Conformational Changes and Stabilization Induced by Ligand Binding in the DNA-binding Domain of the E2 Protein from Human Papillomavirus*

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We are investigating the folding of the 81-residue recombinant dimeric DNA binding domain of the E2 protein from human papillomavirus and how it is coupled to the binding of its DNA ligand. Modifications in buffer composition, such as ionic strength and phosphate, cause an ~5.0 kcal mol\(^{-1}\) stabilization of the domain to urea unfolding, based on very similar conformational changes as measured by far UV circular dichroism. Binding of DNA produces an even greater stabilization, magnitude similar to that caused by the nonspecific polymer ligand heparin, which shifts the urea midpoint 2.5-fold. The DNA-bound complex displays substantial changes similar to those caused by ionic strength and phosphate in terms of overall secondary structure. Bis-8-anilino-1-naphthalenesulfonate provides a very sensitive conformational probe, which shows alterations in the domain caused by the above mentioned compounds. In general terms, binding of DNA involves an overall conformational readjustment in the protein but maintains the \(\beta\)-barrel scaffold intact. This conformational plasticity seems to be of importance in the regulatory functions of this type of DNA-binding protein. The extremely long half-life of the E2-DNA complex, together with its very high stability, suggests that, in the absence of other factors that may affect its stability in vivo, the possibility of dissociation once formed is restricted.

Molecular interaction between proteins and DNA play a central role in the regulation of gene function in the biological world. DNA-binding proteins interact with specific target sequences in the DNA, and the basis for the recognition process at the molecular level has been the focus of intense research (1). Often, the interaction leads to substantial changes in the conformation of both the nucleic acid and the protein, compared with their free forms, with direct implications for their function (2–4). The formation of a protein-DNA complex yields a new and different thermodynamic and structural entity; both processes, DNA binding and conformational changes in the free forms of both macromolecular partners, are highly coupled. This is more dramatic in the case of dimeric transcription factors with intertwined folding topologies such that their dissociated monomers are unfolded, and the process of folding and association and DNA binding are tightly linked (5–7). Thus, DNA-binding domains may undergo local and global folding processes upon binding to their operator sequences.

The E2 transcriptional activator of the human papillomavirus regulates the expression of most viral transcripts and participates in the DNA replication process. The protein consists of a C-terminal DNA-binding and dimerization domain (E2-DBD) and an N-terminal transactivation domain, separated by a flexible region (8). The E2-DBD is an ~80-residue per monomer domain that can be overexpressed recombinantly in bacteria as a stable and soluble dimer (9, 10). Its biological importance is shown by the three forms of the protein produced by alternative splicing: the full-length protein (E2-TA), which acts as a transcriptional activator, and two shorter forms (E2-TR and E2/E8) produced from alternative splicing, acting as repressors (11). All forms have in common the entire C-terminal DNA binding domain.

The structure of a homologous E2-DBD from the bovine papillomavirus (BPV-1) bound to its DNA target was determined by x-ray crystallography and established a new folding topology, the dimeric \(\beta\)-barrel (12). This particular fold is shared by the EBNA1 protein from the Epstein-Barr virus, the crystallographic structures of free and DNA-bound forms were recently described (13, 14). The NMR solution structure of the highly homologous HPV E2-DBD (strain 31) domain free in solution showed a complete conservation of the overall fold of the BPV protein (15).

The HPV-16 E2-DBD was shown to undergo a concomitant dissociation and unfolding following an apparent two-state model, with folded dimers or unfolded monomers, and no intermediates accumulated (10). A highly populated transient intermediate was found on its kinetic folding pathway, possibly of non-native characteristics (16). To approach the problem of coupling between folding and DNA binding processes, in the present work we tackle the analysis of the effect of an oligonucleotide comprising its DNA target and other ligand, such as heparin, on the stability and conformation of the domain. We evaluate the effect of ionic strength and phosphate anions and discuss the relationship between conformational changes and stabilization in the effectors studied. For the analysis of stability and conformation we use fluorescence and circular dichroism spectroscopy, and we probe conformational changes making use of the hydrophobic dye bis-ANS.

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The abbreviations used are: E2-DBD, E2 DNA binding domain; bis-ANS, bis-8-anilino-1-naphthalenesulfonate; HPV, human papillomavirus; BPV, bovine papillomavirus; Bis-Tris, 2-(bis[2-hydroxyethyl]-amino)-2-(hydroxymethyl)-propane-1,3-diol.

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EXPERIMENTAL PROCEDURES

Materials

Chemicals—All reagents and buffers used were of maximal purity available. Water was twice distilled and deionized prior to use. Urea and heparin were purchased from Sigma and bis-ANS from Molecular Probes (Eugene, OR).

Protein and Ligands—The recombinant E2-DBD from HPV-16 was overexpressed and purified as described previously (10). The protein concentration was measured using the extinction coefficient of 41,900 M⁻¹ cm⁻¹, determined from model compounds (17), in very good agreement with the colorimetric method that we used in previous work (10).

We used a synthetic highly palindromic oligonucleotide of 36 base pairs with the following consensus sequence: 5'-TTTGTAGCTCAACCGAGTTGGGTTTCATGCT TT7-3', and the complementary strand (recognition sequence is underlined). Extra bases were included at each side of the consensus sequence to stabilize it further. For the annealing, we mixed equimolar concentrations of the complementary strands in 10 mM Tris-HCl, pH 7.0, and 1 mM dithiothreitol unless otherwise stated. The temperature and was described previously in detail (10). The protein/denaturant solutions were pre-equilibrated at that temperature for 2 h before measurements. In all experiments was 25 °C, and protein/denaturant solutions were made up to 260 nm, out of the range of the analysis of our spectra. Excitation wavelength was at 280 nm, and the emission spectrum was recorded from 300 to 500 nm.

Urea equilibrium denaturation was carried out in 50 mM Bis-Tris, pH 7.0, and 1 mM dithiothreitol unless otherwise stated. The temperature in all experiments was 25 °C, and protein/denaturant solutions were pre-equilibrated at that temperature for 2 h before measurements. In these experiments, fluorescence spectra were recorded on an ISS K2 spectrofluorometer. The excitation wavelength was set at 360 nm, and the base-line subtraction minimizes the contribution of the Raman peak, which nevertheless occurs below 320 nm, out of the range of the analysis of our spectra. Excitation wavelength was at 280 nm, and the emission spectrum was recorded from 300 to 450 nm.

High quality spectra, we accumulated 10 spectra using the software provided by the manufacturer. The spectra were used for the derivative analysis, unsmoothed data were derived successively (stepwise) up to the fourth derivative. In all cases, the buffer plus added compound base line was subtracted, and the temperature was tightly controlled at 25 °C. The base-line subtraction minimizes the contribution of the center of spectral mass \( \langle v_c \rangle \) (Equation 1).

\[
\langle v_c \rangle = \frac{\Sigma F v_c / F}{\Sigma F}
\]

where \( F \) indicates the fluorescence emitted at wave number \( v_c \), and the summation is carried out over the range of appreciable values of \( F \). We observed that following fluorescence intensity at a fixed point is extremely sensitive to small changes in temperature, pH, or ionic strength, most likely due to an exposed tryptophan (Trp-56) in E2-DBD, located facing the solvent in the minor a-helix (Fig. 1).

We want to compare different conditions, in the present work we follow the center of spectral mass that will report only changes on the environment of the two tryptophan residues located at the center of the barrel, which constitutes the dimeric interface. In this manner, we eliminate changes in fluorescence quantum yield at small changes in denaturant concentration, ion strength, or temperature. The quantum yield of E2-DBD is increased by 30% at urea concentrations lower than 0.2 M (not shown); however, the center of mass is not affected. This takes place in a range in which there is no major unfolding, and it is most likely due to the exposed Trp residue. The unfolding parameters obtained using the center of mass are in excellent agreement with those previously obtained (11). The spectra of the unfolding analysis of the unfolding curves was carried out using a two-state model that considers protein concentration and was described previously in detail (10). The \( \Delta G \) values obtained correspond to standard free energy changes.

Circular Dichroism Spectroscopy—CD spectra were obtained with a Jasco J-710 spectropolarimeter, using a 0.1-cm path length quartz cuvette. The spectra were the average of 5 scans at a 50 nm/min speed, and the buffer base lines were subtracted. Only the far UV region from 200 to 260 nm was analyzed, due to increased noise at shorter wavelengths.

Binding of Bis-ANS—Bis-ANS binding was performed at 25 °C in 50 mM Bis-Tris, pH 7.0, and 1 mM dithiothreitol unless otherwise stated. Fluorescence measurements were recorded on a Hitachi F-4500 spectrofluorometer. The excitation wavelength was set at 360 nm, and the emission was measured from 400 to 600 nm. The titrations were performed by adding small amounts of a concentrated solution of bis-ANS to a fixed amount of E2-DBD and allowed to equilibrate for 5 min prior to the measurements. There were no time-dependent changes in fluorescence spectra between 5 and 60 min. The same procedure was applied when titrating with increasing concentrations of protein. In all cases, maximal dilution was 10%, and the data were corrected accordingly. Maximum emission wavelengths were calculated from the first derivative of the spectra.

RESULTS

Structural Considerations of the Dimeric \( \beta \)-Barrel Fold—The structure of the first described E2-DBD from HPV-1 consists of a dimeric 8-stranded \( \beta \)-barrel with 4 strands of each monomer forming the interface, and two a-helices making cross-over contacts on the outside of the barrel (12). The majority of the two helices in E2-DBD is the portion of the protein that binds the palindromic DNA target ACCG-N4-CGGT (Fig. 1). The solution structure of the highly homologous E2-DBD of HPV-31 shows an identical topology to the bovine virus protein (15). The outer strands of the monomers are connected by a hydrogen bonding network, and many hydrophobic side chains protrude to the interior of the barrel. In HPV-16 E2-DBD, as in HPV-31, two tryptophan residues separated by a histidine (residues 34–36) are stacked and interacting, and face inward in the central cavity of the barrel (15), and are most likely responsible for the fluorescence changes that take place upon denaturation/disassociation. Another tryptophan residue is located in the minor helix, facing the solvent. The amino acid sequence of E2-DBD from HPV-16 is 80% homologous to the HPV-31 domain.

Effect of Ionic Strength, Phosphate, Heparin, and DNA Target Oligonucleotide on the Conformation of E2-DBD, Analyzed by Fluorescence Spectroscopy—Tryptophan fluorescence was used as a first approach to investigate conformational changes and stability of the E2 domain. The fluorescence spectrum of E2-DBD at pH 7.0 without any addition is shown in Fig. 2 (inset). We are interested in the study of the effect of compounds such as ionic strength, phosphate, heparin, and the specific DNA oligonucleotide on the conformation and stability of E2-DBD.

Among other parameters in the cellular environment, ionic strength may affect the proteins that bind DNA, since these often present a large number of positively charged residues exposed to the solvent. Modifications in ionic strength also constitute a tool to investigate the contribution of the types of interactions that stabilize specific local or global conformations.

Fig. 1. Schematic representation of the E2 DNA binding domain from BPV-1 bound to DNA (12). The diagram was obtained using the program Molscript (31).
FIG. 2. Fourth derivative analysis of the fluorescence spectrum of E2-DBD under different conditions. a, no additions. The inset shows the untransformed spectra. Concentration of protein was 0.5 μM, in 50 mm Bis-Tris, pH 7.5; the spectral data were recorded as described under "Experimental Procedures." b, in buffer (solid line), plus 100 mm sodium phosphate (dotted line), and in the presence of 0.75 M NaCl (dashed line). c, plain buffer (solid line), 2 μg/ml heparin (dashed line), and 0.25 μg/ml of the DNA 36-mer (dotted line). The concentrations of the added compounds correspond to the amount that is required for the refolding of urea-unfolded E2-DBD, as explained in Fig. 4. These conditions are used throughout the work when the effect of those compounds is tested.

TABLE I

| Spectral area | λ_{max} | Difference in center of mass | [Urea]_{50%} | ΔG_{mol} | m |
|---------------|---------|------------------------------|--------------|----------|---|
| %            | nm      | cm⁻¹            | μ             | kcal mol⁻¹ | x⁻¹ kcal mol⁻¹ |
| Buffer       | 100     | 347             | 0             | 2.2       | 12.5 ± 0.7 | 2.0 ± 0.3 |
| NaCl         | 86      | 347             | 14            | 4.9       | 17.5 ± 0.8 | 1.9 ± 0.2 |
| Phosphate    | 93      | 347             | 19            | 4.3       | 16.9 ± 0.6 | 2.0 ± 0.1 |
| Heparin      | 104     | 345             | 47            | 7.2       | 5       |
| DNA oligonucleotide | 82 | 345 | 77 | 7.0 | 6 |

Stabilization of E2-DBD toward Urea Denaturation by Salts and Ligands—The E2-DBD from human papillomavirus strain 16 showed concerted denaturation and dissociation processes induced by urea (10). We intend to compare the stability of E2-DBD to urea unfolding with addition of salts, phosphate, or its DNA ligand in similar conditions of pH. Fig. 3 shows a urea unfolding curve at pH 7.0 in which changes in the center of spectral mass are followed (see "Experimental Procedures"). The change in the center of mass upon complete unfolding is 10 nm red shift. The inset of Fig. 3 shows the fluorescence spectra of folded and urea-unfolded E2 domain, to illustrate the total change.

For the analysis of the effect of ionic strength on the stability of a particular protein fold. We analyzed the effect of NaCl on the fluorescence spectra of folded E2-DBD. There is a small decrease in the area of the spectrum, but the center of spectral mass and the wavelength maximum remain virtually unchanged (Table I). This suggests that if there were a significant conformational change it does not seem to involve the exposure of the Trp residues of the interface to the solvent and, therefore, no disruption of the dimeric interface or major unfolding. Upon addition of phosphate anion, the fluorescence spectrum shows a minimal change in spectral area, with no apparent changes on its center of spectral mass or wavelength maximum (Table I).

The interaction of the E2-DBD with its DNA target and the changes induced are crucial for understanding the basis of the molecular mechanism for the action of this transcription factor. For this purpose, a synthetic oligonucleotide containing a consensus binding site was incubated with the domain, and a 20% change in the fluorescence spectral area and a small blue shift were observed (Table I). Any significant conformational change it does not seem to involve the exposure of the Trp residues at the interior of the domain.

The glycosaminoglycan heparin is a heteropolymer composed of amino sugar disaccharide units. These type of polymers are highly negatively charged due to the presence of either carboxyl or sulfate groups on the sugar units, which repel each other and cause the polymer to adopt extended conformations. Immobilized heparin is frequently used as the method of choice for purification of nucleic acid binding proteins. In the case of E2-DBD, the protein binds to the heparin-modified matrix and is released only with concentrations higher than 1 M salt (10). This prompted us to study the binding of soluble heparin and to analyze possible effects on the conformation and stability of the folded dimer. Adding heparin to folded E2-DBD led to a 2-nm blue shift in the fluorescence spectra, with no change in the fluorescence intensity.

The possibility to obtain high quality fluorescence spectra allows us to approach a derivative analysis to discriminate different contributions in the spectrum, extending the sensitivity of the technique. A discrete number of bands can be resolved by this procedure, which originates in the transitions of electrons from the excited singlet state returning to different vibrational levels in the ground state (18, 19). Using N-acetyl tryptophanamide as a model compound, three sharp bands were observed in the fourth derivative, at 326, 346, and 364 nm, respectively (20). In that study it was shown that the shift in emission maximum with change in the polarity of the environment is the result of changes in the relative contribution of these bands. The fourth derivative of the fluorescence spectrum of folded E2-DBD is shown in Fig. 2c; the bands are broader than model compounds, and this is due to the different tryptophan residues present whose contributions overlap. Two distinctive wavelength maxima are observed at 332 and 342 nm and a third one at 365 nm. Addition of NaCl and phosphate produced a similar effect, an increase in the band at 332 nm and a decrease at 342 nm (Fig. 2b). Addition of heparin shows a large increase at 342 nm and a decrease at 332 nm (Fig. 2c).

The band at 365 nm does not change in most conditions tested. The E2-DBD-DNA complex shows very little change in the intensity of the peaks, but a small red shift in the 342 nm peak is observed. The band at 365 nm changes slightly, and a small band around 375 nm is also evident in the complex. However, it is not possible to assign these marginal changes to alterations in the conformation at this stage.

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of the domain, we chose the urea concentration that causes near 100% unfolding of E2-DBD (Fig. 3). At 3.0 M denaturant, increasing the salt concentration generates a change in the center of spectral mass, indicative of a recovery of the folded conformation (−29,000 cm⁻¹). Fig. 4a shows that the maximal recovery takes place between 0.5 and 1.0 M NaCl, similar concentrations have a negligible effect on the center of spectral mass of the folded conformation (Table I).

Since the binding site of E2-DBD and other DNA-binding proteins contains a large proportion of positively charged residues, and being DNA a polyphosphate polymer, we wanted to test the effect of phosphate on the conformation and stabilization of the native fold. In Fig. 4b, following the procedure of addition of increasing amounts of the anion to unfolded E2-DBD in 3.0 M urea, we observed a recovery of the spectral properties of the folded conformation, indicating a stabilization with a maximal effect at concentrations higher than 50 mM phosphate.

To estimate the stabilization effect of salts and phosphate we carried out urea denaturation experiments, and the stabilization observed is quite pronounced (Fig. 5). Using a two-state approach as described previously, we obtained a change in the center of spectral mass of 5.0 and 4.5 kcal mol⁻¹ for NaCl and phosphate (10). This corresponds to a stabilization (ΔG_stab) of 5.0 and 4.5 kcal mol⁻¹ for the same compounds. The [U]₅₀%, the urea midpoint, was 4.9 M for NaCl and 4.3 M for phosphate, and the m values, the parameter that measures the cooperativity of the transition, agree very well with the E2-DBD in buffer only (Table I), suggesting that the mechanism of unfolding at equilibrium is not altered.

Effects of DNA on the stability were evaluated by adding increasing concentrations of the oligonucleotide to urea unfolded E2-DBD. Similar to what occurs in the presence of NaCl and phosphate, the DNA is able to fully recover the center of mass of the fluorescence spectrum of the domain, indicative of a folded conformation (Fig. 4c). When unfolded E2-DBD (in 3.0 M urea) was titrated with increasing concentrations of heparin, the center of spectral mass showed a recovery to values corresponding to the tryptophan probes in the folded conformation as in the case of DNA, phosphate, and NaCl (Fig. 4d).

The effect of DNA and heparin on the stability of the E2 domain can be best quantified in urea unfolding experiments, in comparison with the free E2-DBD. Fig. 5 shows the large stabilization caused by both polymer ligands to a similar extent. The [U]₅₀% was determined to be 7.0 M for DNA and 7.2 M for heparin. A detailed mathematical analysis for calculating free energies is not possible since there are several equilibria involved. If we were to assume the unfolding E2-DBD as the only event, we could approximate the analysis to a two-state model and use a linear relationship between [U]₅₀% and ΔG_unf. The stabilization (ΔΔG_unf) caused by DNA and heparin would, therefore, be −9.0 kcal mol⁻¹. This is only useful for a gross

**Fig. 3.** Equilibrium unfolding of E2-DBD by urea. E2-DBD concentration was 0.5 μM, and the procedure is described under “Experimental Procedures.” The inset shows the spectra of folded (solid line) and unfolded (6.0 M urea, dashed line) E2-DBD. The parameters obtained from a two-state analysis are shown in Table I.

**Fig. 4.** Stabilization of E2-DBD by NaCl, phosphate, heparin, and DNA. Unfolded E2-DBD in 3.0 M urea and 50 mM Bis-Tris, pH 7.0, was treated with increasing concentrations of the above mentioned compounds, and spectra were taken 10 min after addition. The center of spectral mass was plotted as an indication of the recovery of the spectral properties of folded E2-DBD. a, NaCl; b, sodium phosphate; c, heparin; and d, DNA.
estimation of the otherwise obvious stabilizing effect.

Conformational Changes Induced by Ionic Strength, Phosphate, and Ligands Determined from Far UV Circular Dichroism—The far UV CD spectrum of E2-DBD showed two minima at 210 and 224 nm and a maximum at 195 nm, all corresponding to α-helix contribution (10). As previously pointed out, the ellipticity minima differ slightly from those observed in most α-helices (208 and 222 nm); a high β-sheet content may influence the spectra and also the fact that the major helix may not be fully formed in the free domain in solution (15). Since we want to investigate effects of NaCl, phosphate, DNA, and heparin on the conformation of E2-DBD, we added concentrations of the above mentioned compounds bearing the maximal effect on the stability toward urea unfolding (see Fig. 4). Sodium chloride concentrations of 0.75 M produced a decrease in negative molar ellipticity ($\Theta_{MWR}$) to approximately half of its value at 210 nm; a much smaller change was observed at 224 nm (Fig. 6a and Table I). An increase of 8,000 deg cm$^2$ dmol$^{-1}$ in $\Theta_{MWR}$ is observed at 200 nm; although it cannot be determined precisely, this could be due either to an increase in the maximum at 195 nm or a shift of the maximum to the red. It is, nonetheless, a considerable spectral change.

Concentrations of 100 mM phosphate cause a decrease of negative $\Theta_{MWR}$ at 210 nm similar to high salt, which uncovers further the minimum at 224 nm, corresponding to the α-helix. The change resembles very much that observed in the presence of NaCl except from the more pronounced change in $\Theta_{MWR}$ at 224 nm in the case of phosphate (Fig. 6a and Table I). Upon binding of the DNA target, a large spectral change also in the region of the minimum at 210 nm becomes evident. The spectrum of E2-DBD-DNA resembles that of the domain in high salt and is virtually identical to E2-DBD in phosphate. All three share the large increase in $\Theta_{MWR}$ at 200 nm.

The assignment of the conformational changes upon DNA binding to a particular type of structure and the extent of that change are not straightforward to interpret from the mere inspection of the far UV CD spectra, except in the case of pure helical proteins. E2-DBD has high β-sheet type structures, including antiparallel β-strands and β-hairpins, as well as many aromatic residues. All this structural heterogeneity precludes an accurate estimation of the precise structural change (21). For example, a β-hairpin or a turn could translate into a positive band between 210 and 220 nm, and this cannot be discerned from a decrease in negative ellipticity that could correspond to helix local unfolding. What is clear is that the domain has a large flexibility in response to salts, phosphate, and ligands, leading possibly to generalized conformational changes. The difference between free and DNA-bound E2-DBD can also be visualized from comparing the sum of the spectra of free E2-DBD and DNA, with the spectrum of the complex (Fig. 6b).

As described in the earlier crystallographic studies of the bovine virus E2-DBD, the DNA binds in a particular bent conformation (12). A conformational change in the DNA oligonucleotide is observed from the difference spectrum of the bound DNA in comparison to the free DNA, shown in the inset of Fig. 6b.

Soluble heparin was also analyzed for its ability to induce conformational changes since it is known to bind to the protein, and it was found to cause a large stabilization toward urea denaturation, described in previous sections. The presence of heparin concentrations similar to those causing maximal stabilization toward urea unfolding (Fig. 4) produces only a small change in the far UV CD spectrum, compared with DNA, phos-
When the bis-ANS (25) was added in excess of E2-DBD, a fluorescence enhancement was observed, as measured by the increase in the spectral area, reaching a plateau at approximately 4-fold excess of dye (Fig. 7a). This fluorescence change depended on the fixed concentration of protein as follows: at 1 μM there is a titration process, indicating that the dissociation constant is below this concentration; at 0.5 and 0.25 μM the dissociation becomes evident (Fig. 7a, inset). If, in the same experiment, we monitor the wavelength maximum of the spectra of bound bis-ANS, a 16-nm red shift is observed (Fig. 7a). However, the change in wavelength maximum, indicative of polarity of the binding site, does not change in parallel with the change in the spectral area, which argues for more than one binding site of different polarity. At 1:1 stoichiometry, there is a break in the plot, suggesting the titration of one site (arrow in the plot).

The increase in the spectral area upon titration with excess protein is shown in Fig. 7b. The relative fluorescence change upon addition of E2-DBD to bis-ANS is superimposable at concentrations of 0.25 and 1 μM dye in terms of molar ratio E2-DBD/bis-ANS (not shown), which suggests similar binding mechanisms at different protein concentrations. The wavelength maximum was also followed in the E2-DBD titration, with a 14-nm total change. There is clearly a displacement between the change in spectral area and maximum wavelength, strongly suggesting at least two sites of different polarity. A high affinity site would involve approximately 40% change in fluorescence intensity and ≤8% change in wavelength maximum, and the low affinity site or sites involve a 60% change in intensity and ≥90% in wavelength. As in the titration with excess bis-ANS, the break in the plot of Fig. 7b is coincident with 1:1 stoichiometry, supporting the hypothesis of the titration of the first, higher affinity, site.

Although not all sites may have the same fluorescence change, the larger total fluorescence change in the titrations with excess protein also suggests more than one binding site,
possibly two (Fig. 7b). For the same reasons, a Scatchard transformation should not be the best alternative for the determination of the exact number of sites since bis-ANS may not show the necessary linear proportionality between fluorescence change and binding at each of the putative different sites. We can use it to estimate the presence of at least two sites that are non-equivalent (Figs. 7b, inset) (26). These sites are different enough so that their dissociation constants can be determined from the slopes to be 0.1 and 0.6 μM, respectively. These values are in agreement with the dissociation curves described in the inset of Fig. 7a. Scatchard plots for bis-ANS binding were used in DNA binding proteins, e.g. in the C-terminal domain of the I repressor (27). Even if the plot extrapolates to a value close to 2 (Fig. 7b, inset), we still cannot be completely sure about the exact number of sites involved.

Probing Conformational Changes Induced by DNA, Heparin, Phosphate, and Ionic Strength—Because bis-ANS is so sensitive to minor changes in the environment of putative-specific and -non-specific sites, our main goal is the use of this probe to sense conformational changes caused by DNA, heparin, phosphate, and sodium chloride, all known to have large effects on stability and secondary structure. Titrations with increasing bis-ANS were carried out at concentrations of the above mentioned compounds that caused maximum stabilization to urea unfolding and conformational changes detected by fluorescence and CD spectroscopy, described in previous sections (Fig. 8). Since the binding of DNA and heparin or the presence of phosphate or large concentrations of salt may affect the bis-ANS binding mechanism, we analyzed the data with a simple binding equation and calculate apparent dissociation constants ($K_{d,a}$) and maximum fluorescence changes for each compound. It would be ideal to study the full mechanism in each case, but in the present study, we focus in using bis-ANS as a probe for conformational changes. Since the maximum wavelength is indicative of the polarity of the binding site, we obtained this parameter from the first derivative of each fluorescence spectra at the saturation point with bis-ANS. The data obtained are listed in Table II.

The large enhancement of the fluorescence quantum yield when the bis-ANS is bound to the protein is accompanied by a wavelength maximum shift of −23 nm with respect to free bis-ANS in buffer (not shown). The maximum fluorescence of the probe bound to E2-DBD is reduced to 50 and 60% in the presence of either phosphate or sodium chloride, respectively, and the wavelength maxima are virtually identical. Binding of bis-ANS by the E2-DBD-DNA complex shows less than 40% fluorescence intensity of the domain in plain buffer. It also shows a significant 3-nm change in the wavelength maximum and a 60% increase in the $K_{d,a}$. The wavelength maximum resulting from the first titrating site with excess bis-ANS (Fig. 7b) is 493 nm, in very good agreement with that from E2-DBD-DNA complex (Table II). Thus, the 60% fluorescence decrease together with the largest change in the apparent binding constant strongly suggests that the DNA is probably blocking one of the sites.

Although it showed similar extent of stabilization to urea unfolding with respect to the DNA-bound form, the E2-DBD-heparin complex shows the smallest change of fluorescence of bound bis-ANS, no change in the wavelength maximum, and no significant change in the apparent binding constant. However, as the far UV CD experiments showed, the heparin bound E2-DBD (Fig. 5a) displays a similar spectrum to that of the free dimeric domain, strongly suggesting that the overall secondary structure remains unchanged. The use of a very sensitive conformational probe such as bis-ANS indicates only a minor change in the protein-heparin complex, and this agrees very well with the conservation of the secondary structure from far UV CD.

DISCUSSION

The formation of protein-DNA complexes is accompanied by conformational changes in the nucleic acid and also by local changes in the protein, and sometimes even association/dissociation of protein subunits, varying according to the many different types of protein-DNA complexes described (4–7). We have used different approaches to evaluate the presence of conformational changes and the stabilization caused by a specific oligonucleotide ligand and heparin on the E2-DBD from HPV-16.

Both ionic strength and phosphate have a strong stabilizing effect on the E2 dimerization domain. There are small changes in the fluorescence properties of the folded form in the presence of these compounds, but no major changes in the exposure of the main tryptophan probes as indicated by fluorescence spectroscopy. A detailed more sensitive analysis using derivative spectra showed small changes upon incubation with the different compounds under study. These results suggest that the changes in folded E2-DBD, especially those induced by heparin, take place on a local basis, possibly mainly in the exposed tryptophan which would not report a large conformational change. The interface would remain unaltered, as indicated by the marginal change in the center of mass and maximum wavelength, strongly suggesting no variation in the exposure of the two internal tryptophan probes to the solvent.

Examination of the secondary structure of E2-DBD by far UV CD shows an evident change in conformation in the presence of either NaCl or phosphate, which appears to be very similar. The molecular basis for this effect could be the stabilization of the many positively charged side chains in the folded conformation; the phosphate requires lower concentration since it may bind more specifically, somehow mimicking the phosphate groups in the DNA. Stabilization by ionic strength may respond to other principles, but the conformation attained and the extent of the effect of salt and phosphate are highly coincident, as judged by circular dichroism and urea unfolding experiments. Nevertheless, solvent-accessible ionic interactions do not seem to be important in the stabilization of this domain.

Although we have no information on the molecular basis for the E2-heparin interaction, we could hypothesize that it is through the negatively charged sulfate groups and the sugar units, the latter also present in nucleic acids. Heparin causes a large stabilizing effect to urea unfolding, of a similar extent to the DNA. However, the conformational change caused by this glycosaminoglycan is minimal, especially so considering the large stabilizing effect it produces. This effect can be explained by the ability of the polymer to lock the conformation of the side chains at the binding site; it is not accompanied, however, by a significant change in secondary structure as in the case of DNA. Heparin also appears not to prevent binding of bis-ANS probably because the complex E2-DBD-heparin does not form a tight structure, allowing the dye to penetrate in the site. Heparin may be viewed as a model for nonspecific binding of a non-nucleic acid polymer, and the lack of conformational change on heparin binding points directly to the functional aspects of the specific DNA-induced conformational change in E2-DBD.

No structural information concerning the location of the binding sites is available. In principle, it is possible that it binds to the DNA binding site and at the central cavity of the barrel, or some other accessible non-polar site. The presence of more than one site is supported by the biphasic changes in the wavelength maximum, indicative of sites of different polarity.
(28). To complement our conformational analysis, we used the extreme sensitivity of bis-ANS to investigate conformational changes caused by ionic strength, phosphate, DNA, and heparin on the E2 domain. Based on studies on ANS, the $\lambda_{\text{max}}$ of these probes goes from 454 to 494 nm, when bound to protein sites (28, and references therein), being the sites less polar as they move to the blue. The $\lambda_{\text{max}}$ of bis-ANS when bound to E2-DBD (497 nm) suggests at least one site of a more polar nature. The presence of two sites of close enough affinity precludes a more detailed analysis of the microscopic binding constants, but in the presence of high salts, phosphate, or heparin, both the $\lambda_{\text{max}}$ and the apparent binding constant increases by 60%. It could be argued that one of the sites is occupied by the DNA or the latter prevents the binding. It is not possible to infer this from the fluorescence intensity since it is not linearly proportional to the number of molecules, and conformational changes may make the analysis difficult.

The wavelength shift could be indicative of a single site in the presence of the nucleic acid or the conformational change affecting the environment of the bound probe. If we assume that the DNA does not allow access of bis-ANS to the site, there is a site elsewhere in the protein for binding of the probe, in line with the biphasic wavelength change discussed above. The shift to the blue in the presence of DNA suggests that the remaining site is less polar. It is not unexpected that the bis-ANS binding site at the DNA site is of a more polar nature. Nevertheless, it is clear that the DNA causes more profound, specific, and persistent changes in the dimer than the other compounds.

Binding of the DNA gives rise to a very large stabilization to urea unfolding, in which the complex starts to fall apart only after 6.0 m urea and unfolds completely at 9.0 m denaturant. This stabilization is accompanied by a conformational change of DNA-bound E2-DBD, and the secondary structure appears to be strikingly similar to those in high salt and phosphate. The major features are a loss in negative ellipticity at $\sim 210-212$ nm and an increase in positive ellipticity at around 200 nm. However, there is little, if any, change at 222–224 nm. There are many factors that affect the contributions to the far UV CD spectra. It would be over simplistic to interpret loss in negative ellipticity as loss of helical content, especially if it only changes at $\sim 210$ nm and not at $\sim 222$ nm and shows a visible increase in the positive ellipticity at lower wavelengths, often indicative of $\alpha$-helix. More specifically, we know that the homologous E2-DBD from BPV1 has the major helix fully formed in the E2-DBN crystal structure (12), and the free E2-DBD from HPV-31 in solution presents evidence of a fluctuating structure in the helix (15). Also, changes in $\beta$-sheet contents or turns are not as homogeneous as changes in $\alpha$-helix and can contribute at different wavelengths. We conclude that there are rearrangements in several structural motifs, involving changes both in $\alpha$-helices and $\beta$-sheets, as well as other contributions from turns and aromatics. The binding of the DNA in solution causes an overall rearrangement of structure in the E2-DBD, i.e. it is not restricted to local changes. However, the tryptophan side chains at the center of the $\beta$-barrel, i.e. the dimer interface, show minimal modifications. This indicates that the core or scaffold of the domain is conserved, and a considerable conformational change takes place, giving a characteristic plasticity to the protein.

Many features appear from the comparison of the backbone of the NMR solution structure of E2-DBD from HPV-31 with the crystal structure of DNA-bound BPV-15 as follows: (i) a systematic deviation of one of the monomers when the other is superimposed, caused by a change in the alignment of the C-terminal $\beta$-strands, which lies at the opposite end of the DNA binding helices in the dimer molecule; (ii) a $\beta$-hairpin between strands 2 and 3, over 15 residues away from the major recognition helix, which is disordered in solution and ordered in the BPV-1 crystal structure due to its contact with the minor groove of the DNA; (iii) an apparently flexible or fluctuating helix in the free HPV-31 domain, as judged from abnormally fast amide proton exchange rates and $^{15}$N dynamics. These observations indicate that a large structural rearrangement must take place on formation of E2-DBD-DNA complex, in line with our observations in this work.

The structure of the highly homologous domain from HPV-31 was determined in sodium phosphate at pH 6.5, and many differences were observed with respect to the crystal structure of the BPV-1, as explained before. Taking account the conformational changes induced by phosphate that we describe here, it could be argued that in the absence of phosphate the flexibility of the folded domain is even larger, making the overall conformational change upon DNA binding more dramatic.

The protein EBNA-1 from Epstein-Barr virus has a similar topology to the E2-DBD from human papillomaviruses (13). Both proteins bind DNA and regulate the expression of various viral genes, yet there is no amino acid sequence homology between them. Since these are the only two proteins sharing this unique dimeric $\beta$-barrel fold, a close relationship between topology and function is likely to exist (14).

The E2-DBD is a highly flexible domain where stability can be largely affected by ionic strength, phosphate ions, and pH (this paper and Ref. 10) and ligands such as its DNA target and heparin. This stabilization is accompanied by a conformational change as measured by far UV CD and binding of bis-ANS, except for the case of heparin. The change produced by DNA seems to be the most profound and persistent as expected according to its natural function. This conformational flexibility and its correlation with stabilization effects need to be defined in detailed molecular terms. Based on the crystal structures of free and DNA-bound EBNA1, a two-step binding was hypothesized (14). There is little, if any, conformational change in both free and bound EBNA1 in the crystal; moreover, a large conformational change is not required for the hypothetical mechanism (29). However, a major difference lies in the fact that in EBNA1 the helix that faces the DNA is not used for binding since it remains 6 Å away from it. Is the conformational change a prerequisite for binding to the DNA operator? Or does the binding to DNA induce a conformational change that alters the physicochemical properties of the rest of the dimer for other purposes? The E2 protein displayed the ability to bind cooperatively to adjacent sites in the trans-regulation of gene expression of bovine papillomavirus (30), which calls for the existence of a conformational change upon binding and the ability of intermolecular interaction with other protein molecules or with DNA.

In the case of $\beta$-barrel dimeric domains, there is a strong evidence linking the topology and, therefore, the folding properties to their key regulatory function in viral gene transcription. The extremely slow half-time for dissociation (> 4 h, see Ref. 9) and the very high stability of the E2-DNA complex suggests a quasi-irreversible reaction, with strong implications...
for its mechanism of action. The conditions under which the dissociation rate was measured include a number of additions to the buffer, including 10% glycerol; these may affect the stability and therefore the off-rate. Nevertheless, it will still be an intrinsically slow process.

As more structural evidence becomes available on this and related domains in addition to the use biochemical and genetic approaches, we will be able to fully understand the many different mechanisms of coupling of protein folding and DNA binding and the connection with their specific biological functions.

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