Role of Tyr-22 in the Binding of Pf3 ssDNA Binding Protein to Nucleic Acids†

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ABSTRACT: A Tyr-22 → Phe-22 (Y22F) mutant of the single-strand DNA binding protein (ssDBP) of the filamentous phage Pf3 was obtained by site-directed mutagenesis. An alignment of protein sequences indicates that Tyr-22 of the Pf3 ssDBP corresponds to Tyr-26 of the fd g5p, a tyrosine within the DNA-binding loop. The mutant Y22F Pf3 protein had a CD spectrum very similar to that of native, wild-type Pf3 ssDBP and could bind to both DNA and RNA polymers. In CD titrations of poly[r(A)], poly[r(C)], and Pf3 ssDNA with the Y22F mutant, the saturation endpoints remained the same as for titrations performed with wild-type Pf3 ssDBP, indicating that the mutant protein retained the same $n = 2$ mode of binding as the wild-type protein. However, a second stoichiometric mode of binding at a ratio of one protein monomer to about four nucleotides ($n = 4$) was observed for titrations of these nucleic acids with the Y22F mutant protein. Both proteins showed only an $n = 2$ mode of binding to poly[d(A)], poly-[d(C)], and poly[d(T)] and only an $n = 3$ mode of binding to poly[r(U)]. Distinctly different CD spectral changes of the nucleic acid were observed in titrations of poly[d(A)] with the Y22F mutant and the wild-type protein. Therefore, the mutant and wild-type ssDBP interact differently with some nucleic acids, depending on the base and sugar composition, providing evidence that Tyr-22 is indeed in the DNA-binding loop and may be important in the sequence discrimination of the binding of the Pf3 ssDBP.

The single-strand DNA binding protein (ssDBP)[1] encoded by the *Pseudomonas* phage Pf3 is functionally similar to the gene 5 protein (g5p) of the *Eschericha coli* filamentous Ff phages (M13, f1, fd). Based on phage coat symmetry, Pf3 is a class II phage, while the Ff phages are in class I (Day et al., 1988). Pf3 ssDBP has 78 amino acids (M_r = 8907; Putterman et al., 1984), compared with 87 amino acids for the fd g5p (M_r = 9700; Cuypers et al., 1974). The Pf3 ssDBP exists as a dimer in solution (Powell & Gray, 1993) and forms a protein−ssDNA complex that is a precursor to the mature phage (Casadeval & Day, 1985). By CD spectroscopy, the Pf3 ssDBP appears to have little α-helical content (<1%; Powell & Gray, 1993), consistent with the Pf3 protein being similar in overall secondary structure to the known predominantly β-sheet structure of fd g5p (Brayer & McPherson, 1983; Skinner et al., 1994).

Although fd g5p and Pf3 ssDBP share little overall amino acid homology (15%), there is some conservation of amino acid residues, particularly in the region of the fd g5p sequence known to be involved in DNA binding (Peeters et al., 1983). Of the five tyrosines present in fd g5p (see Figure 1), three (Tyr-26, Tyr-56, Tyr-61) are conserved in Pf3 ssDBP (Tyr-22, Tyr-53, Tyr-57) while the remaining two (Tyr-34 and Tyr-41) are conservatively substituted by phenylalanine in Pf3 ssDBP (Phe-28 and Phe-36). The roles that tyrosines might play in the binding of fd g5p to nucleic acids have been the subject of investigation by protection studies and NMR spectroscopy (Anderson et al., 1975; King & Coleman, 1988; Stassen et al., 1992a,b). The results of these studies show that only Tyr-26 is directly involved in the binding of fd g5p to nucleic acids. Since this residue is conserved in Pf3 ssDBP (Tyr-22), it seemed likely that Tyr-22 of the Pf3 protein might also play a direct role in the binding of Pf3 ssDBP to nucleic acids.

In this study, we describe the binding properties of a mutant of Pf3 ssDBP in which Tyr-22 was substituted by phenylalanine (Y22F). Since tyrosine can interact with nucleic acids through either stacking interactions or hydrogen bonding via its hydroxyl group, but phenylalanine can only stack with nucleic acid bases, a Y22F mutant allows us to investigate whether the loss of a hydrogen bond (plus a possible increase in stacking) affects the CD properties and interactions of Pf3 ssDBP with nucleic acids. Stoichiometries and salt sensitivities of complexes of the Y22F mutant protein with the homopolymers poly[d(A)], poly[r(A)], poly-[d(C)], poly[r(C)], poly[d(T)], and poly[r(U)], as well as with Pf3 ssDNA, were determined from the nucleic acid CD changes during protein titration and salt-dissociation experiments. The results are compared with data previously obtained for wild-type Pf3 ssDBP.

MATERIALS AND METHODS

Plasmid Construction. The region coding for the Pf3 ssDBP was isolated from Pf3 phage replicative form (RF) DNA by cleavage with the restriction enzymes *Eco*RI and *Hin*dIII. The resulting 537 bp *Eco*RI−*Hin*dIII fragment was

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[1] Abbreviations: ssDBP, single-strand DNA binding protein; g5p, gene 5 protein; CD, circular dichroism; OD, optical density; cell buffer, 10 mM Tris-HCl plus 70 mM NaCl at pH 8.2; IPTG, isopropyl D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; P/N, [protein monomer]/[nucleotide] molar ratio; RF, replicative form.

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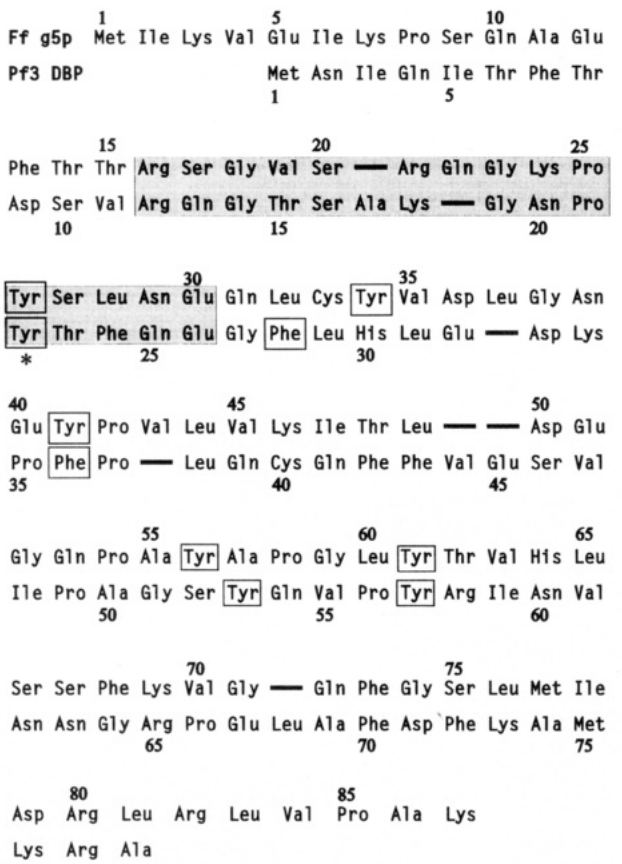

FIGURE 1: Amino acid sequences of fd g5p (top) and Pf3 ssDBP (bottom) aligned according to Peeters et al. (1983). The highlighted region denotes the DNA binding loop of the fd g5p. The Tyr of each protein and the conservatively substituted Phe of Pf3 are highlighted by single boxes. The asterisk denotes Tyr-22.

then cloned into the multicloning site of a pUC18 derivative which had previously had the unique *Afl*III restriction site destroyed by site-directed mutagenesis. The plasmid pMDP6 contained the entire coding region of Pf3 ssDBP under the control of the *lac* promoter and contained a unique *Afl*III restriction site in the coding region at Tyr-22.

The tyrosine at position 22 in Pf3 ssDBP was changed to phenylalanine by site-directed mutagenesis using the method of Deng and Nickoloff (1992). The oligonucleotide used for the mutagenesis (synthesized by R. L. Ratliff, Los Alamos National Laboratory, Los Alamos, NM) had the following sequence: 5′ CCC TTC TTG GAA CGT GAA AGG ATT CCC TTT AGC 3′. Mutagenesis changed the Tyr-22 codon from TAC to the Phe codon TTC (underlined position) which substituted a phenylalanine at position 22 and destroyed the unique *Afl*III site present while creating a unique *Xmn*I site. Since this particular mutation destroyed a unique restriction site, only one primer was needed for the protocol, which uses the destruction of a restriction site as a means of selection against the wild-type strand. The resulting plasmid pMDP16 contained the mutant Pf3 ssDBP protein with phenylalanine substituted for tyrosine at amino acid position 22 (Y22F). The sequence of pMDP16 was confirmed by the dideoxynucleotide chain termination method (Sanger et al., 1977), using [α-^{35}S]dATP and Sequenase.

Phage Reconstruction Experiments. Attempts were made to replace the coding region of wild-type Pf3 ssDBP with the Y22F mutation in the phage genome. Wild-type RF DNA was digested with *Hin*dIII and partially digested with *Eco*RI to release the *Hin*dIII-*Eco*RI fragment containing the ssDBP coding region. The fragment containing the rest of the Pf3 genomic sequence was then ligated to the *Hin*dIII-*Eco*RI fragment containing the Y22F mutation using T4 DNA ligase (Promega). As a control, the wild-type *Hin*dIII-*Eco*RI fragment was also ligated in a separate reaction. The recombinant RF DNAs were transformed into *Pseudomonas aeruginosa* (PA02), which was plated for plaque assays. In repeated attempts, no plaques were obtained from the constructs containing the Y22F mutation, while the wild-type control recombinant DNAs produced from 100 to 200 PFUs per 100 ng of RF DNA.

Protein Isolation. Y22F protein was expressed in *E. coli* strain K561 (Davis et al., 1985) containing pMDP16. Cells were grown in a medium of 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 100 mg/L ampicillin at 37 °C. IPTG was added to a final concentration of 1 mM to induce expression when the cell density of the culture had reached an OD(650) = 1. Cells were harvested after three additional hours of incubation by centrifugation for 10 min in a GS3 (Sorvall) rotor at 4000*g*, 4 °C. Pellets were combined and stored at −20 °C until needed. Isolation of protein from frozen cells was by the same procedure used for isolating wild-type Pf3 ssDBP from phage-infected cells (Powell & Gray, 1993). Purified protein was kept in cell buffer (10 mM Tris-HCl, 70 mM NaCl, pH 8.2) at 4 °C for immediate use or stored at −20 °C. Protein concentration was determined by absorbance measurements at 276 nm using an extinction coefficient of 2754 L·Mol^{-1}·cm^{-1}, calculated from the extinction coefficients of tyrosine and phenylalanine (CRC Handbook of Biochemistry, 2nd ed., 1970). The proteins were >95% pure as determined by polyacrylamide gel electrophoresis.

Absorption and CD Measurements. Absorption spectra were taken with a Cary Model 118 spectrophotometer, and CD spectra were obtained using a Jasco Model J500A spectropolarimeter calibrated and operated as described by Antao et al. (1988). CD data are plotted in nanometer intervals and are shown as $\epsilon_L - \epsilon_R$ in units of L·Mol^{-1}·cm^{-1}, per mol of amino acid or per mol of nucleotide. In all experiments, the temperature was maintained at 20 ± 0.5 °C.

Nucleic Acids. All the polynucleotides used in this study were purchased from Sigma Chemical Co. Calculation of the concentrations and the physical parameters of the individual polynucleotides were the same as previously described (Powell & Gray, 1993). As in the previous work, the polymers were dialyzed into cell buffer.

Titrations and Salt Dissociations. Titrations of nucleic acids with the Y22F ssDBP were done as previously described for titrations with the wild-type ssDBP (Powell & Gray, 1993) in cell buffer. Also as in the previous work, salt dissociation of complexes was accomplished by additions of aliquots of 4 M NaCl. Exact concentrations were derived from the change in volume, determined by weighing the sample before and after each addition, taking into account the density of 4 M salt. For the salt dissociation experiments, complexes were made by saturating each polynucleotide with ssDBP to give a [protein monomer]/[nucleotide] molar ratio (P/N) of 0.5, with the exception of poly[r(U)], which was saturated at a P/N = 0.33 molar ratio. The complexes were formed such that the total concentration of protein monomer was 23 μM in each case. Dissociation was monitored by

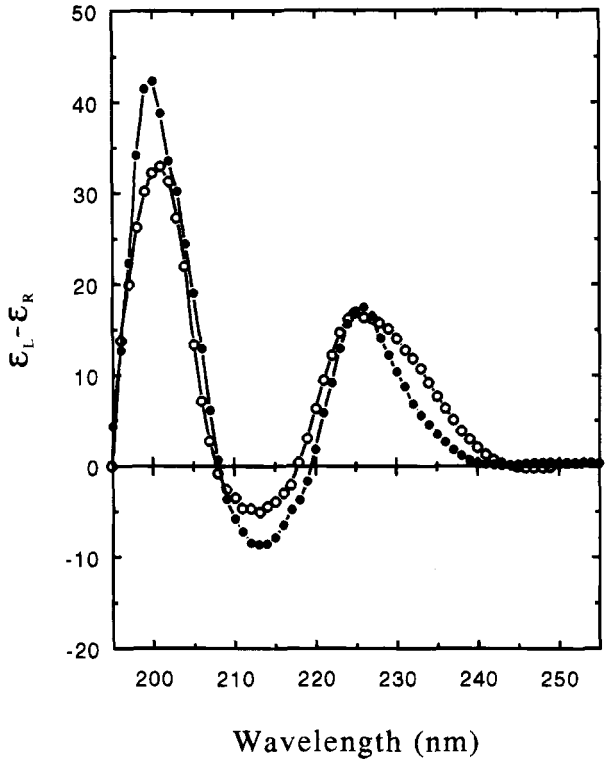

FIGURE 2: Representative CD spectra of wild-type Pf3 ssDBP (●) and the Y22F mutant ssDBP (○). Spectra are plotted in units of $L \cdot mol^{-1} \cdot cm^{-1}$ ($\epsilon_L - \epsilon_R$) per mol of protein monomer. Spectral data in this and the following figures were taken with samples in 10 mM Tris-HCl, 70 mM NaCl, pH 8.2, 20 °C. Both proteins were isolated from cloned genes. The CD band below 210 in the spectrum of the wild-type PF3 ssDBP shown here had a maximum at 200 nm, while the band maximum was at 202−204 nm for the wild-type protein isolated from infected cells (Powell & Gray, 1993), presumably due to a slight difference in purity.

the change in CD at 275 nm after each addition of NaCl for all of the nucleic acids except for poly[d(A)], which was monitored at 250 nm. The percent dissociation was calculated from a comparison of the CD spectrum of partially dissociated complex to the spectrum of free nucleic acid corrected for any change in the CD spectrum of the nucleic acid due to the addition of salt.

RESULTS

Y22F Protein Isolation. Isolation of Y22F protein from *E. coli* K561 containing pMDP16 was essentially the same as the isolation of wild-type protein from phage-infected *Ps. aeruginosa* PA02 (Powell & Gray, 1993). Expression of Y22F protein from the plasmid pMDP16 was substantially greater (>5-fold) than that obtainable from the wild-type plasmid pMDP6. Stassen et al. (1992a) similarly observed large increases in the amount of protein expressed from a plasmid carrying a copy of an analogous mutant (Y26C) in M13 g5p.

The mobility of Y22F on SDS-PAGE gels was indistinguishable from that of wild-type ssDBP. They both migrated at an apparent molecular weight of 6000 even though the known molecular weight of the monomer is 8907 (Putterman et al., 1984). The molecular weight of Y22F in solution as determined by gel filtration on a Sephadex G-50 column was approximately 18 000, close to the predicted molecular weight of a dimer (M_r = 17 814); the elution profile was indistinguishable from that of the wild-type ssDBP.

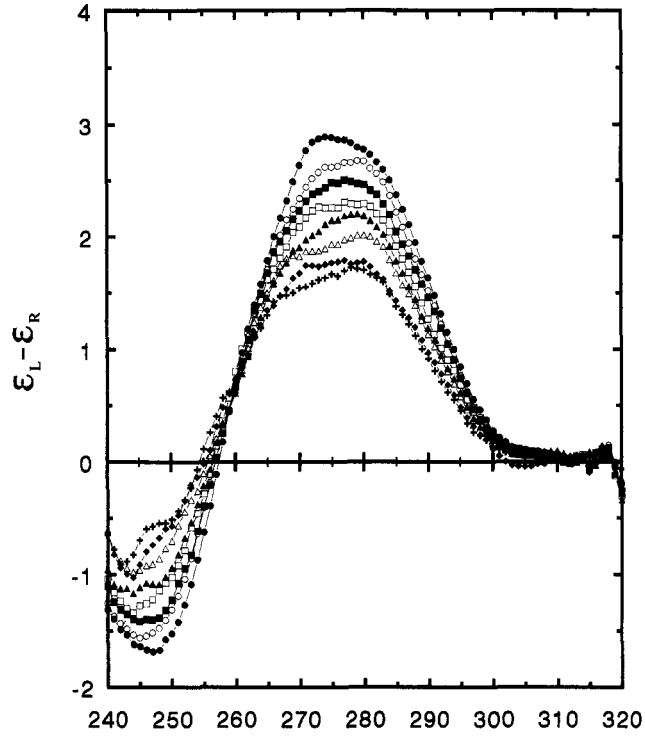

FIGURE 3: Titration of Pf3 ssDNA with wild-type ssDBP. Spectra are for free DNA (●) and DNA • protein complexes at P/N ratios of 0.07 (○), 0.14 (■), 0.21 (□), 0.28 (▲), 0.36 (△), 0.43 (◆), and 0.50 (+). Note the isodichroic point at 260 nm.

CD of Free Y22F Protein. Figure 2 shows a comparison of the CD spectra of wild-type and Y22F ssDBPs. CD spectra of the fd g5p have a band centered about 229 nm, which is primarily due to the contributions of the tyrosines in this protein (Day, 1973). As seen in Figure 2, the spectra of Y22F and wild-type Pf3 ssDBPs had tyrosyl CD bands of similar magnitude at 226 nm, and, in fact, the spectra were similar throughout the 190−240 nm region. The magnitude of the CD band centered at about 226 nm was approximately the same for both proteins, even though the Y22F ssDBP had only two of three tyrosines present in the wild-type. The CD band at 226 nm of the Y22F protein did not change upon binding to any of the nucleic acids in this study (data not shown). This was consistent with the lack of a CD change in this band of the wild-type Pf3 ssDBP during complex formation (Powell & Gray, 1993). Overall, the CD spectrum of the mutant Y22F protein indicated that the single amino acid substitution did not significantly alter the secondary structure of the protein.

CD Titrations. Figure 3 shows typical spectra from a titration of Pf3 ssDNA with wild-type Pf3 ssDBP. On this scale, the CD of the protein was negligible in the region of 240−310 nm. Therefore, CD changes in this region are likely due to changes in the nucleic acid structure upon binding. Figure 4 shows a similar graph of spectra from a titration of Pf3 ssDNA with Y22F ssDBP. Note that there was an isodichroic point at approximately 260 nm in the spectra of the titration with the wild-type protein (Figure 3). The same isodichroic point was present in the spectra of the titration of Pf3 ssDNA with Y22F (Figure 4), but only for the spectra obtained for [protein monomer]/[nucleotide], or P/N, ratios up to 0.2−0.25. Spectra of samples at P/N >

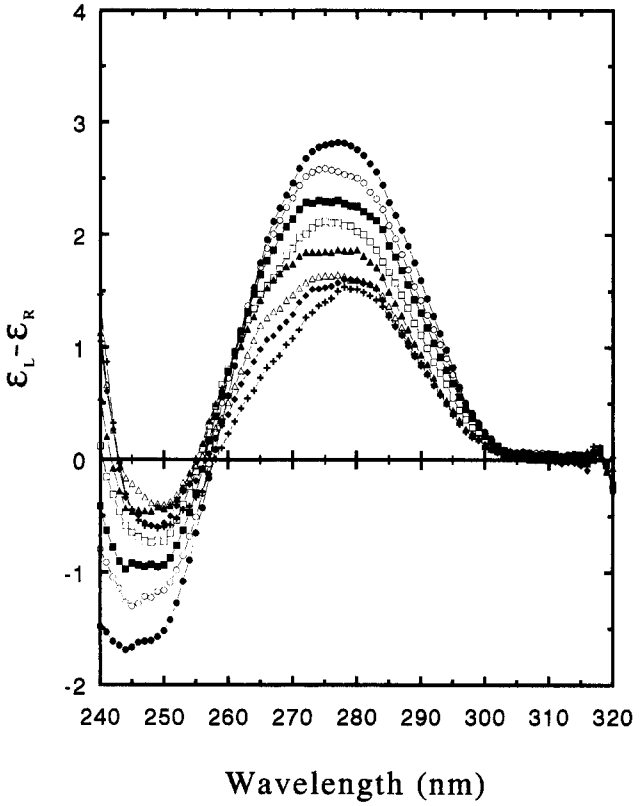

FIGURE 4: Titration of Pf3 ssDNA with Y22F mutant ssDBP. Spectra are for free DNA (●) and DNA • protein complexes at P/N ratios of 0.07 (○), 0.11 (■), 0.16 (□), 0.28 (▲), 0.33 (△), 0.38 (◆), and 0.50 (+). Spectra of samples up to a ratio of 0.28 have an isodichroic point at 260 nm. Spectra of samples with P/N ratios of 0.28 or higher are not in the same family of curves that cross through the isodichroic point.

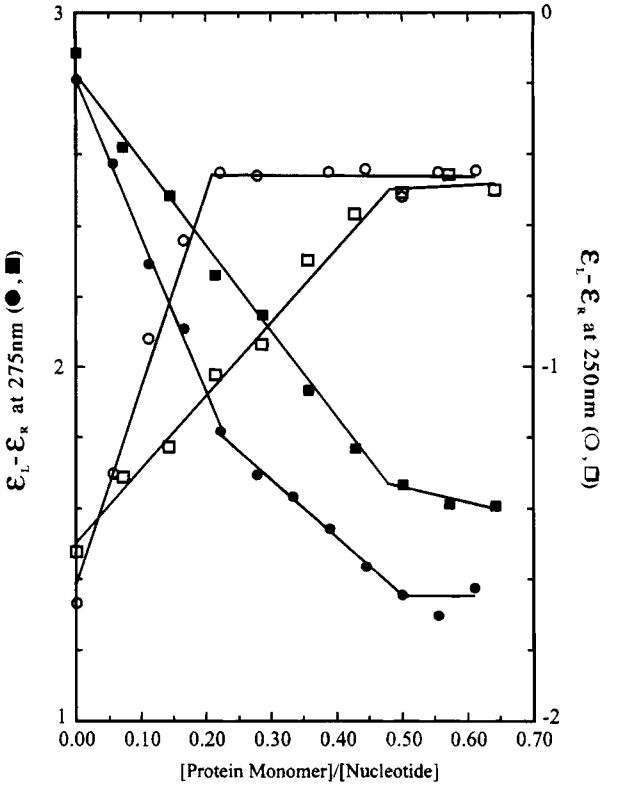

FIGURE 5: Titration plots of Pf3 ssDNA with wild-type ssDBP at 250 nm (□, right axis) and at 275 nm (■, left axis). Also shown is a similar plot of Pf3 ssDNA with Y22F mutant ssDBP at 250 nm (○, right axis) and at 275 nm (●, left axis).

0.25 did not intersect each other at wavelengths above 250 nm. That is, the spectra obtained from a titration with the mutant fell into two classes, one of which resembled spectra obtained from a titration with the wild-type protein.

Titration plots were constructed by plotting the change in the molar CD of a nucleic acid at a particular wavelength versus the [protein monomer]/[nucleotide] molar ratio. Figure 5 shows the results of titrations plots at two wavelengths (275 and 250 nm) for the titrations of Pf3 ssDNA with wild-type ssDBP and the Y22F mutant protein. The titration plots could be fit with straight lines, indicating that binding was stoichiometric. Endpoints were reached at P/N $\cong$ 0.5, with the exception of the CD change at 250 nm for the titration with Y22F, which reached an endpoint at P/N $\cong$ 0.2−0.25. In addition, the plot of the 275 nm CD from the titration with the Y22F mutant showed a breakpoint at P/N $\cong$ 0.2−0.25 as well as an endpoint at P/N $\cong$ 0.5.

Similar plots were constructed for titrations of poly[d(A)], poly[r(A)], poly[d(C)], poly[r(C)], poly[d(T)], and poly[r(U)] to determine the endpoints of these titrations. Results of these titrations are summarized in Table 1. When titrations were performed with the mutant Y22F protein, the homopolymers poly[r(A)] and poly[r(C)] showed an additional breakpoint in their titration curves at P/N $\cong$ 0.25 (Figures 6 and 7) . In these cases, the total CD changes at P/N $\cong$ 0.5 were the same as seen with titrations using the wild-type protein. The types of CD changes were also qualitatively the same for titrations with both proteins (not shown). However, the slopes of the CD titration curves with the mutant protein were greater up to P/N $\cong$ 0.25. This resulted in an additional breakpoint, analogous to that seen in titrations of Pf3 ssDNA with Y22F ssDBP.

The homopolymers poly[d(A)], poly[d(C)], poly[d(T)], and poly[r(U)] showed no intermediate breakpoints in the titration plots with Y22F ssDBP. They all had single endpoints at P/N $\cong$ 0.5 or 0.33 (Table 1). The CD changes of poly[d(C)], poly[d(T)], and poly[r(U)] caused by the mutant protein were all comparable to the changes caused by the wild-type ssDBP. The change in the CD spectrum of the poly[d(A)] homopolymer, however, was qualitatively quite different (Figure 8) with the two proteins. During the titration with the Y22F mutant protein, the CD bands of poly[d(A)] above 260 nm slightly decreased, while during the titration with the wild-type ssDBP there was a decrease of the 280 nm band but a substantial increase in the small band at about 265 nm. In addition, the magnitude of the change in the CD band at 250 nm was less in titrations with Y22F ssDBP than with wild-type ssDBP. A comparison of the CD titrations of poly[d(A)] with Y22F and wild-type ssDBP provided the clearest example of structurally different nucleic acid changes during complex formation with the two proteins.

Salt Dissociation of Complexes. To determine if the Y22F substitution had any affect on the salt sensitivity of the complexes, salt dissociation experiments were performed. Complexes were formed at a P/N ratio of 0.5 for each nucleic acid, with the exception of complexes with poly[r(U)], which were formed at a P/N ratio of 0.33. All of the complexes were made such that the total protein concentration was the same, 23 μM, as previously used in salt-dissociation experiments with wild-type Pf3 ssDBP (Powell & Gray, 1993). Therefore, the concentrations of NaCl needed to dissociate the complexes to 50% could be used to compare the salt

Table 1: Comparison of Pf3 Wild-Type and Y22F Mutant ssDBP Binding Characteristics[a]

	WT			Y22F		
nucleic acid	absorbance % change (260 nm)	binding modes (n)	[NaCl] (M) at 50% dissociation	absorbance % change (260 nm)	binding modes (n)	[NaCl] (M) at 50% dissociation
Pf3 ssDNA	+12.5[b]	2[c] (4)[e]	0.52[d]	+16.7[b]	2, 4[c]	0.31[d]
poly[d(A)]	+21.9	2	0.33	+18.4	2	0.20
poly[r(A)]	+23.5	2	0.51	+19.2	2, 4	0.43
poly[d(C)]	−5.9	2	0.12	+0.6	2	0.20
poly[r(C)]	+6.9	2	0.21	+10.9	2, 4	0.22
poly[d(T)]	−9.9	2	>4.0	−9.4	2	>4.0
poly[r(U)]	−7.3	3	1.28	−6.1	3	1.34

[a] All titrations were done in 10 mM Tris-HCl, pH 8.2, plus 70 mM NaCl at 20 °C. Salt dissociations were begun with the complexes in the same buffer, and small aliquots of concentrated NaCl were added to increase the [NaCl]. Values for WT Pf3 ssDBP are the averages from three or more titration or dissociation experiments. Values for Y22F mutant ssDBP are the averages from two or more titration or dissociation experiments. All of the salt dissociation experiments were done starting with complexes at the same 23 μM total protein concentration. [b] Percent absorbance changes are for the total change, neglecting any intermediate breakpoints in the titration curves. The standard deviation for experiments in which there were three or more titrations was ±1.5% or less for both WT and Y22F measurements. [c] Values shown for n for both WT and Y22F are rounded to the nearest whole number. Errors were ±0.3, taken as the ranges of the endpoints of two or more linear regressions. [d] Salt dissociation values are the 50% dissociation points of data fitted by a third-order polynomial regression. The values are averages of two or more regressions and the standard deviations from the individual regressions were ±0.04 or less. [e] A second mode of binding has been identified by gel mobility shifts (Powell & Gray, 1993).

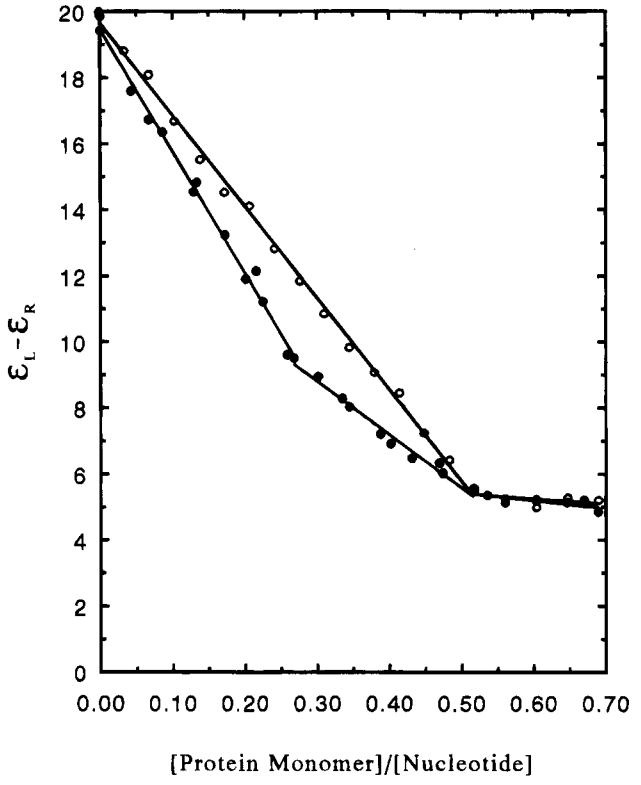

FIGURE 6: Titration plots of poly[r(A)] with wild-type ssDBP (○) and Y22F mutant ssDBP (●) at 265 nm.

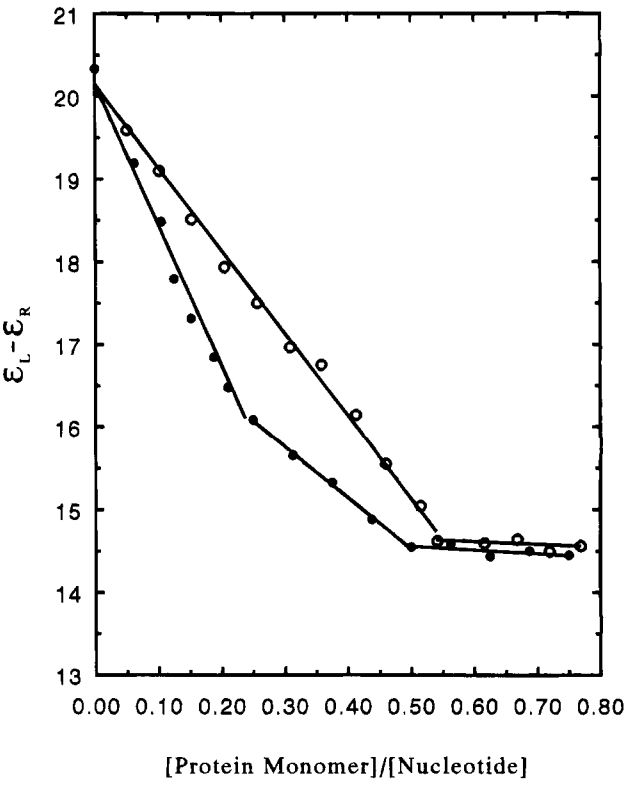

FIGURE 7: Titration plots of poly[r(C)] with wild-type ssDBP (○) and Y22F mutant ssDBP (●) at 265 nm.

sensitivities of complexes with the two proteins. The results of the salt dissociations are summarized in Table 1 along with the values previously determined for the wild-type ssDBP complexes.

Complexes of poly[d(T)] with the Y22F mutant protein would not dissociate in 4 M NaCl, like complexes of poly[d(T)] with the wild-type protein. Likewise, complexes of poly[r(U)] and poly[r(C)] with Y22F ssDBP had nearly identical salt sensitivities to those of complexes with the wild-type protein. However, both poly[r(A)] and poly[d(A)] formed complexes with Y22F ssDBP that were more salt-sensitive, while poly[d(C)] formed a less salt-sensitive complex with the mutant protein. The largest difference in salt dissociation values for complexes formed with the two proteins was seen with Pf3 ssDNA, which formed a more salt-sensitive complex with the mutant protein.

DISCUSSION AND CONCLUSIONS

Protein Isolation. As mentioned in Results, the expression of the Y22F mutant protein was substantially greater than that of wild-type ssDBP in the same system. Expression of the wild-type protein eventually resulted in cell death, as evidenced by the failure of *E. coli* K561 cells containing copies of the wild-type gene to grow on LB (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with 10 mM IPTG. Expression of the Y22F ssDBP under the same conditions resulted in poor growth on the 10 mM IPTG

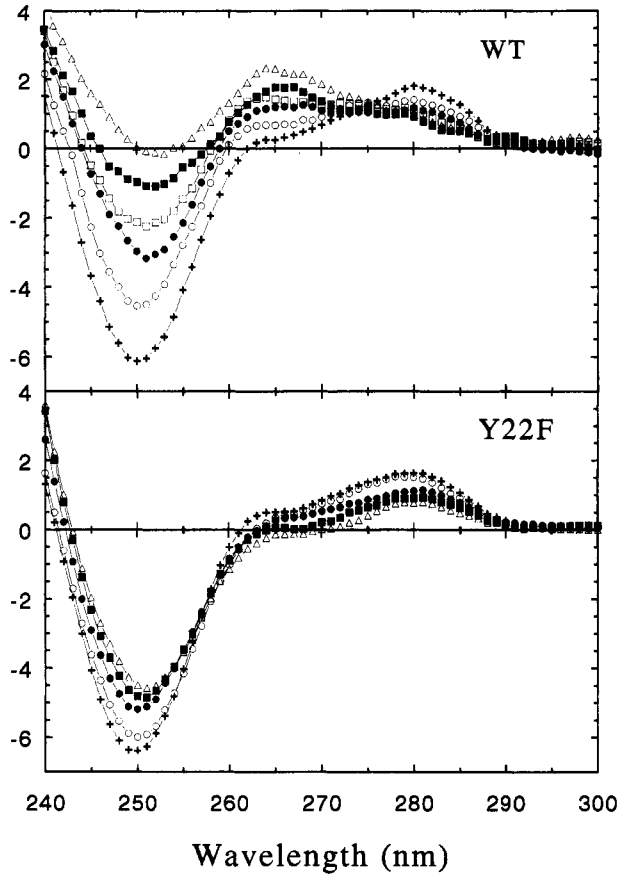

FIGURE 8: Comparison of the poly[d(A)] CD changes upon binding wild-type ssDBP (upper panel, WT) and Y22F mutant ssDBP (lower panel, Y22F). Spectra in the upper panel are for free poly[d(A)] (+) and DNA • protein complexes at WT [protein monomer]/[nucleotide] molar ratios of 0.13 (○), 0.25 (●), 0.32 (□), 0.38 (■), and 0.50 (△). Spectra in the lower panel are for free poly[d(A)] (+) and DNA • protein complexes at Y22F mutant [protein monomer]/[nucleotide] molar ratios of 0.08 (○), 0.25 (●), 0.42 (■), and 0.50 (△).

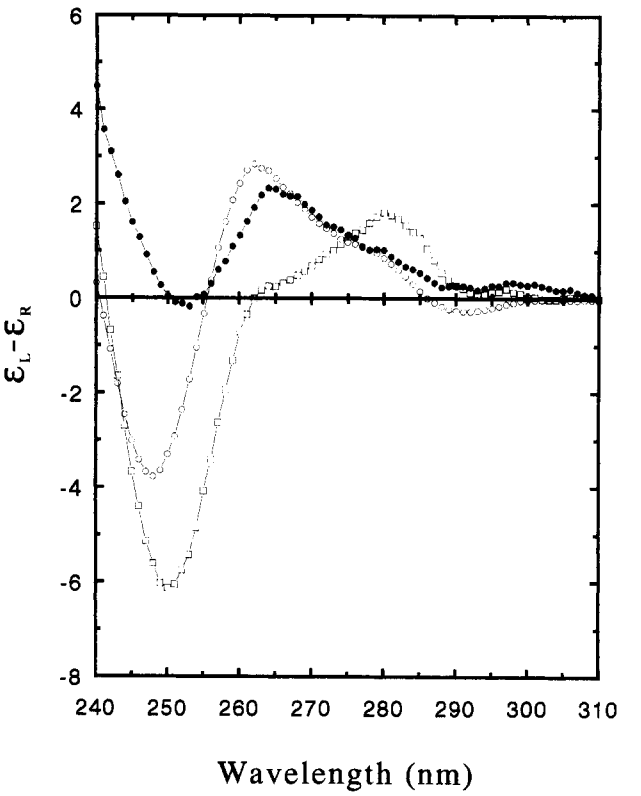

FIGURE 9: CD spectra of free poly[d(A)] at pH 8.2 (□), free poly[d(A)] at pH 4.8 (○), and poly[d(A)] complexed with wild-type Pf3 ssDBP at P/N = 0.5 (●).

plates, but small colonies did eventually form. It is possible that, in the absence of phage ssDNA, the Pf3 ssDBP binds to either DNA replication forks or free mRNA and inhibits or prevents cell growth. The reduced toxicity of the Y22F protein could reflect a reduced affinity of this protein to ssDNA and the adenine-containing homopolymers (Table 1).

Spectroscopy of the Free Y22F ssDBP. The CD spectrum of the wild-type protein is dominated by contributions from tyrosine (Powell & Gray, 1993). The CD spectra of the free Y22F ssDBP and wild-type ssDBP were very similar. The molar magnitude of the 226-nm band was almost the same for both proteins. This suggested that the contribution of Tyr-22 to the CD of this band is negligible in the wild-type protein and that the contributions of Tyr-53 and Tyr-57 dominate the 226-nm band. The analogous two Tyr of the fd g5p (Tyr-56 and Tyr-61) also apparently dominate the Tyr 229-nm CD band of g5p (B. Mark, UT Dallas, personal communication). It is interesting that these two Tyr are close to each other in the crystal structure of the fd g5p (Skinner et al., 1994).

The similarity in overall shape of the Y22F protein CD spectrum to that of wild-type ssDBP indicated that the change in Tyr-22 did not drastically alter the protein secondary structure. Likewise, the wild-type and mutant proteins both formed dimers, similarly changed the CD of the homopolymers studied, except poly[d(A)], and had similar stoichiometric endpoints. These observations indicated that the secondary and tertiary structures of the Y22F and wild-type ssDBPs were similar.

CD Titrations. The most dramatic CD change on binding was evident in the titrations done with poly[d(A)]. Titrations of poly[d(A)] with Y22F and wild-type ssDBPs gave qualitatively different spectra (Figure 8) due to differences in the nucleic acid structure upon binding. This suggested that the hydroxyl group of Tyr-22 in wild-type ssDBP had a unique interaction with deoxyadenosines. We noted that the change in CD above 255 nm of the poly[d(A)] when protonated at low pH was similar to the change in CD of the poly[d(A)] when complexed with wild-type ssDBP. Figure 9 shows the acid-induced structure of poly[d(A)] at pH 4.8 (Antao & Gray, 1993) compared with poly[d(A)] complexed with wild-type Pf3 ssDBP. According to Bush and Scheraga (1969), the pH-dependent changes seen in the spectra of poly[d(A)] are consistent with an increased solvation, protonation, or hydrogen bonding and a shift of an n $\rightarrow \pi^*$ band from 280 nm to shorter wavelengths. It appears that poly[d(A)] interacts with the hydroxyl of Tyr-22 of the wild-type protein to cause a CD change above 255 nm similar to protonation at low pH. In the case of the Y22F mutant protein, the hydrogen of the hydroxyl is no longer available for such an interaction, resulting in a general reduction in the magnitudes of the poly[d(A)] CD bands, consistent with a more simple loss of base stacking.

The Tyr-22 $\rightarrow$ Phe-22 substitution did not affect the final endpoints of any of the titrations with the nucleic acids used in this study. All endpoints were at $n = 2 \pm 0.3$, with the exception of the titration of poly[r(U)], which had an endpoint at $n = 3$ for titrations with both wild-type and

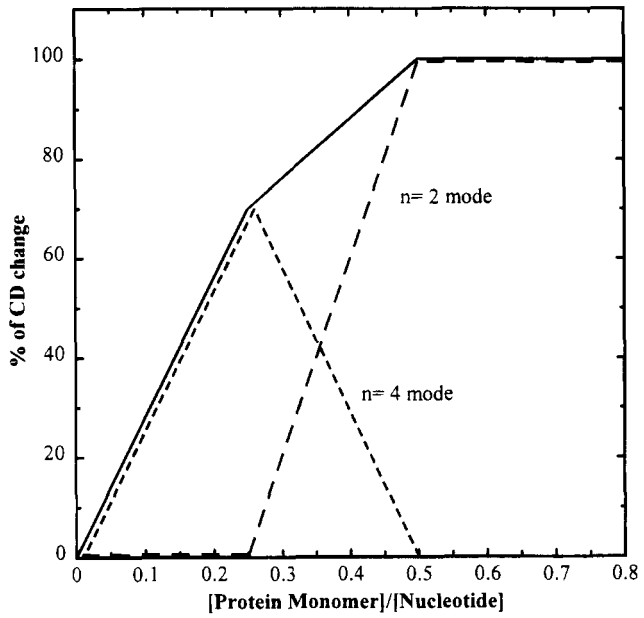

FIGURE 10: Schematic of how two exclusive modes of binding ($n = 2$ and $n = 4$ modes) with large intermode cooperativities and different CD contributions could lead to a breakpoint at a [protein monomer]/[nucleotide] ratio $\cong$ 0.25 in CD titration curves under stoichiometric binding conditions. In this illustration, the CD change per nucleotide bound in the $n = 4$ mode is 0.7 that of a nucleotide bound in the $n = 2$ mode. It should be noted that a maximum CD effect at P/N $\cong$ 0.25 by binding in an $n = 4$ mode does not require that the DNA lattice be saturated at this P/N ratio (Kansy et al., 1986; Bulsink et al., 1988).

mutant ssDBPs. There was, however, an additional breakpoint at $n = 4 \pm 0.3$ in the titration of Pf3 ssDNA, poly[r(A)], and poly[r(C)] (Figures 5, 6, and 7). In the cases of poly[r(A)] and poly[r(C)], the slopes of the CD titration plots were greater for the titrations with the Y22F mutant ssDBP up to P/N $\cong$ 0.25. As the ratio of [protein monomer]/[nucleotide] increased above 0.25, the slopes of the titration plots with the mutant protein decreased, to give total CD changes at saturation that were qualitatively the same as for titrations with the wild-type protein. Titrations of Pf3 ssDNA with the Y22F protein also showed enhanced CD changes up to P/N $\cong$ 0.2−0.25 (Figure 5). Thus, CD measurements were able to detect, in several cases, two apparent stoichiometric modes of binding for the mutant protein. Since an additional mode of binding was evident in titrations with poly[r(A)] and poly[r(C)], but not with poly[d(A)] or poly[d(C)], the ability of the Y22F ssDBP to bind in two distinguishable modes to homopolymers depended on the sugar−phosphate backbone.

We previously showed in gel electrophoresis band shift experiments (Powell & Gray, 1993) that complexes between Pf3 ssDNA and the wild-type protein undergo the most change in electrophoretic mobility at P/N ratios up to about 0.25. As more protein is added, there is much less change in mobility until the saturation endpoint (P/N = 0.5) is reached. Once the DNA is saturated, the mobility of the complex is unchanged with a further addition of protein.

The CD and mobility shift experiments are consistent with two exclusive modes of binding for the Pf3 ssDBP wild-type and Y22F proteins, as previously has been shown to be the case for the fd g5p (Kansy et al., 1986; Bulsink et al., 1988). That is, the binding may be qualitatively described as being consistent with two modes having large intramode cooperativity factors $\omega_1 \cong \omega_2$, but for which there is no intermode cooperativity ($\omega_{12} = \omega_{21} = 1$) [see Schwarz and Stankowski (1979)]. In this case, ligands bound in one mode will essentially be excluded by those bound in the other mode, if the intramode cooperativities are large. Details will depend on the actual values of ω_1 and ω_2, as well as the relative values of the intrinsic binding constants for the two modes, which cannot be determined from the present data obtained under stoichiometric binding conditions. In any case, the nucleic acid polymer will always eventually be saturated with protein in the binding mode of lowest n, if the protein concentration is high enough. Figure 10 illustrates how our CD titration data for poly[r(A)] and poly[r(C)] may be interpreted according to this type of model. For these two polymers, about 70% of the maximum CD change occurs at P/N $\cong$ 0.25, when the nucleic acid is predominantly bound in the $n = 4$ mode. As P/N increases above this value, the nucleic acid CD is proportionately perturbed by protein bound in the $n = 2$ mode. All of our stoichiometric CD titration data may be expressed in analogous plots, if it is recognized that the nucleic acid CD change caused by binding in the two modes depends on the nucleic acid sequence being bound and the wavelength at which the CD is plotted. For example, the $n = 4$ mode may cause no CD change at a given wavelength (such as would be seen in titration plots at 260 nm of the binding of either protein to Pf3 ssDNA), or the protein bound in the two modes may cause the same CD change in the nucleic acid (such as seen in Figure 5, where the titration plot at 250 nm for the binding of the Y22F protein to Pf3 ssDNA becomes constant above P/N $\cong$ 0.25). For other polymers like poly[d(A)] and poly[d(C)] there may not be an evident breakpoint in the titration curve at $n = 4$.

We cannot exclude the possibility of binding in two nonexclusive modes that have significant intermode cooperativity. However, the fact that the electrophoretic mobility of the complex of the wild-type protein with ssDNA is maximally shifted at P/N $\cong$ 0.25 (Powell & Gray, 1993) implies that, if the intermode and intramode cooperativities are similar ($\omega_{12} \cong \omega_{21} \cong \omega_1 \cong \omega_2$), the first mode has to be close to an $n = 6$ mode so that the ssDNA becomes saturated in an effective, or average, "$n = 4$" mode. Even though we cannot exclude the possibility of two such nonexclusive modes of binding, it seems unlikely that the same mixture of two modes, with different site sizes and presumably different intrinsic binding constants K_1 and K_2 for various sequences, would occur to give breakpoints at P/N $\cong$ 0.25 for two very different homopolymer sequences (poly[r(A)] and poly[r(C)]) and for heterogeneous Pf3 ssDNA.

Therefore, we believe that both the wild-type and Y22F mutant Pf3 ssDBPs probably bind to nucleic acids with two stoichiometric binding modes that are exclusive, as does the fd g5p. The exact stoichiometries of the two modes for the fd and Pf3 proteins are, however, different and are summarized in Table 1 of Powell and Gray (1993). In the case of the Pf3 wild-type ssDBP, the $n \cong 4$ mode is optically silent during CD titrations. That is, changes in the CD of nucleic acids, per protein bound, caused by binding of the wild-type protein apparently are the same for proteins bound in the $n = 4$ mode and for proteins bound in the $n = 2$ mode. This is also the case for the binding of ssDNA by the wild-type fd g5p under low-salt conditions, where the $n = 3$ and 4 modes of binding are not distinguished by CD measure-

ments (Kansy et al., 1986). However, for the Y22F mutant protein, the perturbation of nucleotides of ssDNA, poly[r(A)], and poly[r(C)] was different enough in the two binding modes that breakpoints were observed in the CD titration curves. Since the CD changes were enhanced for the first protein additions, it seems that the increased hydrophobicity, and lack of a hydroxyl, for the Phe-22 allowed an increased perturbation of these nucleic acids in the first ($n = 4$) mode of binding.

Salt Dissociation of Complexes. The salt disociation curves for the Y22F mutant were like the dissociation curves previously published for the wild-type protein (Powell & Gray, 1993) in that only one transition was detectable. That is, the binding modes detected during the CD titration experiments with a given nucleic acid could not be distinguished by their salt-sensitivity.

The salt-dissociation data in Table 1 for the two proteins give the salt concentrations at which the $K\omega$ values are equal, at $\cong 8.7 \times 10^4\ M^{-1}$, since the dissociations were all obtained for complexes at the same starting total protein concentration. [With the assumption that ω is much larger than n, $K\omega$ = 1/[free protein] at 50% dissociation, and [free protein] is $^1/_2$ the starting complexed protein concentration (McGhee & von Hippel, 1974).] The salt sensitivities do not necessarily imply corresponding similarities or differences in binding affinities at another salt concentration, unless the log $K\omega$ versus log [NaCl] for each type of complex is the same (Record et al., 1978). However, published data on complexes of fd g5p with nine different RNA and DNA homopolymers and ssDNA show that the slope d(log $K\omega$)/d(log [NaCl]) remains relatively constant and is in the range of -4.0 ± 0.8 (Bulsink et al., 1985). Moreover, nine of eleven mutants of fd g5p have the same [NaCl] dependence as the wild-type protein, given by d(log $K\omega$)/d(log [NaCl]) = -4.0 ± 0.5 (Stassen et al., 1992b). Therefore, the salt sensitivities listed in Table 1 can cautiously be relied on to compare the binding affinities of the wild-type and Y22F mutant Pf3 ssDBPs. Assuming that it would be ideal to have all of the $K\omega$ values at about 0.2 M NaCl (in the physiological range), particular confidence can be placed on the comparisons for the poly[d(A)], poly[d(C)], and poly[r(C)] substrates, since 0.2 M is the NaCl concentration for 50% dissociation of complexes with one or both of the proteins. In any case, the point to be made is that the replacement of Tyr-22 by Phe did affect the salt sensitivities of four of the complexes studied, and three of the complexes with Y22F dissociated at lower [NaCl].

The largest differences in the salt concentrations needed to dissociate the Y22F mutant and wild-type proteins were for complexes with poly[d(A)] and Pf3 ssDNA (Table 1). Interestingly, these two nucleic acids were the only ones that had a qualitative difference in the CD of the nucleic acid upon binding the two proteins. Clearly, the effect of the substitution of phenylalanine for tyrosine depended on the nucleic acid being bound. Either the hydrogen bonding possibilities available with tyrosine are important in binding of the wild-type protein to some nucleic acids and not others, or the enhanced hydrophobic interactions with phenylalanine change the selectivity of binding of the Y22F protein relative to the wild-type protein.

Complexes of either protein with poly[d(T)] could not be dissociated with NaCl concentrations as high as 4 M, and complexes with poly[r(U)] required over 1 M NaCl for 50% dissociation. The change of Tyr-22 to phenylalanine did not seem to appreciably affect the binding of Y22F protein to these nucleic acids. Apparently the hydroxyl of Tyr-22 does not contribute significantly, in comparison with all the other interactions, to the salt sensitivity of the binding of ssDBP to these polymers.

The concentrations of NaCl necessary to dissociate the Y22F • poly[r(C)] and wild-type ssDBP • poly[r(C)] complexes were essentially the same. However, it was evident from a comparison of the titration plots of these two proteins that they interacted with poly[r(C)] in a different manner (Figure 7). Therefore, even though the first ($n = 4$) mode of binding may have been optically enhanced for these polymers during binding with the mutant protein, the overall binding affinities did not appear to change (at the salt concentrations in Table 1) with the wild-type protein.

The concentration of NaCl needed to dissociate Y22F • poly[d(C)] complexes was greater than the concentration needed to dissociate wild-type ssDBP • poly[d(C)] complexes. In this case there were no noticeable differences in the change in the CD of the poly[d(C)] upon binding either wild-type or Y22F ssDBP, although there were significant differences in the change in absorbance upon binding wild-type or Y22F ssDBP (Table 1). While binding of wild-type ssDBP to poly[d(C)] resulted in an approximate 6% decrease in absorbance, binding of Y22F to poly[d(C)] resulted in almost no change in absorbance. This suggests that even though there were no detectable differences in the changes in nucleic acid structure by CD, the base stacking was different during formation of complexes with the two proteins.

SUMMARY

In this study we have shown that the substitution of a phenylalanine for Tyr-22 in Pf3 ssDBP selectively affects its interactions with nucleic acids and, yet, does not appear to drastically alter the protein's secondary or tertiary structure. Thus, it appears likely that Tyr-22 is indeed located in the binding site of the Pf3 ssDBP and is directly involved in nucleic acid interactions. This is consistent with the idea that the binding site of Pf3 ssDBP may be structurally similar to the binding site of the fd g5p, and Tyr-22 may be analogous to Tyr-26 in fd g5p.

The change of Tyr-22 to Phe-22 in the mutant ssDBP affected its binding to nucleic acids in a sequence-dependent fashion. In some cases (poly[d(T)] and poly[r(U)]), there was no apparent change in the interactions. In other cases (poly[r(A)], poly[r(C)], and Pf3 ssDNA), there was an enhanced change in the nucleic acid CD in the first ($n \cong 4$) mode of binding. Binding of Y22F to poly[d(A)] resulted in a qualitatively different type of nucleic acid structure from that in the wild-type ssDBP • poly[d(A)] complex. Yet another type of change was observed in the binding of the Y22F mutant to poly[d(C)], which produced CD changes similar to those caused by the wild-type ssDBP, but which resulted in a nucleic acid that was less hypochromic in the complex with the mutant protein. Together with the altered salt sensitivities exhibited by some complexes formed with the Y22F mutant, these data indicate that Tyr-22 contributes to the ability of Pf3 ssDBP to discriminate among various sequences.

We have been unable to isolate viable phage which carry the Y22F mutation in the ssDBP (see Materials and

Methods). It appears that even this conservative change is able to disrupt the normal function of the protein. Since the complex of Pf3 ssDNA with the Y22F protein is more salt-labile than the complex with the wild-type protein, it is possible that under physiologic conditions the affinity of the mutant protein is not adequate to form and maintain a proper complex or that exchange of the ssDBP for coat protein at the membrane has been adversely affected.

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