Characterization of a Partially Folded Monomer of the DNA-binding Domain of Human Papillomavirus E2 Protein Obtained at High Pressure*

(Received for publication, September 19, 1997, and in revised form, December 31, 1997)

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The pressure-induced dissociation of the dimeric DNA binding domain of the E2 protein of human papillomavirus (E2-DBD) is a reversible process with a Kd of 5.6 × 10^-6 M at pH 5.5. The complete exposure of the intersubunit tryptophans to water, together with the concentration dependence of the pressure effect, is indicative of dissociation. Dissociation is accompanied by a decrease in volume of 76 ml/mol, which corresponds to an estimated increase in solvent-exposed area of 2775 Å^2. There is a decrease in fluorescence polarization of tryptophan overlapping the red shift of fluorescence emission, supporting the idea that dissociation of E2-DBD occurs in parallel with major changes in the tertiary structure. The dimer binds bis(8-anilinonaphthalene-1-sulfonate), and pressure reduces the binding by about 30%, in contrast with the almost complete loss of dye binding in the urea-unfolded state. These results strongly suggest the persistence of substantial residual structure in the high pressure state. Further unfolding of the high pressure state was produced by low concentrations of urea, as evidenced by the complete loss of bis(8-anilinonaphthalene-1-sulfonate) binding with less than 1 M urea. Following pressure dissociation, a partially folded state is also apparent from the distribution of excited state lifetimes of tryptophan. The combined data show that the tryptophans of the protein in the pressure-dissociated state are exposed long enough to undergo solvent relaxation, but the persistence of structure is evident from the observed internal quenching, which is absent in the completely unfolded state. The average rotational relaxation time (derived from polarization and lifetime data) of the pressure-induced monomer is shorter than the urea-denatured state, suggesting that the species obtained under pressure are more compact than that unfolded by urea.

The interaction of proteins with DNA constitutes the basis for the regulation of key biological functions such as gene expression, replication, and recombination. DNA-binding proteins recognize specific stretches of DNA, and the molecular basis for the interactions is currently the focus of intensive research (1, 2). The domains of proteins that interact with DNA are highly variable in folding topology, and thus they can accommodate a large number of functions for the different complexes in both eukaryotic and prokaryotic systems (1, 3). One characteristic of many DNA-binding proteins is that the structures of the monomers are often highly intertwined (4-8). Studies on several complexes have revealed that both DNA and protein undergo conformational changes upon interaction, especially at the interface, and there is an important free energy coupling among folding, dimerization, and DNA recognition (9-12). In cases where these DNA-binding proteins have been studied, their unfolding by denaturing agents occurs simultaneously with dissociation in a highly concerted manner (13-17).

Human papillomavirus (HPV)1 infection of the anogenital tract is associated with several premalignant and malignant lesions, especially dysplasia and carcinoma of the uterine cervix (18). The E2 transcriptional transactivator protein (E2-TA) participates in the regulation of the expression of viral genes in papillomavirus (19, 20). The products of the E2 gene are crucial to the life cycle of the virus because they regulate transcription from all viral promoters, which makes E2-TA a potential target for antiviral therapy. The E2 protein is comprised of an N-terminal transactivation domain separated from the C-terminal DNA binding and dimerization domain by a flexible region rich in proline residues (7). The solution structure of the C-terminal DNA binding domain (E2-DBD) from human papillomavirus strain-31 (HPV-31) was recently determined by NMR spectroscopy (21). The urea-induced denaturation of the recombinant E2-DBD from HPV-16 was shown to proceed through a concerted two-state unfolding and dissociation process, with no detectable intermediate species (17). However, investigation of its kinetic folding pathway reveals the presence of a short lived monomeric intermediate (22).

Noncovalent interactions can be reversibly perturbed using high hydrostatic pressure, which allows a thermodynamic characterization of protein folding, protein-protein, and protein-ligand interactions (23, 24). Hydrostatic pressure drives the structure of proteins to a thermodynamic state of smaller volume (23, 25, 26). Protein folding and protein-protein interactions are normally accompanied by an increase in volume because of the combined effects of the formation of solvent-excluding cavities and the release of bound solvent (24). Water is released as nonpolar amino acid residues are buried, as well as when salt linkages are formed. Arc repressor, a small DNA-

* This work was supported in part by Howard Hughes Medical Institute International Grant 75197-553402 (to J. L. S.) and by grants from Programa de Apoio ao Desenvolvimento Cientifico e Tecnologico, Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), and Financiadora de Estudos e Projetos of Brazil (to J. L. S. and D. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Dedicated to the memory of Gregorio Weber.

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1 The abbreviations used are: HPV, human papillomavirus; E2-TA, E2 transcriptional transactivator; E2-DBD, E2 DNA binding domain; bis-ANS, bis(8-anilinonaphthalene-1-sulfonate).
binding dimer protein from the bacteriophage P22, has been studied in detail by high pressure, in an attempt to understand interrelationships among protein folding, dimerization, and DNA recognition (9, 11, 15, 16).

In this paper, we study the reversible dissociation of HPV-16 E2-DBD using high pressure in combination with fluorescence spectroscopic techniques. We present evidence for a persistent residual structure in the monomeric denatured state at high pressure and are able to characterize it by Trp fluorescence spectra, polarization, lifetime distribution, stability to urea unfolding, and binding of bis(8-anilino-naphthalene-1-sulfonate) (bis-ANS). The existence of a folded monomeric state of E2-DBD in the absence of denaturants may represent an important target to drug development in addition to being highly relevant to the understanding of the basic principles underlying protein folding mechanisms.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**All reagents were of analytical grade. bis-ANS was purchased from Molecular Probes (Eugene, OR). Distilled water was filtered and deionized through a Millipore water purification system.

The C-terminal 80-amino acid (positions 286–365) DNA binding domain of HPV-16 E2 protein was overexpressed in Escherichia coli and purified as a soluble, folded dimeric protein (17). As shown previously, the isolated C-domain still retains the ability to dimerize and bind to the DNA (27). Protein concentration was determined using an extinction coefficient of 41,900 M⁻¹ cm⁻¹ at 280 nm (28).

**Spectroscopic Measurements under Pressure—**The high pressure cell equipped with optical windows has been described (15) and was purchased from ISS (Champaign, IL). Fluorescence spectra were recorded on an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). Fluorescence spectra were recorded at the standard buffer: 50 mM bis-Tris-HCl containing 1 mM dithiothreitol (pH 7.0). Samples were excited at 295 nm with a 300-W xenon lamp, and emission was collected using 7–52 and 0–52 filters. For pressure experiments, light scattered from ficol particles was used as reference (15). The choice of fitting with Lorentzian components was based on χ² values and plots of weighted residuals.

**Fluorescence Anisotropy Measurements—**Values of fluorescence anisotropy were measured according to the equation,

\[ A = I_1 - I_0 + 2I_2 \]

where \( I_1 \) and \( I_2 \) are the intensities of the emission when the polarizers are oriented parallel or perpendicular to the direction of the polarizer of the excitation, respectively. The errors for the polarization measurements were less than ±0.005.

Average rotational relaxation times (\( <\tau_p> \)) were calculated from the anisotropy values (A) and from the average lifetime experimentally determined by using the Perrin equation,

\[ \frac{Ao}{A} = (1 + 3f(p)) \]

where \( Ao \) is the limiting anisotropy of the fluorophore (0.240), and \( \tau \) is the fluorescence decay lifetime.

**Urea Unfolding of the High Pressure State—**To a solution containing the E2-DBD dimer at 1 μM, 50 mM bis-Tris-HCl, 1 mM dithiothreitol, pH 5.5, and 5 μM bis-ANS at 22 °C, different concentrations of urea were added. The mixture was introduced in the high pressure cell, and after a 30-min period of incubation, fluorescence spectra of bis-ANS were recorded. Next, the pressure was taken to 2860 bars, required for attaining the maximum change in the center of spectral mass when monitoring Trp fluorescence in pressure titrations in the absence of bis-ANS. Since the decrease of the fluorescence of E2-bound bis-ANS at high pressure is a slow reaction, we followed the changes in the total fluorescence intensity with time until the completion of the reaction.

**RESULTS**

**Structural Considerations of the E2-DBD—**The crystal structure of the E2-DBD from bovine papillomavirus strain-1 revealed a new class of folding topology (7, 32), only shared by the EBNA1 DNA binding domain from the Epstein-Barr virus with no amino acid sequence homology (33). The fold consists of a dimeric eight-stranded β-barrel, with each monomer contributing half of the barrel. Two helices/monomer interact with the outside of the barrel, forming hydrophobic cores: a major α-helix, which forms the DNA binding site, and a minor α-helix at the opposite side (Fig. 1A). The solution structure of the E2-DBD from HPV-31 (80% homology with E2-DBD from HPV-16; Fig. 1B) has been solved recently by NMR methods and has identical folding topology, although some significant differences between solution and crystal structures were observed (21). The dimeric interface is stabilized by intersubunit β-sheet hydrogen bonding and by the packing of hydrophobic residues at the center of the barrel. The formation of the dimer buries 2,567 Å² of solvent-accessible area in the bovine papillomavirus strain-1 domain, which strongly suggests that any partly folded monomer would have to undergo substantial accommodations in structure (7).

The HPV-16 E2-DBD has three tryptophan residues/monomer, two of which face the central cavity of the barrel that forms the interface between the two monomers in a stacked conformation as shown in the HPV-31 E2-DBD structure (Ref. 21; Fig. 1). The position of tryptophan residues in E2-DBD is unique, in that the indole rings of Trp37 (monomer 1) and Trp39 (monomer 1) stack in a characteristic antiparallel fashion; the same occurs with Trp37 (monomer 2) and Trp39 (monomer 2). All four base-stacking interactions occur within the dimer interface, giving rise to a favorable aromatic-aromatic interaction. This makes it possible to follow dissociation and unfolding processes using fluorescence, with substantial sensitivity. The third tryptophan residue, Trp38, which is present in HPV-16 E2-DBD but absent in HPV-31 E2-DBD faces the solvent and is located in the smaller α-helix (Fig. 1).

**Pressure Dissociation of the E2-DBD: Concentration Dependence, Reversibility, and Thermodynamic Parameters—**To mon-
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Fig. 1. A, ribbon representation of the solution structure of E2-DBD of HPV-31. The monomeric subunits are colored differently. The trypophan (residues 37 and 39) and histidines (residues 8, 38, 46) are shown in green and yellow, respectively. The coordinates are from Liang et al. (21). The schematic representation was produced by using the program Rasmol. B, sequence alignment of the E2-DBD proteins from human papillomaviruses 31 and 16 (Ref. 21). The schematic representation was produced by using the program Rasmol.

The equilibrium dissociation constant and the accompanying volume change for the dimer ⇔ monomer equilibrium were calculated using the equation for dissociation by pressure (Equation 4). Fig. 3A shows the degree of dissociation at each pressure, based on the center-of-mass data at pH 5.5 from Fig. 2B (Equation 2). The values derived from fluorescence anisotropy (squares) coincide with those obtained from the changes in fluorescence spectra (circles). The intercept of the semilogarithmic plot of these data (Equation 4) yields the dissociation constant \( K_d \); the slope provides the volume change of association \( \Delta V \) (Fig. 3A, inset). The \( K_d \) values obtained in this way from both fluorescence spectra and anisotropy data are in excellent agreement with that calculated by Mok et al. (17) from urea unfolding experiments (Table I), especially so considering the difference in techniques and the extrapolations to atmospheric pressure and absence of denaturant, respectively. These results show that the previous use of an empirical approach \( \Delta G_m = \Delta G_p + m(\text{urea}) \) agrees very well with the thermodynamic relation \( \Delta G_m = \Delta G_p + p\Delta V \) at pH 5.5.

Measurements of changes in volume are inaccessible to most denaturation techniques but are of great importance, since they measure the differences in packing of the states involved, adding valuable information to the thermodynamic characterization. The calculated volume change for the dissociation of E2-DBD by high pressure at 1 mM and pH 5.5 is 76 ml/mol (slope of plot in Fig. 3A), which falls within the range found for other dimers (23).

An independent assessment of dissociation can be obtained from the concentration dependence of the process (Fig. 3B). An increase in E2-DBD concentration promotes a displacement in the \( p_{1/2} \) value (the pressure that promotes 50% change) from 1300 bars at 1 mM E2-DBD. The difference in \( p_{1/2} \) between these two experiments \( \Delta p_{1/2} = 770 \text{ bars} \) allows us to calculate \( \Delta V \) (Equation 5). The \( \Delta V \) value was 73.2 ml/mol, in very good agreement with the value obtained for \( \Delta V \) at a fixed protein concentration (76 ml/mol; Fig. 3). A ratio of \( \Delta V/\Delta V_e \) that is close to 1 indicates that the dissociation...
process complies with the law of mass action in the concentration range analyzed and that there is no significant conformational heterogeneity as observed in multisubunit complexes (34), tetramers (35), and more recently in a dimeric protein (36).

The reversibility of the dissociation process induced by pressure was confirmed by following the spectral change on decompression. The value of center of spectral mass for the Trp emission returns to the initial value (prior to pressure application), with negligible hysteresis (Fig. 3B, open circles).

The stability of E2-DBD toward pressure denaturation depends markedly on the pH (Fig. 4A). At pH 6.0 or 7.0 the process is incomplete, with a decrease in the center of mass of only 200–300 cm\(^{-1}\) at 2.86 kilobars, the highest pressure applied. At pH 5.8, the center of mass shift was greater, but only at pH 5.5 is the final value (28,600 cm\(^{-1}\)) compatible with the value obtained in urea, indicating complete exposure of tryptophan residues to the solvent. The urea unfolding curves also showed a strong dependence on pH (17). It is noteworthy that at atmospheric pressure, the center of mass of E2-DBD increases from 29,050 cm\(^{-1}\) at pH 5.5 to 29,280 cm\(^{-1}\) at pH 7.0 (Fig. 4A). This result clearly indicates that pH causes a change in the environment of at least one of the Trp residues. The changes in the center of spectral mass with an increase in pH from 5.5 to 7.0, although rather small (≈200 cm\(^{-1}\)), are not significantly dependent on protein concentration (not shown).

Besides, as previously shown by analytical ultracentrifugation (17), E2-DBD is still a dimer, with no detectable monomers at pH 5.5. These data suggest that an isomerization reaction rather than a dissociation reaction is taking place when the pH

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**TABLE I**

| Dissociation constant at atmospheric pressure (K\(_d\)) and the volume change of association (\(\Delta V\)) for the equilibrium E2\(_x\) \(\rightarrow\) 2 E2 at different pH conditions |  |
|---|---|---|
| E2-DBD, pH 5.5 | \(K_d\) | \(\Delta V\) |
| Values obtained by the center of spectral mass shift | \(5.6 \times 10^{-5}\) | 76.0 ± 3.5 (5)a |
| Values obtained by the anisotropy change | \(4.3 \times 10^{-5}\) | 78.5 ± 4.2 (3)b |
| E2-DBD, pH 6.0 |  |  |
| With 0.5 M urea | \(6.0 \times 10^{-5}\) | 93 |
| With 0.75 M urea | \(1.0 \times 10^{-5}\) | 100 |
| With 0.95 M urea | \(5.4 \times 10^{-5}\) | 86 |
| Extrapolated value at 0 M urea (17) | \(5.5 \times 10^{-5}\) |  |

a Average and S.D. of five experiments.
b Average and S.D. of three experiments.
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Fig. 5. Effects of subdenaturing concentrations of urea on the dissociation of E2-DBD by pressure at pH 6. The center of mass shift was used to follow dissociation in the absence of urea (●) or in the presence of 0.5 M (●), 0.75 M (■), and 0.95 M (▼) urea. Other conditions were as in Fig. 2A. The isolated symbol at the bottom left corresponds to the center of mass value of 1 μM E2-DBD in the presence of 6 M urea at atmospheric pressure at the same pH.

is lowered from 7.0 to 5.5. The existence of a predissociated dimer (17, 37) and of a denatured dimer (38) has been demonstrated for the Arc repressor. Fig. 4B shows the change in volume change as a function of pH. At high pH values, the changes induced by pressure are less steep, which results in a smaller volume change (~43 ml/mol). Between pH 6.0 and 5.5 there is an abrupt transition, and ∆V increases to ~80 ml/mol. The presence of a predissociation transition explains the smaller volume changes (less steep dependence on pressure) at high pH values, where the presence of a dimeric intermediate stretches the dissociation curve (16, 23).

Eight histidines out of 10 per dimer of HPV-16 E2-DBD lie at the interface between the two subunits (histidines 8, 38, and 46 are indicated in the E2-DBD structure; Fig. 1). The histidines of each subunit face each other at the interface, especially histidines 8 and 38; if they are protonated, they could destabilize the dimer. This may account for the greater tendency to dissociate at lower pH. Proton dissociation of the histidines, with a pK around 5.8 from Fig. 4B, would suppress the charge repulsion at the interface and engender a tight dimer. These histidines may exert a crucial regulatory effect on the stabilization with a potential effect on sequence-specific DNA binding.

Since the pressure dissociation process was not fully complete at pH 6.0 or 7.0 at the maximum pressures that were attained in Fig. 4A, we repeated the experiment at pH 6.0 with subdenaturing concentrations of urea added to the buffer (Fig. 5). In the presence of 0.5–0.95 M urea, pressure induced a decrease in the center of spectral mass to a value similar to that observed at high urea concentrations at atmospheric pressure or at high pressure at pH 5.5 in Fig. 4A, i.e., complete exposure of the Trp side chains to the solvent. From these curves, we calculate the ∆V of association and the Kd at pH 6.0 in the presence of different concentrations of urea (Table I). The Kd values are in good agreement with the value previously obtained from urea unfolding curves (17). Interestingly, the data show that ∆V increases dramatically with the addition of a small concentration of urea (0.5 M urea). The increase in ∆V observed when small amounts of urea are added could reflect an extra transition from “native-like” monomer to an unfolded monomer.

Residual Structure in the High Pressure Monomeric State of E2-DBD—In Fig. 2A, it was shown that the urea-induced denaturation of E2 shifts the spectrum to the red but does not promote any decrease in intensity, whereas dissociation induced by high pressure affects both parameters. Fig. 6A shows the changes in the spectral area of the tryptophan fluorescence emission as a function of pressure or urea. As it is seen, over a broad range of concentrations, urea did not promote changes in tryptophan emission intensity, while pressure provoked a gradual decrease in the fluorescence intensity. The changes in tryptophan fluorescence intensity under high pressure resemble those induced by acid pH (17).

Time-resolved fluorescence measurements allow us to probe the tryptophan environment in the nanosecond time frame, providing a dynamic characterization of the conformational state of a protein (30, 31). Fluorescence lifetime measurements were performed to compare the excited state of the Trp residues of E2-DBD at pH 5.5 in three different states: native state (atmospheric pressure), pressure-dissociated state (2.8 kilobars), and urea-denatured state (Fig. 6B). In the native state, the best fit for the data is achieved by a Lorentzian distribution of lifetimes, rather than discrete exponential decays, centered at 1.745 ns. For the pressure-dissociated state, the distribution of lifetimes shifts to shorter lifetimes, centered at 0.727 ns (Fig.
6B and Table II). The decrease of 2.2-fold in the lifetime value is consistent with the decrease in tryptophan emission that takes place under these conditions (Fig. 6A). On the other hand, the center of the distribution of lifetimes for the urea-denatured state (1.951 ns) is very similar to the native state, consistent with the identical intensities of the native and urea-denatured states. However, for both pressure-dissociated and urea-denatured states, the width of the distribution increases dramatically in comparison with the control at atmospheric pressure and in the absence of denaturant (Fig. 6B; Table II). The dramatic differences between the distribution of lifetimes of the pressure-dissociated and urea-denatured states indicate that the phase space explored by the Trp residues are very different. The shorter lifetimes experienced by the Trp residues in the pressure-dissociated state suggest that a conformation-dependent quenching takes place.

Hydrodynamic Evidence for Residual Structure in the Pressure-dissociated Monomer—To compare the rotational hydrodynamic properties of E2 in the pressure-dissociated and urea-denatured states, fluorescence anisotropy measurements were performed. The steady-state anisotropy value for the dimer at atmospheric pressure was 0.12. The anisotropy decreases to 0.0548 at 2.5 kilobars, suggesting a decrease in the rotational hydrodynamic radius (Fig. 7A). Fig. 7A also shows the decompression curve (open circles), indicating complete reversibility of the dissociation process. Fig. 7B shows the change in anisotropy induced by the increase in urea concentration. At 5 M urea, the fluorescence spectrum was already shifted completely to the red, the anisotropy was 0.078, significantly higher than the value observed for the pressure-dissociated protein. This result indicates that the urea-denatured form of E2 is more expanded than the pressure-induced monomer. From the changes in anisotropy, in combination with the lifetime measurements, the average rotational relaxation times (\(\langle \rho \rangle \)) were determined according to Equation 7 (Table II). The value of \(\langle \rho \rangle \) at atmospheric pressure is consistent with the expected value for a combination of local motions with global rotation of the Trp residues in a protein with the dimensions of E2-DBD (7, 21). The tryptophans of the pressure-dissociated monomer rotate faster, indicating a more compact state than is seen with the urea-denatured form (Table II). The \(\Delta V \) and \(K_d \) values were 78.5 ml/mol and 4.3 \(\times 10^{-8}\) M, respectively, very close to those obtained from the center of mass data (Fig. 3A and Table I).

Persistent Binding of bis-ANS in the Pressure-dissociated State—As part of the characterization of the high pressure state, we made use of the hydrophobic dye bis-ANS, which binds to accessible hydrophobic patches in structured proteins, which translates into a large increase of its fluorescence emission (15, 39, 40). At pH 7.0, the E2-DBD dimer appears to bind two molecules of bis-ANS (40). When the E2-DBD was pressurized in the presence of bis-ANS, there is a 30% decrease in the bis-ANS fluorescence (Fig. 8), whereas the change in the center of spectral mass of the tryptophan residues (not shown) is the same as before. Urea denaturation causes a much larger decrease in the bis-ANS fluorescence, which is compatible with the lack of long range interactions in an unfolded polypeptide (Fig. 8). These data indicate that the protein under pressure retains some degree of long range tertiary interactions, whereas high concentrations of urea (5 M) cause a more extensive unfolding.

Probing the Stability of the Pressure-dissociated State to Urea Unfolding—To gain more insight on the pressure-dissociated state, we analyzed its stability toward urea denaturation. We reasoned that adding increasing concentrations of urea to the state at high pressure, in the presence of bis-ANS, would further decrease the amount of bound bis-ANS consistent with a fully unfolded polypeptide. In this way, we could obtain an estimate of the stability of the species trapped under high pressure. Fig. 9A shows typical traces of the time course of changes in bis-ANS fluorescence of E2-DBD after pressure (2.86 kilobars) is applied, at increasing concentrations of urea.

The bis-ANS fluorescence values obtained after reaching the equilibrium were plotted against urea (Fig. 9B). The structure present at high pressure is completely unfolded at 1 M urea, with an apparent \([U]_{50\%}\) of \(-0.25\) M. In contrast, the stability of the folded dimer at atmospheric pressure but otherwise similar conditions is much higher (\([U]_{50\%}\) of \(-2.5\) M, Fig. 9). The species present at 2.6 kilobars in the presence of 1 or 2 M urea could be defined as a largely unfolded state in which the tryptophan residues are completely exposed to the solvent (center of mass equal to 28,600 cm\(^{-1}\)) and its bis-ANS binding capacity is almost abolished, as expected for a completely unfolded polypeptide.

**DISCUSSION**

When studying the folding of dimeric proteins, it is important to determine the hierarchy of stability of possible structures formed in the reaction and to be able to distinguish, when possible, between folding and association events (41). In the particular case of several DNA-binding proteins with structures that are highly intertwined, folding and association are
highly coupled processes. A good example is the Arc repressor, in which a highly concerted folding and association process is observed, as determined by fluorescence spectroscopy and NMR (13, 15, 16, 37, 38).

High pressure constitutes a noninvasive technique in which reversible dissociation or denaturation processes can be analyzed on a thermodynamic basis (24). In the present work, we describe the reversible dissociation of E2-DBD by high pressure, and we characterize the partially folded monomers. The Trp fluorescence changes that take place under pressure allow us to determine the volume change of association by applying the thermodynamic principles associated to pressure effects. A substantial fraction of the increase in volume on folding and association of proteins results from the formation of solvent-excluded void volumes. The structural importance of the volume change can be evaluated by comparison with other dimer-monomer dissociation reactions. The volume change of association for E2-DBD (76 ml/mol) is within the same range of that obtained for other dimers (55–170 ml/mol) (for a review, see Ref. 23). However, most of the dimers previously studied were much larger than E2-DBD. Only the Arc repressor was smaller (15). An appropriate way to express the volume change is to normalize to the molecular weight of the dimer (15, 23), which furnishes the specific volume change. The value obtained for Arc and E2-DBD is much higher than for other dimers. The specific volume change of association is 4.2 ml/g for E2-DBD; 7.69 ml/g for Arc; and 0.688, 1.25, 1.88, and 4.73 ml/g for enolase, hexokinase, tryptophan synthase β subunit, and R17 coat protein dimer, respectively (23). The large change in volume per mass of protein found in Arc, R17 coat protein, and E2-DBD dissociation can be explained by a high degree of interaction of buried amino acid side chains with the solvent under dissociation. The hydration of charges that were involved in salt bridges or the hydration of polar and nonpolar groups results in volume contraction. It is suggested that the partially unfolded states of these three proteins in the monomeric state favor a higher degree of hydration when compared with other dissociation systems. However, the Arc repressor undergoes an almost 2 times greater volume change per mass of protein than does E2-DBD, indicating that in the case of Arc the disruption of the structure under dissociation is more drastic.

An estimate of the solvent-excluded surface can be made from the volume change (42). The volume change obtained experimentally (76 ± 4 ml/mol) for E2-DBD at pH 5.5 corresponds to a decrease in volume of 126 Å²/dimer dissociated. Considering a nonpolar solvent as a model for calculating linear compressibility, this value corresponds to exposure of 2775 ± 256 Å² when E2-DBD dissociates. This value is compatible with the x-ray diffraction structure, which led to the conclusion that 2,467 Å² of solvent-accessible area is buried on formation of the dimer (7). This remarkable similarity (within experimental error) between the pressure-dissociated state and that calculated from x-ray diffraction for the native monomer presents further evidence that the monomer retains some tertiary structure; although it is less stable, it still maintains a substantial proportion of hydrophobic residues buried in its interior.

Although the structure of E2-DBD from HPV-16 has not yet been solved, a structure of an equivalent protein from a strain with 80% homology (HPV-31) has been solved by NMR methods and shows the same topology as in the crystal structure of the bovine virus E2-DBD (Fig. 1; Refs. 7 and 21). This confirms that one of the three tryptophan residues/monomer is effectively located at the surface in the minor α-helix, facing the solvent, and the other two are located at the dimeric interface, facing the center of the barrel. It is, therefore, very likely that the buried tryptophan residues are responsible for the change in the fluorescence spectral mass, as they become exposed to the solvent at high pressure or high urea concentration. However, although the tryptophan residues in the pressure-dissociated state undergo solvent relaxation (resulting in a spectral shift as large as that caused by urea denaturation), the lifetime

![Figure 8](image)

**FIG. 8.** Decrease in binding of bis-ANS to E2-DBD as dissociation/denaturation is induced by urea (●) or pressure (○). The area of the bis-ANS fluorescence spectrum is normalized to $A_{0}$, the area obtained at atmospheric pressure in the absence of urea. The difference between the values at 3000 bars and in 5 M urea indicates that different conformations are attained. Protein concentration was 1 μM, and bis-ANS was 5 μM. Excitation was set at 360 nm, and emission was collected in the range 400–600 nm.

![Figure 9](image)

**FIG. 9.** Effects of subdenaturing concentrations of urea on the intermediate species trapped under pressure. A, kinetics of E2-DBD denaturation under pressure (2860 bars), based on the decrease in bis-ANS binding in the absence of urea (○) or in the presence of 1 M urea (●) at pH 5.5. The fluorescence of bis-ANS was measured as in Fig. 8. B, urea-induced denaturation of the species trapped under high pressure (●) and of the protein at atmospheric pressure (▲). The end point of each kinetic experiment (see panel A) is plotted as a function of urea concentration. Note that in the presence of 0.75–1.00 M urea, bis-ANS decreases to a value similar to that seen in the presence of 4–5 M urea at atmospheric pressure (▲). Protein concentration was 1 μM, and bis-ANS concentration was 5 μM.
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distribution is different from that in the urea-unfolded state. The width of the distribution is broader than in the native state but narrower than in the completely unfolded state. Four lines of evidence distinguish the states attained by pressure and urea: Trp intensity, excited-state lifetime, average rotational relaxation time, and bis-ANS binding. This clearly shows the presence of a state with persistent structure at high pressure. The conformation of E2 trapped under high pressure is more compact than the urea-denatured state, and it retains the ability to bind bis-ANS, features compatible with molten globule-like conformations (43, 44). High pressure has been used to trap molten-globule conformations of different protein systems (15, 24, 45, 46).

The urea-unfolded state virtually binds no bis-ANS, whereas the pressure-dissociated state retains ~70% of its bis-ANS binding capacity. This strongly suggests that there are persistent long-range interactions that allow for the formation of sites where the dye can be inserted. The nature of those sites is unclear. They might lie in an accessible core or in the DNA binding site, since folded E2-DBD binds both ANS and bis-ANS (22, 28). It can be argued that the bis-ANS binding is in fact due in part to a residual native-like conformation of the DNA binding site.

The structure of E2-DBD in the monomeric state under pressure is very unstable: urea titration of the bis-ANS binding reveals a midpoint around 0.25 M urea, in contrast with the 2.5 M for the native state (Fig. 9B). This decreased stability to urea explains why the dissociation constant obtained from extrapolation to zero in the urea-unfolding curves (atmospheric pressure) is similar to that obtained from the pressure-dissociation experiment. The lack of resistance to unfolding of the high pressure state at low urea concentrations can be interpreted as high sensitivity of the global structure to the chemical denaturant or noncooperative unfolding of the local structure that binds bis-ANS. Although we cannot discern between these two explanations due to experimental limitations, the lack of cooperativity is a defining characteristic in molten globule-like conformations (43, 44).

Altogether, our data suggest the following pathway for the reversible dissociation and unfolding of E2-DBD at pH 5.5.

\[ (E2)_2 \leftrightarrow 2A \leftrightarrow 2U \]

**REACTION 1**

Equilibrium 1 encompasses the process of dissociation of the dimer to partially folded monomers (A) at pH 5.5. Unfolding by high concentrations of urea involves both equilibria. Equilibrium 2 per se was monitored by the experiment of Fig. 9, where the pressure-dissociated state was denatured by urea to a completely unfolded species (U). The free energy change for reaction 2 (\( \Delta G_U \)) is less than 2.0 kcal/mol, indicating that A is relatively unstable.

With the evidence accumulated in the present work, it is tempting to suggest that the monomeric high pressure-dissociated state is related to the nonnative monomeric intermediate species found in kinetic refolding studies (22). The subsequent association of E2-DBD from the monomeric intermediate is slow, consistent with a rearrangement that must take place before the final association/folding step leading to the native dimer, which must involve the acquisition of precise geometrical features and tight side chain packing (22). Further experiments will be needed to test this hypothesis.

The overall characteristics of the pressure-dissociated state strongly suggest a molten globule-like conformation. The implications of a "structured" monomer in the absence of chemical denaturants or extreme pH conditions contribute to the understanding of the equilibrium between folded and unfolded states in conditions that are more compatible with the cellular environment, since pressure is a thermodynamic variable that strictly affects the position of that equilibrium. Further detailed characterization of the state at high pressure will require the use of high pressure NMR experiments (16). Provided that enough structural detail can be obtained from NMR experiments, future prospects will include the possibility of developing synthetic compounds capable of trapping the monomeric state, of potential therapeutical value against papillomaviruses.

**Acknowledgments—**We thank Emerson R. Gonçalves for competent technical assistance, Y.-K. Mok and M. Bycroft for the expressing E2-DBD clone, Marta Sorensen for critical reading of the manuscript, and Fabio Almeida for the kind assistance with molecular modeling.

**REFERENCES**

1. Pabo, C. O., and Sauer, R. T. (1992) *Annu. Rev. Biochem.* 61, 1053–1095

2. von Hesper, P. H. (1994) *Science* 263, 769–770

3. Travers, A. (1993) *DNA-Protein Interactions, Chapman & Hall, Publishers, London*

4. Scheck, R. W., Otwinowski, Z., Joachimiak, A., Lawson, C. L, and Sigler, P. B. (1985) *Nature* 317, 782–786

5. Jordan, S. R., and Pabo, C. O. (1988) *Science* 242, 893–899

6. Breg, J. N., van Opheusden, J. H. J., Burgering, M. J. M., Boelens, R., and Kaptein, R. (1990) *Nature* 346, 586–589

7. Hegde, R. S., Grossman, S. R., La summarize, the authors discuss the stabilization of E2-DNA-binding protein to high pressure. They present experimental evidence that suggests a molten globule-like conformation under high pressure conditions. The authors also acknowledge the importance of future experiments to test this hypothesis and the potential therapeutic value of synthetic compounds that could trap the monomeric state.