Positive Contribution of Hydration on DNA Binding by E2c Protein from Papillomavirus

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Protein-nucleic acid interactions are responsible for the regulation of key biological events such as genomic transcription and recombination and viral replication. However, the recognition mechanisms involved in these processes are not completely understood. Here, we investigate the dominant forces involved in protein-protein and protein-DNA interactions for the 80-amino-acid C-terminal domain of the E2 protein (E2c) from human papillomavirus (HPV-16). The E2c protein is a homodimer that specifically binds to double-stranded DNA containing the consensus sequence ACCG-N$_6$CGGT, where N is any nucleotide. DNA binding affinity is reduced by lowering water chemical potential, accompanied by an increase in cooperativity. Wyman linkage relations between affinity and water chemical potential indicate that 11 additional water molecules are bound in the formation of the complex between E2c and DNA. Salt dissociation isotherms showed that 10 countercations are released upon association, even at low water activity, indicating that this latter variable does not change the electrostatic component of the interaction. Further analysis demonstrates a strong dependence of cooperativity of binding on the protein concentration. Altogether, these results reveal a novel binding pathway in which the consolidated complex may achieve its final form via a monomer-DNA intermediate, which favors the binding of a second monomer. This molecular mechanism reveals the contributions of multiple conformers in a tight virus genome modulation that seems to be important in the cell infection scenario.

Human papillomavirus (HPV)

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Experimental Procedures

Reagents—All reagents were of analytical grade. Distilled water was deionized to less than 1.0 μS cm$^{-1}$ and filtered through a 0.22-μm membrane in a water purification system prior to use. All solutions were prepared just before use. All solutions were checked for exact glycerol concentration by refractive index (34). Changes in buffer osmolality due to the addition of glycerol were calculated from tabulated data (34).

E2c Expression and Purification—The C-terminal 80-amino-acid DNA-binding domain of HPV-16 E2 protein (E2c) was cloned into pTz18U, overexpressed in Escherichia coli BL21(DE3)pLysS, and purified as described (13). The concentration of the protein stock solution was determined using the extinction coefficient of 41.900 M$^{-1}$ cm$^{-1}$ at 280 nm (14).

**Synthetic Oligonucleotides**—The single-stranded synthetic oligonucleotides, high pressure liquid chromatography-purified, were pur-
chased from Integrated DNA Technologies (Coralville, IA). The oligonucleotide concentrations were calculated from their extinction coefficients at 260 nm. The double-stranded 18-bp oligonucleotides containing one E2 recognition sequence (site 35 in the HPV-16 genome; E2-DNA-binding site, E2DBS) were prepared as follows. The single-stranded oligonucleotide E2DBS-seA, 5′-GTA ACCG AAAT CGGT TGA-3′ (recognition sequence underlined), was paired with E2DBS-seB, the complementary strand. Oligonucleotides were annealed in a PCR apparatus (PTC-100™ programmable thermal controller, MJ Research, Inc.) at equimolar concentrations in 10 mM Tris-Cl, 50 mM NaCl, 1 mM EDTA, pH 7.8, by heating at 90 °C for 10 min and cooled slowly to 25 °C at 5 °C h⁻¹. Annealed oligonucleotides were stored in the annealing buffer at −20 °C and checked for complete hybridization by subjecting the products to native polyacrylamide electrophoresis. The labeled double-stranded oligonucleotide (F-E2DBS) contained a fluorescein molecule attached to the 5′ end via a six-carbon linker in the E2DBS-seA strand and was annealed as described above.

Fluorescence Spectroscopy—All fluorescence measurements were performed in an ISS-PC1 spectrofluorimeter (ISS, Champaign, IL), assembled in “L” geometry. Excitation was set to 475 nm, and emission was recorded through an orange short wave cut-off filter WG3–69 (cut-off 50% at 520 nm). Anisotropy values were calculated by the ISS program according to the equation,

\[ A = \frac{(I_{p\uparrow} - I_{p\downarrow})(I_{p\uparrow} + 2I_{p\downarrow})}{(I_{p\uparrow} + I_{p\downarrow})} \]  (Eq. 1)

where \( I_{p\uparrow} \) and \( I_{p\downarrow} \) represent the emission intensity with the polarizer oriented parallel or perpendicular, respectively, to the incident polarized light. For each sample, anisotropy was measured until standard errors were less than 0.005. Data were corrected for fractional contributions of fluorescence over anisotropy.

Titrimetric Assay of E2c-DNA Interaction—Isothermal titrimetric assay of DNA binding by E2c was performed by adding small amounts of a concentrated NaCl solution to a fixed amount of DNA. Following this addition, the solution was homogenized and allowed to equilibrate for 5 min prior to anisotropy measurements, as described above. In all cases, maximal dilution was less than 10%. Changes in fluorescence intensity and DNA and E2c concentrations were corrected for dilution. Binding reactions were carried out at 25 ± 1 °C using 5 nM F-E2DBS, 50 mM bis-Tris-Cl, 200 mM NaCl, 1 mM dithiothreitol, pH 7.0, and different amounts of glycerol. Glycerol belongs to a chemical class of low molecular weight polyols that have long been employed to adjust water chemical potential, similar to other neutral solutes (23, 35–40). It was the polyol of choice because: (i) it does not noticeably change ionic strength or the macroscopic dielectric constant of buffer solutions (41); (ii) it can induce large changes in solution osmolality and thus in water activity, without drastic changes in viscosity (34) that could interfere with fluorescence anisotropy measurements; and (iii) it is preferentially excluded from the complementary strand. Oligonucleotides were annealed in a PCR apparatus (PTC-100™ programmable thermal controller, MJ Research, Inc.) at equimolar concentrations in 10 mM Tris-Cl, 50 mM NaCl, 1 mM EDTA, pH 7.8, by heating at 90 °C for 10 min and cooled slowly to 25 °C at 5 °C h⁻¹. Annealed oligonucleotides were stored in the annealing buffer at −20 °C and checked for complete hybridization by subjecting the products to native polyacrylamide electrophoresis. The labeled double-stranded oligonucleotide (F-E2DBS) contained a fluorescein molecule attached to the 5′ end via a six-carbon linker in the E2DBS-seA strand and was annealed as described above.

Cooperativity Linkage on E2c-DNA Interaction

Thus, we define the dissociation constant \( K_d \) of the above reaction according to

\[ K_d = \frac{(D^2/E)c}{(E)cD} \]  (Eq. 2)

where \( D \) is the molar concentration of the free double-stranded DNA-binding site, \( E \) is the molar concentration of the free E2c dimer, and \( (E)cD \) is the molar concentration of the complex formed between dimeric E2c and DNA.

The complex is related to the anisotropy measurements by,

\[ E_cD = D\alpha + (A_f - A_i)(K_d + Et + Dt) \]

where \( \alpha \) is the fraction of DNA (or E2c) bound and \( A_{obs} = (A_f - A_i)/(A_f - A_i) \)

\[ (K_d + Et + Dt)^2 - 4Et^2Dt^2/(2Dt) \] (Eq. 5)

where \( Dt \), the total molar concentration of DNA employed in the assay, corresponds to the sum of free (D) and bound (E, D) DNA concentrations, and \( Et \) is the molar concentration of total dimeric E2c protein.

In cases where the binding isotherms presented cooperative behavior (Hill \( n+1 \)) and thus could not be fit by Equation 5, we employed the Hill formalism (44). For this analysis, the formalism is,

\[ A_{obs} = A_i + (A_f - A_i)(L^2/K_d^2)(1 + (L^2/K_d^2)) \]  (Eq. 6)

where \( L \) is the free E2c concentration and \( K_d \) is the apparent dissociation constant for interacting sites. Combining Equations 4 and 6 gives

\[ A_{obs} = A_i + (A_f - A_i)(L^2/K_d^2)(1 + (L^2/K_d^2)) \]  (Eq. 7)

Adjusting Equation 6 to the binding data, one can thus obtain the cooperative Hill coefficient \( n \) and \( K_d \). For noncooperative binding isotherms, Equations 5 and 7 give the same result.

Salt Dissociation Isotherms—Isothermal salt dissociation was induced by adding small amounts of a concentrated NaCl solution to a fixed concentration of E2c-DNA complex (equimolar). Following this addition, the solution was homogenized and allowed to equilibrate for 5 min prior to anisotropy measurements, as described above. In all cases, maximal dilution was less than 10%. Changes in both fluorescence intensity and DNA and E2c concentrations were corrected for dilution. Experiments were conducted at 25 ± 1 °C. All solutions (sample and salt stock) contained 50 mM bis-Tris-Cl, 1 mM dithiothreitol, pH 7.0, and the indicated amount of NaCl. For both isothermal binding and salt dissociation, a delay much longer than 10 min between additions and measurements led to no significant difference.

Analysis of E2c-DNA Interaction as a Function of Ligand Activity—Modulation of E2c-DNA association/dissociation by a third solution component (ligand X) can be interpreted according to Wyman (45),

\[ \ln K_d/\ln a_i = \Delta N_x \]  (Eq. 8)

The slope of \( \ln K_d vs. \ln a_i \), the natural log of ligand X activity, gives \( \Delta N_x \), the difference between the number of X associated with free versus bound E2c and DNA. In this study, X refers specifically to water (W) or salt (NaCl), according to the analysis. According to Lima et al. (16), for the equilibrium condition,

\[ a = (K_d/4)^2*\Delta N_x*(1 + 8*2^D/K_d^0.5 - 1) \] (Eq. 9)

Combining Equations 4, 8, and 9, \( A_{obs} = A_i + (A_f - A_i)*(K_d*exp(\Delta Nx*ln a_i))*(1 + (16*D)/(K_d*exp(\Delta Nx*ln a_i)))^0.5 - 1/\sqrt{8*D}) \) (Eq. 10)

Equation 10 allows direct evaluation of a single ligand-induced thermodynamic of complexes dissociation by nonlinear least squares fitting of raw data, without the need for performing several binding isotherms with different concentrations of salt (46). This approach can be extended to any ligand in which Wyman linkage relation (45) is applied, as in the case of OSA and the binding of water molecules (or other cosolutes) to macromolecules.

High Hydrostatic Pressure Isomerization—E2c undergoes concerted pressure denaturation-dissociation (15, 16). Thus, the equilibrium between E2c dimers (E) and E2c monomers (E) can be described according to

\[ E_2 := 2E \] (Eq. 11)

The equilibrium constant for unfolding at atmospheric pressure (Kd) is

\[ K_d = \langle p_{obs} \rangle - \langle p \rangle - \langle \psi \rangle = (K_d*exp(p*ΔV/R*T^2))*1 + (8*Pt/\sqrt{K_d*exp(\Delta V/R*T^2)})*1/\sqrt{2^D} \] (Eq. 11)

where \( \langle p_{obs} \rangle \) is the observed (measured) center of spectral mass and \( \langle \psi \rangle \) and \( \langle \psi \rangle \) are the asymptotic limits of the fitting, respectively, the initial and final values of the center of spectral mass, \( ΔV \) is the standard volume change upon association (in milliliters/mole), and Pt is the total protein concentration expressed in terms of the monomer.

Fitting—Equations were adjusted to data by nonlinear least-squares.
Cooperativity Linkage on E2c-DNA Interaction

FIG. 1. Binding isotherms for E2c binding to DNA containing the palindromic recognition sequence ACCgN4cGG. Isothermal titration of DNA (5 nM) by E2c in the presence of 10% v/v (●), 20% v/v (■), 30% v/v (▲), and 40% v/v (▼) glycerol. Titrations were conducted in 50 mM bis-Tris-Cl, pH 7.0, 200 mM NaCl, 1 mM DTT at 25 °C. Solid lines are nonlinear regression fit to the raw data by using Equations 5 and 7. Raw anisotropy data were converted into a fraction bound with Equation 4 as described under “Experimental Procedures.”

regression using SigmaPlot 2002 (version 8.0, Jandel Scientific Co.). All data were obtained using at least two different batches of protein.

RESULTS

To investigate changes in hydration involved in the association of E2c with a specific DNA-binding site, we performed binding isotherms in solutions that differ in osmolality, and thus in water activity (aW). E2c was used to titrate an 18-bp DNA fragment bearing a specific E2c recognition sequence with a fluorescein moiety attached to one 5′ DNA strand (F-E2DBS). Data representative of several binding isotherms, demonstrating the effect of water activity on the complex formation between E2c and DNA, are shown in Fig. 1. The increasing solution osmolality shifts the curves to a higher protein concentration, indicating a decrease in binding affinity and, thus, that both E2c and DNA had undergone a net hydration upon complexation. It can also be seen clearly from Fig. 1 that the decrease in affinity reveals the evident positive cooperativity of the binding event. The fact that the protein binds cooperatively to the DNA demonstrates that there is protein-protein interaction linked energetically to the formation of the complex.

We designed isothermal binding assays under stoichiometric conditions with a high DNA concentration (250 nM), both in the presence (Fig. 2, open circles) and in the absence (Fig. 2, closed circles) of 30% v/v glycerol. This condition ensures that the amount of DNA complexed with E2c approaches 100%. This titration would give access to any changes in binding stoichiometry due to the high solution osmolality, although the DNA fragment used in these measurements contains only one DNA-binding sequence, allowing us to evaluate the existence of putative nonspecific E2c-DNA binding. The similarity between the two binding profiles with different solutions conditions clearly indicates that high solution osmolality (in this case, 6.5 osmolality) does not change the stoichiometry of the complex (Fig. 2, inset), meaning that only one dimer can bind a single DNA consensus sequence, ruling out the possibility of association of E2c to species with assembly states greater than dimers.

Before attributing the changes in affinity in Fig. 1 to changes in hydration, we considered the possibility that increasing solution osmolality would be changing specific protein-DNA contacts (46, 47). To address this question, we performed salt dissociation isotherms of E2c-DNA in solutions of different osmolality (Fig. 3). Keeping the concentrations of both E2c and DNA at a constant value (200 nM), we measured the changes in anisotropy as a function of salt concentration. In Fig. 3, these data are normalized to show the fraction of complex remaining due to dissociation by salt. It can be observed that increasing glycerol concentration makes a lower salt concentration necessary to dissociate the complex (Fig. 3). These data corroborate the measured decrease in E2c-DNA affinity due to the presence of glycerol (Fig. 1). The parallel shift in the dissociation curves indicates that the decrease in water activity only diminishes the affinity, with no significant changes in electrostatic contacts involved in recognition. Using Equation 10 to fit the data in Fig. 3, we obtained the number of ionic contacts in the E2c-DNA complex and thus the number of salt molecules released to solution upon complexation: 10.2 ± 1.1 with no added glycerol, 10.6 ± 1.5 in 5% glycerol, and 11.1 ± 1.0 in 10% glycerol. These values are essentially the same, within experimental error.

In the process of assembling a macromolecule, there is a decrease in solvent-accessible surface area corresponding to the contact area between the ligands. This has been demonstrated for many biological systems, including complex formation between protein and DNA (48, 49), ferredoxin binding to...
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ferredoxin-NADP⁺ reductase (50), and epitope recognition by a monoclonal antibody (51). A molecular reaction accompanied by a decrease in solvent-accessible surface is favored by decreasing the water activity. Thus, analysis of an affinity constant as a function of water activity on a log-log scale gives the total number of water molecules bound in the process (22–24).

Changes in solution water activity (\(a_w\)) were calculated according to (25),

\[
\ln a_w = -\frac{\text{Osm}}{55.5} \quad (\text{Eq. 12})
\]

where Osm is the change in solution osmolality due to the presence of glycerol.

Analysis of data in Fig. 4A according to Equation 7 indicates that there is an apparent binding of 11 water molecules to the consolidated complex upon E2-DBS recognition by E2c. At first inspection, it might appear a surprising result since if one supposes the simple interaction of two rigid bodies, exclusion of water molecules upon complex formation would be expected. However, our data clearly indicate the opposite; there is a small increase in the hydration of the complex when compared with free ligands (E2c and DNA). Similar demonstrations of positive hydration upon protein and DNA association have been published elsewhere (36, 37).

Considering the results presented so far, we considered the possibility that changes in water activity would be additionally changing monomer-dimer equilibrium. The next experiments were designed to address this issue. To further explore the effects of water activity variation on E2c structure and stability, we subjected E2c to high hydrostatic pressure at different glycerol concentrations. As reported previously (15), E2c can be dissociated by high hydrostatic pressure into molten globule monomers (Fig. 5, circles). Increasing the glycerol concentration leads to a shift in the midpoint transition of the pressure isotherm (Fig. 5, triangles and squares), suggesting at first visual inspection an increase in protein stability. However, as observed in Table I, our data indicated that the dissociation constant did not change, within error, over the range of osmolality tested, whereas volume changes associated with the pressure denaturation of E2c decreased greatly with increasing solution osmolality (Table I). This can be attributed to the reduced hydration of the monomers that are formed as pressure is applied and the dimers dissociate.

As shown in Fig. 1, the decrease in binding affinity is accompanied by an increase in cooperativity, as revealed by the Hill coefficient (Fig. 4B). In most reported work with E2c binding to DNA, equilibrium between free and DNA-bound dimers is assumed. However, in a detailed kinetic study (21), Ferreiro and de Prat-Gay showed that the DNA binding by E2c presents two phases, and the amplitude involved in the slower association phase disappears as the protein concentration is increased. These authors suggested a pre-equilibrium of DNA-binding conformers in the dimeric state of E2c, in which different conformers correspond to two different binding modes. To evaluate the protein concentration dependence for DNA binding in equilibrium, we plotted the effects of water activity variation on E2c structure and stability. The next experiments were designed to address this issue. To further explore the effects of water activity variation on E2c structure and stability, we subjected E2c to high hydrostatic pressure at different glycerol concentrations. As reported previously (15), E2c can be dissociated by high hydrostatic pressure into molten globule monomers (Fig. 5, circles). Increasing the glycerol concentration leads to a shift in the midpoint transition of the pressure isotherm (Fig. 5, triangles and squares), suggesting at first visual inspection an increase in protein stability. However, as observed in Table I, our data indicated that the dissociation constant did not change, within error, over the range of osmolality tested, whereas volume changes associated with the pressure denaturation of E2c decreased greatly with increasing solution osmolality (Table I). This can be attributed to the reduced hydration of the monomers that are formed as pressure is applied and the dimers dissociate.

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![Figure 4: Linkage plot of the natural logarithm of the dissociation constant \(K_d\) (A) and Hill cooperativity parameter \(n\) for the binding of E2c to DNA as a function of the natural logarithm of the water activity (B). The dissociation constant \(K_d\) refers to the fitting of Equation 5 or 7 to the isothermal titration curves represented in Fig. 1, expressed as a function of water activity (Equation 12) as described under “Experimental Procedures.”

![Figure 5: Effects of increasing solution osmolality on pressure dissociation/denaturation isotherms of E2c. E2c (500 nM) was subjected to high hydrostatic pressure in 50 mM bis-Tris-Cl, 1 mM DTT, pH 5.6, and in the absence (●) or in the presence of 10% v/v (■) and 30% v/v (▲) glycerol at 25°C. Continuous lines represent nonlinear regression fits according to Equation 11, which considers the equilibrium between dimers and monomers. The final spectral center of mass obtained from fitting control curves (●) was used as the end point for the other curves. Details can be found under “Experimental Procedures.”

![Table I: Osmotic effects on E2c dissociation by high hydrostatic pressure dissociation constant and volume changes of the pressure isotherms were calculated according to the legend for Fig. 5.](http://www.jbc.org/)

| Glycerol (v/v) | \(K_d\) | \(\Delta V\) | \(\Delta ASA\) |
|---------------|--------|-------------|----------------|
| %             | \(\text{nM}\) | \(\text{ml \times mol}^{-1}\) | \(\Delta\text{Å}^2\) |
| 10%           | 15.4 ± 2.5 | 81.8 ± 2.2 | 3,000 ± 81 |
| 30%           | 11.5 ± 6.5 | 30.3 ± 5.5 | 1,108 ± 201 |

\(\Delta ASA\): Increase in accessible surface area (ASA) calculated according to Foguel et al. (54).
curves at different protein concentrations (Fig. 6A). Extending this interpretation, data in Fig. 6A were converted into E2c-DNA complex dissociation at different protein concentrations as a function of water activity. These data show a progressive change in slope as the protein concentration increases, indicative of different hydration changes for each protein concentration tested. By adjusting Equation 10 to the experimental data in Fig. 6B, we obtained the hydration change $\Delta N_W$ that accompanies E2c dissociation from DNA. In Fig. 6C, a plot of the resulting $\Delta N_W$ versus E2c concentration shows that the lower the protein concentration, the higher the hydration change upon complex formation. Thus, in the range of E2c concentrations investigated, the cooperative mechanism of binding appears to be highly modulated, meaning that a conformational equilibrium that might be attributed to the equilibrium between monomers and dimers holds. The decrease in hydration change seems to reach a plateau of $\Delta N_W = 11$ at about 50 nM E2c, the same concentration range in which the slow kinetic binding phase is no longer detected (21). We emphasize that the kinetic experiments (21) and the present equilibrium assays (Figs. 1 and 6) were conducted in exactly the same pH, solution conditions, and DNA concentration. Taking together our previous work (16), the kinetic binding study (21), and our results from Figs. 4, 5, and 6 (this study), we suggest the existence of an active monomeric species of E2c, able to recognize and bind the DNA and induce the binding of second monomer, cooperatively linked to E2c dimerization and folding.

**DISCUSSION**

Investigations conducted on the E2c protein from papillomavirus have demonstrated its surprising structural and functional plasticity in DNA binding (52). A more detailed characterization of the recognition mechanism should aid, for example, in the design of lead compounds able to block DNA binding and thus papillomavirus infectivity. With this aim, we have studied the equilibrium pathways of E2c binding to a cognate DNA.

Different authors have described the binding of different DNA sequences with E2c from HPV-16 and BPV-1 and with anti-double-stranded DNA monoclonal antibodies. To obtain consolidated complexes between the E2c protein and its consensus DNA sequence, specific ionic contacts are required. Our analysis clearly demonstrates that 10 specific ionic contacts are required to the formation of the consolidated complex from E2c and the DNA sequence used here, even in the presence of a high glycerol concentration, suggesting a significant contribution of electrostatic interactions to the specific sequence recognition (Fig. 3).

Ferreiro and de Prat-Gay (21) have interpreted the sensitivity of the E2c-DNA interaction to the presence of 500 mM sodium chloride as an osmotic effect. This interpretation was based on the concept that a high concentration of any solute leads to decreased thermodynamic activity of water (53). However, 500 mM NaCl decreases the water activity to about 0.983 (34), equivalent to $\ln a_W$ of $-0.017$ (Equation 12). According to our measurements, this small change in osmolality would have almost no effect on binding affinity (Figs. 4 and 6). Thus, it is more likely that disruption of the E2c-DNA complex is due to protein concentration dependence (increasing from bottom to top in A and B) of complex dissociation due to changes in glycerol concentration (A) and water activity (B) using Equation 5 (as described in the legend for Fig. 1). Data in B were fitted using Equation 10, which considers the equilibrium between free and associated E2c and DNA, and using fixed values of 0 and 1 for complete (100%) dissociation and association, respectively. In C, the hydration changes for the process obtained from panel B are plotted as a function of E2c concentration, and in this panel, the solid line has no analytical value.

**FIG. 6.** Dependence of hydration changes upon complex dissociation on E2c concentration. Isothermal DNA binding by E2c was conducted in the presence of different concentrations of glycerol, as described in the legend for Fig. 1. Raw anisotropy data were converted to protein concentration dependence (increasing from bottom to top in A and B) of complex dissociation due to changes in glycerol concentration (A) and water activity (B) using Equation 5 (as described in the legend for Fig. 1). Data in B were fitted using Equation 10, which considers the equilibrium between free and associated E2c and DNA, and using fixed values of 0 and 1 for complete (100%) dissociation and association, respectively. In C, the hydration changes for the process obtained from panel B are plotted as a function of E2c concentration, and in this panel, the solid line has no analytical value.
electrostatic shielding by salt, with minor contribution from osmotic effect.

We used glycerol to adjust the water activity of the solution, according to the principle of the OSA (22–24). This osmolyte is preferentially excluded from the protein surface and thus is considered to be a “noninteracting” solute (42, 43). In this way, one might expect $\Delta N_W$ to contribute more importantly to $d\ln K_{obs}/d\ln a_W$ than does $\Delta N_{glycerol}$. A similar conclusion was reached for other systems in which $d\ln K_{obs}/d\ln a_W$ was obtained with glycerol and other osmoties that differ structurally and chemically (37, 38, 48, 54, 55).

Using the osmotic stress approach, we estimate that an additional 11 water molecules become associated with the E2c and DNA structures as they bind to each other. We call attention to the fact that, for some DNA complexes with E2c from other papillomaviruses, water was observed to be mediating contacts in the complex interface. Although no structure is yet available for the DNA complex with E2 proteins from HPV-16, it seems possible that some of the remaining water molecules might mediate certain contacts in the interface between E2c and DNA, as observed for complexes obtained with DNA and E2c from BPV-1 (8) and HPV-18 (56). In addition, the positive hydration changes upon E2c-DNA binding might arise from the reported DNA unwinding, base unstacking, and protein conformational changes (14, 19, 20).

The difference in the number of water molecules bound to the free and associated E2c and DNA ($\Delta N_W$) is a measurement of cooperativity in this equilibrium transition (45). Here, we demonstrated that, depending on protein concentration, complex formation can engender different changes in hydration (Fig. 6). As depicted in the reaction scheme in Fig. 7, we can extend this interpretation as described in the following paragraph.

1) At low E2c concentrations, the left side of the reaction would prevail. Thus, hydration changes (and thus, cooperativity of binding) are large when compared with the observed $\Delta N_W$ at high E2c concentrations, indicating that, in the light of the classic interpretation of cooperativity, the binding of a second monomer and consolidation of the complex are facilitated by the preliminary binding of the first monomer. Monomer-DNA complex (Fig. 7, ED) facilitates the interaction with the second monomer following the formation of the consolidated E2c-DNA complex (Fig. 7, E,D). Such equilibria are possible since in this range of protein concentration, the solution can be described as an ensemble of conformers (monomers and dimers). 2) At high E2c concentration, the protein would bind predominantly as a preexisting dimer (right side of the reaction), as was clearly the case in our stoichiometric measurements in Fig. 2 (high protein and high DNA concentrations).

Dimer is the major form of the E2c DNA-binding domain. However, this is valid for the micromolar range and above. Since a concentration dependence is observed for equilibrium unfolding of E2c (13, 15), we can describe the unfolding as a transition between dimer and monomer. Therefore, a simple dilution of the E2c from micromolar to nanomolar concentrations would produce free monomers in solution. The dissociation constant for the dimer-monomer equilibrium of E2 at our conditions was $15 \text{nM}$ (Table I), a value above the protein-DNA dissociation constant measured in Fig. 1 (5–$5\text{nM}$). This is not surprising since the association-dissociation of oligomers due only to dilution has been demonstrated for several other macromolecules (57–60).

A partially folded monomeric structure is formed in the kinetic refolding pathway from unfolded monomers to folded dimers (61). Clusters of residual structure in regions corresponding to the DNA-binding helix and the second $\beta$-strand in the folded conformation have been found by NMR measurements for the urea-denatured E2c monomer (62). At this solution condition (high urea concentration), DNA can rescue the unfolded monomers to a folded specie (14). Also, even at high hydrostatic pressure, persistent DNA-bound monomers can be found (16). These published data strongly indicate that a monomeric species: (i) can be kinetically populated and (ii) can be populated at equilibrium, at high and low protein concentration, respectively, in the presence or absence of chemical or physical perturbation. The data also indicate that the monomeric species, although not “native-like,” does present persistent structure, mainly in the recognition helix. Taking together the present investigation and previously published results, evidences are given to support the hypothesis of a DNA-bound monomer specie, both kinetically and in equilibrium. In a broader context, it is not surprising to observe subunit association and protein folding coupled to ligand binding since coupling has already been described for E2c folding induced by DNA and other unrelated polyelectrolytes (14), for cooperative binding of E2 dimers to adjacent binding sites (63), and(10) for many other systems, from oxygen binding by hemoglobin (57) to protein-DNA interaction (64–67).

We do not claim that the E2c-DNA molecular recognition is mediated only by a pathway containing a DNA-bound monomer of the E2c protein. However, it is plausible that the monomer-DNA complex exists at low protein concentrations and that it contributes to the cooperative binding of a second monomer, leading to a protein-protein interaction (folding and dimerization) energetically coupled to DNA binding. Taking into account that the behavior of macromolecules and their mutual interactions are commanded by local chemical potentials (68–70), our evidences indicate that regulation of papillomavirus transcription involves a more complex mechanism that goes beyond the interactions of E2-full with cell factors. Moreover, this seems to be one more case in which cooperativity dictates biological regulation.

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