon binding i.e., the slopes for additions of free protein. Up to P/N ratios of 0.29−0.32, titrations of fd ssDNA with WT, Y26F, and Y41F gene 5 proteins all showed reduced CD(229) magnitudes of 32−40% relative to that expected for additions of free protein. Breakpoints in the CD(229) titration curves with these three proteins were found at P/N ratios of 0.29−0.31 (Figure 7A,B,D and Table 1A), beyond which the slopes of the titration curve more closely paralleled that expected for additions of free protein. Increased scatter in the data points at P/N ratios above 0.3 obscured any additional breakpoint. However, the fact that the measured and expected slopes were not exactly parallel hints that the tyrosyl CD band at 229 nm continued to change slightly along with the CD of the nucleic acid (Figure 6).

The key point to be made from Figure 7 is that, in the case of Y34F g5p (Figure 7C), the 229 nm titration curve approximately paralleled the slope expected for addition of free Y34F g5p throughout the entire titration range. That is, the CD at 229 nm was not reduced during binding of Y34F g5p to fd ssDNA, as was the case for all the other proteins. With the tyrosine absent from position 34, there was no change in the optical activity of the tyrosyl 229 nm band of the protein upon binding to ssDNA, although the Y34F protein saturated and perturbed fd ssDNA to give CD changes like those induced by the WT g5p (Figures 4 and 6). The titration curve in Figure 7C also supports our assumption that there was a negligible CD change of the nucleic acid at 229 nm and that the CD changes shown in the other titration curves in Figure 7 were from the protein tyrosyl 229 nm band.

The CD changes at 229 nm from titrations of poly[d(A)] with all four proteins (not shown) were qualitatively similar to those reported for fd ssDNA. The background signal from poly[d(A)] was substantial at 229 nm. Nevertheless, for WT, Y26F, and Y41F gene 5 proteins, there was an initial decrease in the 229 nm CD band upon binding to poly[d(A)]. The breakpoints were at about the same P/N ratios as for titrations of fd ssDNA and were intermediate to the breakpoints determined by monitoring the CD changes of the poly[d(A)] nucleic acid (see Table 1). The apparent maximum decrease in the 229 nm CD band was between 38% and 50% for these three proteins upon binding poly[d(A)]. Moreover, as in the case of binding to fd ssDNA, Y34F did not show any initial decrease in CD when binding to poly[d(A)] and essentially paralleled the line expected for the addition of free protein throughout the titration.

Salt Dissociations and Binding Constants. In one type of experiment, complexes were formed between fd ssDNA or poly[d(A)] and each protein at a fixed protein concentration of 2.3×10^{-5} M and at a relatively high P/N ratio of 0.47. The CD change at 270 nm was monitored as aliquots of 4.0 M NaCl were added to increase the Na^+ concentration of the solutions and cause dissociation of the complexes. As demonstrated in the main panel of Figure 8 for dissociations from complexes with poly[d(A)], the most saturated forms of the complexes were dissociated with the initial additions of salt, resulting in increases in the percentage of maximum CD change (i.e., the CD at 270 nm became more

Table 1: (A) Titration Breakpoints, (B) 50% Salt Dissociation Values, and (C) Salt Dependence of Binding Constants for WT and Mutant Gene 5 Proteins

	Part (A) P/N ratios at the titration breakpoints (in 2 mM Na^+)			
	WT	Y26F	Y34F	Y41F
fd ssDNA, 270 nm				
$n = 3$ mode[a]	0.35 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.34 ± 0.01
$n \approx 2$ mode[b]	0.46	0.45	0.50	–[c]
fd ssDNA, 229 nm				
$n = 3$ mode[d]	0.29	0.30	no breakpoint	0.31
poly[d(A)], 270 nm				
$n = 4$ mode[a]	0.25 ± 0.01	0.25 ± 0.02	0.25 ± 0.01	0.30 ± 0.01
$n \approx 2.5$ mode[b]	0.39	0.37	0.42	0.44
poly[d(A)], 229 nm				
$n = ?$ mode[d]	0.29	0.34	no breakpoint	0.32
	Part (B) NaCl (M) for 50% dissociation from primary mode			
	WT	Y26F	Y34F	Y41F
fd ssDNA[e]	0.36 ± 0.07	0.34	0.20 ± 0.05	0.16 ± 0.06
poly[d(A)] [e]	0.21 ± 0.02	0.17	0.10 ± 0.01	0.08 ± 0.01
	Part (C) Salt dependence of binding constants to poly[d(A)]			
	WT	Y26F	Y34F	Y41F
$-d \log(K\omega)/d \log [NaCl]$[f]	4.1 ± 0.8	4.1 ± 0.5	6.6 ± 1.7	4.9 ± 0.6
$K\omega$ ($\times 10^{-5}$ M^{-1})[g]				
at 0.2 M NaCl	1.7 ± 0.4	1.2 ± 0.2	0.05 ± 0.01	0.05 ± 0.007
at 0.1 M NaCl	31 ± 6	20 ± 3	5.0 ± 1.3	1.5 ± 0.2

[a] Mean and range of values from triplicate titrations. [b] Range of values was ±0.05, from triplicate titrations. [c] $n \approx 2$ binding mode was much less distinct in this case. [d] The breakpoint ratios were derived with the assumptions that the CD contribution from the ssDNA remained negligible and that the data could be fit with two straight-line regressions. For values determined from triplicate titrations, the range was ±0.04. [e] Mean and range of values from at least two dissociations, except for single dissociations with Y26F. [f] Slope and standard error from linear regression. [g] Error on $K\omega$ was calculated from standard error on $\log(K\omega)$.

negative), as expected for the dissociation of weak, salt-sensitive complexes that were formed at high P/N ratios under low salt conditions. See Figure 6. This was followed by the dissociation of the $n = 4$ mode of binding for poly[d(A)], and a complete loss of any change in CD. In the case of fd ssDNA, there were also two phases of CD change during salt dissociations (not shown). Dissociations of the most saturated forms of the g5p•fd ssDNA complexes again resulted in a more negative CD at 270 nm at very low salt concentrations (<0.05 M), followed by the dissociation of the $n = 3$ mode of binding. The dual transitions observed during salt dissociations also illustrated that the CD spectra found at the highest P/N ratios (Figures 4–6) were properties of particular conformations of the nucleic acids and were not artifacts, caused, for example, by precipitation.

The salt concentrations needed for 50% dissociation of complexes formed with the same protein concentration are summarized in Table 1B. The order of salt stabilities of complexes of the four proteins with either fd ssDNA or poly[d(A)] was WT > Y26F ≫ Y34F > Y41F.

The relative apparent binding constants $K\omega$ can be estimated at a given salt concentration by first determining the slopes of $\log(K\omega)$ *versus* log [NaCl] plots (Record *et al.*, 1976). With the assumption that ω is much larger than n, it has been shown that the binding constant $K\omega$ (the product of the intrinsic binding constant K and a cooperativity factor ω) equals 1/[free protein] at 50% dissociation (McGhee & von Hippel, 1974). Therefore, a second group of experiments was carried out to compare the binding constants of the proteins for poly[d(A)] in the $n = 4$ mode. Complexes between poly[d(A)] and each protein were formed at a range of concentrations and at a P/N ratio of 0.25 (i.e., in the $n = 4$ binding mode). These were then subjected to saltdissociations as above and as in previous experiments (Sang & Gray, 1989).

The inset to Figure 8 shows the plots, which were essentially linear, of $\log(K\omega)$ *versus* log [NaCl] from the salt dissociations of complexes formed over a range of concentrations. The negative slopes of these plots ($-d \log(K\omega)/d \log [NaCl]$) are given in Table 1C. These slopes indicated that about four ions are released during formation of the complexes of poly[d(A)] plus WT, Y26F, or Y41F proteins (Record *et al.*, 1976), consistent with previous work (Bulsink *et al.*, 1985; Stassen *et al.*, 1992b). Binding of Y34F may result in a greater ion release, but the uncertainty in slope was greatest for the dissociation data for this protein. Values of $K\omega$ extrapolated to 0.1 M NaCl for WT and Y41F proteins (Table 1C) were in substantial agreement with those reported by Stassen *et al.* (1992b), even though different buffer systems were used.

DISCUSSION AND CONCLUSIONS

CD Spectroscopy of the Mutant Gene 5 Proteins. Near-UV CD spectra gave discrete fingerprints of each of the proteins, as shown in Figure 2A. Although Tyr-26, Tyr-34, and Tyr-41 all have >10% solvent exposure on the protein surface (33.7%, 32.9%, and 83.0%, respectively; Terwilliger *et al.*, 1994), their environments vary enough to be reflected in observable differences in near-UV optical activity. The most dramatic difference resulted from the replacement of Tyr-34 with phenylalanine, which yielded a significant

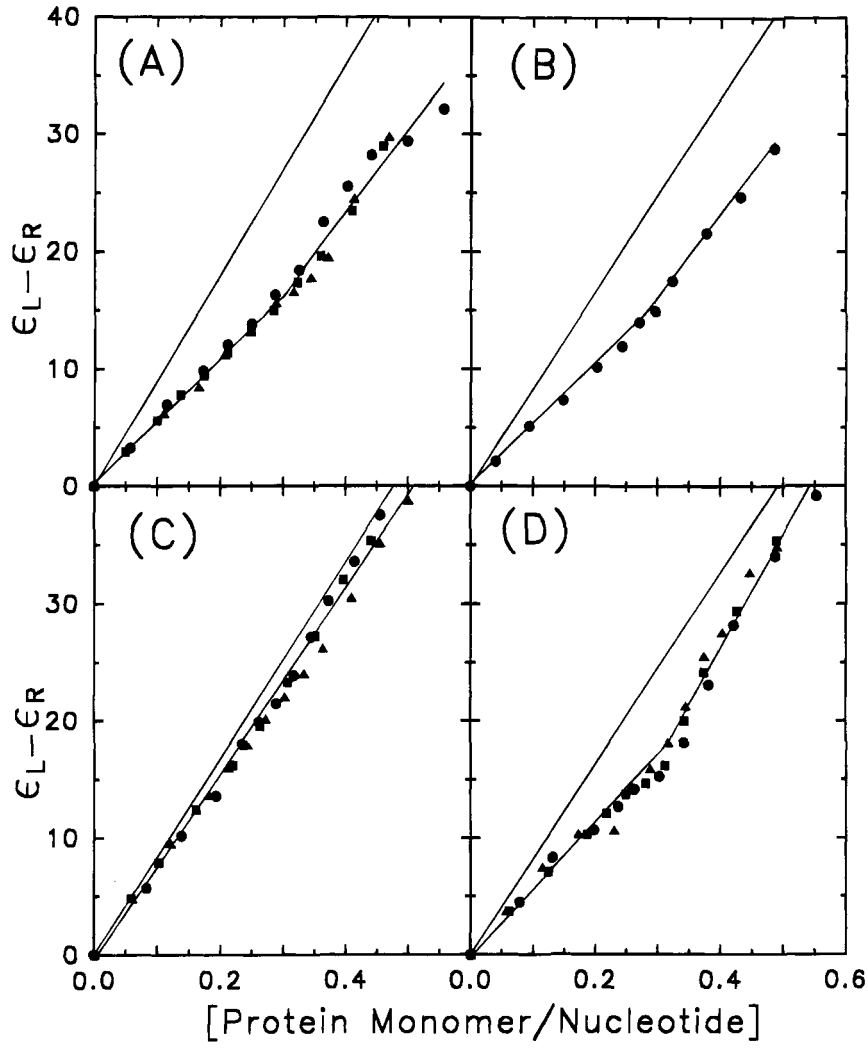

FIGURE 7: CD titration plots at 229 nm of fd ssDNA with (A) WT, (B) Y26F, (C) Y34F, and (D) Y41F gene 5 proteins. The minor contribution of the ssDNA was subtracted from each point. The data-free line represents the CD at 229 nm expected for the addition of free, unperturbed protein.

positive CD band at 280 nm. This suggested that Tyr-34 contributes a relatively large negative CD band to this region of the CD spectrum of the free protein. Replacement of Tyr-26 or Tyr-41 with phenylalanine did not lead to large differences in the magnitudes of the near-UV CD bands. Thus, the positive CD of Y34F in the 270−290 nm region is apparently contributed by Tyr-56 and/or Tyr-61, and this positive CD is largely compensated for by the negative CD of Tyr-34 to give the measured small CD bands shown in Figure 2A for the WT protein. Phenylalanine does not typically have CD bands above 270 nm (Shiraki, 1969; Strickland *et al.*, 1974; Durand *et al.*, 1975).

Figure 3 shows that the magnitudes of the far-UV 229 nm CD bands for Y26F, Y34F, and Y41F gene 5 proteins were essentially equally decreased (9%, 8%, and 10%, respectively) from the 229 nm CD magnitude of the WT g5p. Thus, assuming additive effects, deletion of three of the five tyrosines of gene 5 protein and the addition of three extra phenylalanines would decrease the 229 nm CD of the WT g5p (94 $M^{-1}\cdot cm^{-1}$) by only 27%. A substantial part of the remaining 73% contribution to this CD band is therefore most likely made by Tyr-56 and Tyr-61. The three indigenous phenylalanines (Phe-13, Phe-68, and Phe-73) probably contribute only slightly at 229 nm. The CD of phenylalanine is 10−18% that of tyrosine at 229 nm (Shiraki, 1969; Durand *et al.*, 1975).

Binding Modes. In addition to the two well-established binding modes of fd g5p ($n = 3$ and $n = 4$; reviewed by Kansy *et al.*, 1986; Bulsink *et al.*, 1986, 1988), a third binding mode of $n \approx 2-2.5$ was identified. In this mode, the CD change at long wavelengths was less than the change measured for binding in the penultimate binding modes ($n = 3$ mode for fd ssDNA and $n = 4$ mode for poly[d(A)]). That is, the magnitude of the nucleic acid CD change reversed (i.e., became less negative) upon addition of protein to saturate either nucleic acid in the $n \approx 2-2.5$ mode (Figures 4−6). Since the first mode of binding in the case of poly[d(A)] is an $n = 4$ mode, the $n \approx 2.5$ mode of binding to poly[d(A)] has been assumed, by ourselves and others (Kansy *et al.*, 1986; Bulsink *et al.*, 1988), to be structurally equivalent to the $n = 3$ mode of binding to fd ssDNA. However, we now believe that the second, or $n \approx 2.5$, mode of binding to poly[d(A)] is structurally different from the $n = 3$ mode of binding to fd ssDNA and that it is actually more analogous to the $n \approx 2$ mode of binding to fd ssDNA.

The most straightforward explanation of the reversal of the CD change is that the nucleotides of poly[d(A)] or of fd ssDNA are less perturbed in their most saturated states than

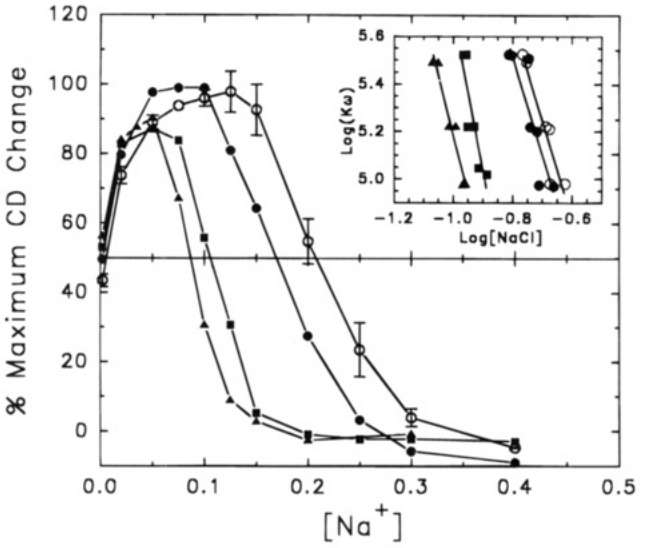

FIGURE 8: Plots of $(\Delta CD_{meas}/\Delta CD_{max})(100)$ at 270 nm *versus* the concentration of NaCl for the dissociation of WT (○), Y26F (●), Y34F (■), and Y41F (▲) gene 5 proteins from complexes with poly[d(A)]. Complexes were formed at a P/N ratio of 0.47 and a final protein concentration of 2.3×10^{-5} M. Error bars show the range of values from two salt dissociations using WT g5p. The initial buffer was 2 mm Na^+ (phosphate, pH 7.0). The inset shows plots of log($K\omega$) *versus* log [NaCl] for duplicate sets of salt dissociation experiments for each protein; symbols as in the main panel. For each set of experiments for the inset, complexes with poly[d(A)] were formed at a P/N ratio of 0.25 and final protein concentrations of about 6, 12, and 21×10^{-6} M.

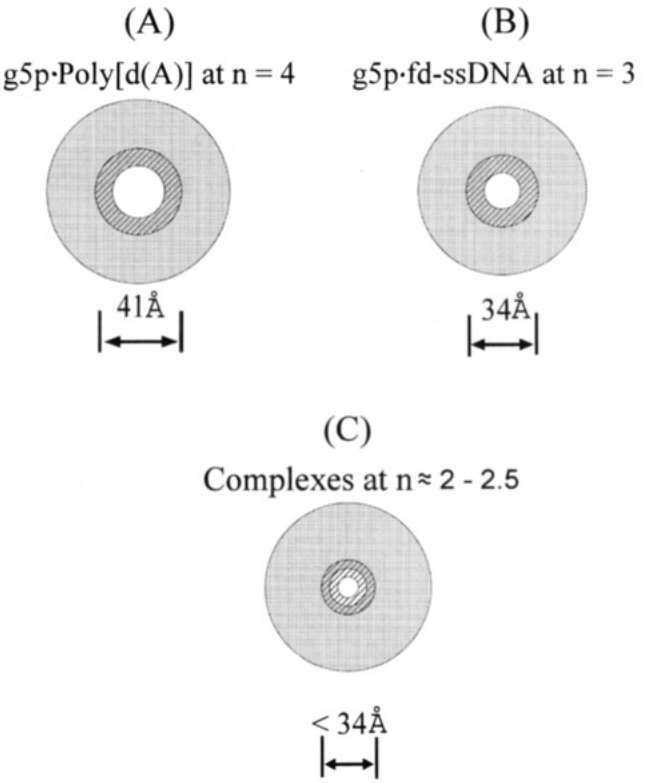

FIGURE 9: Cross-sectional views of helical complexes. The stippled area is the approximate cross-sectional area covered by the g5p protein backbone. The cross-hatched area represents the cross-sectional area covered by the nucleic acid. (A) g5p•poly[d(A)] at $n = 4$; a very similar view holds for g5p•fd ssDNA at $n \approx 4$. (B) g5p•fd-ssDNA at $n = 3$. Views A and B are from small-angle X-ray scattering data of Olah *et al.* (1995). View C is a sketch with the same protein cross-sectional area as in (B) but with the nucleic acid only partially overlapping the protein. This illustrates the concept that a reversal of the nucleic acid CD change (as protein is added in going from the $n = 4$ or $n = 3$ binding mode to the $n \approx 2-2.5$ binding mode) would be consistent with a partial release of the nucleic acid from the protein binding sites. That is, if the overall structure of the superhelix remains substantially unchanged as the nucleic acid becomes saturated in an $n \approx 2-2.5$ binding mode, then the nucleic acid is likely to become more axially extended, at a reduced radius, resulting in only partial contact with the DNA-binding sites of the protein.

in their respective $n = 4$ or $n = 3$ binding modes.

Figure 9 illustrates the type of change in the association of the nucleic acid with g5p that could account for the CD data. The cross-sections in Figure 9A,B are from models of the superhelical complex based on small-angle X-ray scattering of g5p•poly[d(A)] and g5p•fd ssDNA complexes in the $n = 4$ and $n = 3$ binding modes, respectively (Olah *et al.*, 1995). The nucleic acid, represented by cross-hatching, has a radial extent of ±5 Å. The radius of the center of the nucleic acid strands decreases from 15 to 12Å, and the pitch of the helical complex decreases, as the P/N ratio increases from 0.25 to 0.33. In both cases, the phosphate−phosphate distance between nucleotides is 5.0 ± 0.3 Å (Olah *et al.*, 1995).

Electron microscopy of stained g5p•poly[d(A)] complexes visualized by the procedures of Gray (1989, 1994) has shown that complexes formed at P/N ratios of 0.25 and 0.40 are both left-handed helical structures having the same apparent diameter and overall appearance. The mean pitch of g5p•poly[d(A)] complexes measured on electron micrographs decreases from 79 ± 9 to 66 ± 9 Å as the P/N ratio increases from 0.25 to 0.40 (Olah *et al.*, 1995; C. W. Gray, unpublished observations). Therefore, it seems that the superhelical complexes formed with different nucleic acids and at different P/N ratios are all fundamentally the same structures, but with added protein causing a decrease in helical pitch.

If the radial extent of the protein component and the number of protein monomers/helical turn remain constant for the helical superstructure, there are two possibilities for accommodating a reduction in stoichiometry from $n = 3$ to $n \approx 2$. (1) The path of the bound nucleic acid could remain within the protein's DNA-binding site but with the phosphate−phosphate distance being substantially stretched from 5 ± 0.3 to 6.8 ± 0.4 Å (i.e., by (3/2) × (0.91); the latter factor takes a possible reduction in pitch to 66 Å into account). (2) An obvious alternative is that the radius of the path of the nucleic acid could be reduced. If we assume that the pitch is reduced to 66 Å for complexes at $n \approx 2$, that the phosphate−phosphate distance remains ≈5.0 Å, and that the number of nucleotides per helical turn of each strand is 14−15 (i.e., equal to 2 nucleotides/monomer times 7.2−7.6, the number of protein monomers/turn; Olah *et al.*, 1995), it is straightforward to calculate that the radius of the path of the nucleic acid backbone would have to decrease to 4−6 Å . If the nucleic acid has a reduced radius, it may be partially released from the DNA-binding sites of the protein, as sketched in Figure 9C. We propose that binding in the $n \approx 2-2.5$ mode is at least partially accomplished by such a reduction in the radius of the path of the nucleic acid and the release of the nucleic acid from the protein, since this would account for a partial restoration of the optical activity to be more like that of the free nucleic acid. For poly[d(A)], the competition between nucleotide stacking and binding to the protein may be more in favor of stacking than in the case of fd ssDNA, since the reversal of the CD effect is

greater and the reversal occurs at a lower P/N ratio for poly[d(A)] than for fd ssDNA.

Optical Activity of Tyr-34. Day (1973) originally suggested that the initial reduction in the 229 nm CD band of g5p upon binding to nucleic acid (see Figure 7A) was the result of a change in environment or orientation of tyrosine(s). Also, Woody (1978) has shown by calculations involving dipeptides that, depending upon the proximity of the aromatic ring to the peptide bond, the environment can significantly affect the tyrosine L_a transition. Our data indicate that a single tyrosine may be responsible for this reduction in CD and that the tyrosine that undergoes this environmental change, and thus a change in optical activity, is Tyr-34.

If so, the CD of Tyr-34 appears to actually change sign upon binding to ssDNA. That is, free (unbound) WT protein had a CD value at 229 nm of $\approx$89 $M^{-1}\cdot cm^{-1}$, while the CD at 229 nm of bound WT protein (complexed with fd ssDNA at a P/N of 0.33) was reduced to $\approx$54 $M^{-1}\cdot cm^{-1}$ (which may be derived from Figure 7A). The CD at 229 nm of either bound or free Y34F g5p was $\approx$79 $M^{-1}\cdot cm^{-1}$ (Figures 3 and 7C), which was less than that of the free WT protein. Therefore, Tyr-34 contributed a positive CD to the 229 nm band of the free WT protein. Since the CD magnitude of the 229 nm band was much less for the WT protein bound to nucleic acid than it was for the free Y34F mutant protein, and since none of the remaining tyrosines in Y34F mutant protein contributed to a change in optical activity upon binding ssDNA, we conclude that the 229 nm band of Tyr-34 changed sign and magnitude upon binding ssDNA. This change in sign could perhaps be caused by a rotation of the Tyr-34 side chain relative to adjacent peptide bonds (Woody, 1978).

Salt Dissociations. Two binding modes were evident in salt dissociation experiments for all four proteins with poly[d(A)] (Figure 8) and with fd ssDNA (not shown). Whereas the $n = 3$ binding mode for ssDNA and the $n = 4$ binding mode for poly[d(A)]) showed significant differences in salt sensitivity among the four proteins, we did not resolve differences in the salt sensitivities of the $n \approx 2-2.5$ mode of binding with the [NaCl] increments used in the present experiments.

The hydrogen bonding abilities of Tyr-34 and Tyr-41 made significant contributions to the salt stabilities of the protein bound to both fd ssDNA and poly[d(A)]. Binding of the Y34F and Y41F mutant proteins to fd ssDNA was markedly more salt-dependent than was that of the other two proteins (Table 1B). This was in agreement with salt stability studies done by Turner and Kneale (1995) using ssDNA−agarose chromatography on g5p with mutations at positions 26 and 41. They found that Y26F and WT gene 5 proteins both dissociated from ssDNA−agarose columns at the same salt concentration, while Y41A dissociated at half the salt concentration required to dissociate the WT g5p.

Apparent binding constants, $K\omega$, were determined as a function of NaCl concentration (Figure 8 inset and Table 1C) for binding to poly[d(A)] ($n = 4$ mode). By extrapolating the data, we found that the binding constants were ranked WT > Y26F $\gg$ Y34F $\geq$ Y41F for the $n = 4$ binding mode at 0.1−0.2 M NaCl. That is, the greatest decreases in the $K\omega$ for binding to poly[d(A)] at these NaCl concentrations were caused by the loss of the hydroxyls from Tyr-34 and Tyr-41. The substitution of Tyr-26, a residue in the DNA-binding site, with phenylalanine caused a much less dramatic reduction in $K\omega$.

An important conclusion from our work is that Tyr-34 plays a role that is not yet obvious from models of the g5p•nucleic acid superhelix. Tyr-41 and Tyr-34 are respectively within 5 and 7 Å of the dimer−dimer interface in models of the superhelix (Figure 1B). Tyr-34 was the only tyrosine whose CD at 229 nm appeared to change upon binding to nucleic acids. Its substitution by phenylalanine resulted in an increase in salt sensitivity, and a decrease in binding affinity, similar to that caused by substitution of phenylalanine for Tyr-41. In the case of Y41F, the cooperativity factor ω has been shown to be less by a factor of about 3 for the mutant protein compared with the WT protein (Stassen *et al.*, 1992b). Our data suggest that Tyr-34 is likewise involved in stabilizing the dimer−dimer interface and that its hydroxyl may also make an important contribution to ω. It will be interesting to learn from future work on the superhelical complex whether Tyr-34 is indeed directly involved in dimer−dimer interactions or whether it is involved in an intramolecular allosteric change during binding to single-stranded nucleic acids. In either case, the alteration in the environment of Tyr-34 upon binding nucleic acids is one to which CD measurements happen to be exquisitely sensitive.

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