The bZIP Region of the Plant Transcription Factor Opaque-2 Forms Stable Homodimers in Solution and Retains Its Helical Structure upon Subunit Dissociation†

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ABSTRACT: Opaque-2 (O2) is a plant bZIP transcription factor that regulates the expression of α and β prolamines, the main storage proteins in seeds of cereals such as maize and *Coix*. One of the main processes modulating O2 activity is the heterodimerization with other bZIP transcription factors, but the primary mechanism underlying the partner choice is still unknown. In this paper, we have characterized the bZIP domain of O2 by nuclear magnetic resonance (NMR), circular dichroism (CD), and size-exclusion chromatography. Results obtained from CD measurements suggested that the native O2bZIP has about 40 of its 49 leucine-zipper residues in helical structure, while the DNA-binding domain is completely unstructured. Diffusion-ordered NMR spectroscopy and size-exclusion chromatography showed that O2 forms homodimers in solution. Thermal denaturation experiments indicate that O2 reversibly undergoes dissociation and unfolding in a process that is fully dependent on the protein concentration. Subunit dissociation of O2bZIP dimers, upon dilution of the protein, led to partially folded monomers that retained ∼80% of the native CD ellipticity at 222 nm. We believe that the existence of partially folded monomers could decrease the entropic penalty for helix formation involved in the DNA binding and in the subunit association of O2bZIP. Stabilization of partially folded monomers may also play a significant role in the dimerization of O2 with other bZIP transcription factors and, consequently, can be important for the regulation of the biological functions of O2 in plants.

Leucine zippers, the simplest motif of protein association in nature, are formed by two parallel coiled coils with a heptad repeat, denoted $(abcdefg)_n$. Hydrophobic residues form the subunit interface, which usually comprehends the interaction of leucine residues in positions *d* with hydrophobic residues in positions *a* of the complementary subunit (*d* with *a*′ and *d*′ with *a*). Although hydrophobic contacts provide the driving force for leucine-zipper association, interhelical salt bridges also contribute to dimer stability, because residues with opposite charges frequently occupy neighboring positions *e* and *g* (*1*, *2*). Leucine zippers have received considerable attention over the past decade because of their fundamental role in determining the activity of eukaryotic transcription factors. Transcription factors that have a basic DNA-binding region and a leucine-zipper oligomerization domain are known as bZIPs.[1] They usually form either homo- or heterodimers in solution. Heterodimerization is essential for the biological activity of bZIP transcription factors, because it appears to modulate transcriptional activity of target genes (*3*). Because subunit association should be under strict control to allow bZIP heterodimerization, the study of dimer stability can contribute to the understanding of the molecular mechanisms of protein specificity and gene regulation that are not yet completely known. For instance, the presence of an asparagine residue in position *a* of a heptad can determine the dimerization specificity (*4*, *5*). Yet, studies with c-Fos and c-Jun zippers showed that repulsive interhelical interactions in either the c-Jun and c-Fos homodimers are the driving forces in the formation of the c-Jun−c-Fos heterodimer (*6*).

A common structural feature among eukaryotic transcription factors is the small amount of secondary and tertiary structures. This is true for GCN4 (*7*), c-Fos bZIPs (*8*), and also other classes of transcription factors such as zinc fingers (*9*). Frequently, in bZIP transcription factors, the dimerization domain forms a coiled coil, while the rest of the protein is unstructured. In most cases, the helical content of the proteins is around 40%.

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[1] Abbreviations: bZIP, basic leucine zipper; O2, Opaque-2; OHP, Opaque-2 heterodimerization protein; IPTG, isopropyl thiogalactopyranoside; PEI, polyethyleneimine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple-quantum coherence; D_2O, deuterated water.

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Opaque-2 (O2) is a plant bZIP transcription factor that regulates the expression of storage proteins, called prolamines, in the endosperm of maize and *Coix* seeds. Homozygotic deletion of the O2 gene leads to a phenotypic change in maize seeds from hard glassy kernels to opaque seeds and a decrease in the content of α and β zeins in the endosperm (*10*). O2 also seems to be involved in the regulation of lysine catabolism and synthesis of growth precursors in seed (*11*). Similar to other plant bZIP transcription factors, O2 can form heterodimers with other bZIP proteins and recognizes many sequences containing the ACGT box (*12*, *13*), therefore displaying a broad binding specificity and recognizing a variety of target sites in several distinct genes (*14*). O2 displays similarity with other bZIPs such as GCN4, c-Fos, and c-Jun. Recently, similar transcription factors were also identified in the *Arabidopsis* genome (*15*); they share with O2 the presence of a long region of the leucine-zipper domain (*14*).

Although several bZIP proteins with long dimerization domains have been identified, relatively little is known about the thermodynamic properties of the folding and subunit association of such proteins. In this paper, we performed diffusion-ordered nuclear magnetic resonance (NMR) experiments and circular dichroism (CD) spectroscopy to characterize the O2 biophysical properties in solution. We report stability studies on the folding and association of the O2 bZIP region (O2bZIP) and on the isolated leucine-zipper region (O2Z), the domain most likely responsible for the oligomerization of O2. The data showed that O2bZIP forms homodimers in solution, at concentrations above 70 μM, and about 30% of the protein is in the helical conformation. The thermal stability of O2bZIP is strongly dependent on pH, being more stable at pH 4 than at pH 7, suggesting that there is an important electrostatic contribution for subunit association. The results with the isolated leucine-zipper domain suggested that all the ellipticity signal present in O2bZIP comes from the leucine-zipper region of the protein, corroborating the notion of an unfolded DNA-binding domain, in the absence of the target DNA.

EXPERIMENTAL PROCEDURES

Purification of O2. Large-scale purification of O2bZIP has not been reported before. Recombinant O2 was obtained by expression in *Escherichia coli* (BL21 (DE3)/pLysS) transformed with plasmid (pET) containing *Coix* (*Coix lacryma-jobi*) O2 bZIP region (O2bZIP) or its isolated leucine-zipper domain (O2Z). Bacterial cells were grown in LB or M9 medium until the OD_{660} reached ~0.8, when protein expression was induced with 1 mM IPTG. After 4 h, cells were harvested by centrifugation, resuspended in (50 mL/L of medium) 50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 5 mM DTT, and 5 mM benzamidine-HCl. Cells were lysed by five cycles of freezing in liquid N_2 and thawing in a water bath at 37 °C. Five fast bursts of sonication were performed to reduce the viscosity of the medium, and the supernatant was separated by centrifugation, incubated at 80 °C for 3 min, and quickly cooled in an ice bath. After centrifugation, the remaining proteins in the supernatant were precipitated with 80% saturated ammonium sulfate, centrifuged, and resuspended in 20 mM Tris-HCl at pH 7.5, 5 mM EDTA, 2 mM DTT, and 1 M NaCl. Precipitation of nucleic acid was done by slow addition of PEI to a final concentration of 0.5%. The supernatant was separated by centrifugation and precipitated again with 80% saturated ammonium sulfate. The pellet was washed twice with 80% saturated ammonium sulfate to remove residual PEI and resuspended in 50 mM Tris-HCl at pH 7.2 and 1 mM EDTA (buffer A). The O2-enriched solution was diluted to reduce the conductivity to about 300 μS and injected in a Pharmacia Sulfopropyl column (400 mL of bed volume) in a Pharmacia GradFrac system. The flow rate used was 20 mL/min. After the solution was washed with about 1 L of buffer A, a 2.6-L gradient from buffer A to 50 mM Tris-HCl at pH 7.2, 1 mM EDTA, and 2 M NaCl (buffer B) was performed. Fractions containing O2 eluted with about 1 M NaCl. The fractions were pooled and precipitated with 80% saturated ammonium sulfate. The ammonium sulfate pellet was then resuspended in 2% acetonitrile and 0.06% trifluoroacetic acid (~4 mL/L of growth medium). The resulting solution was spun to remove solid residues and applied in a Poros 50 R2 (5.65 mL) column using a BioRad-System Interface HPLC. Purified O2 was eluted with an acetonitrile gradient from 2% to 98%. The concentration of acetonitrile in which O2 eluted was about 45%. Protein was then lyophilized and stored at −20 °C.

Protein concentration and purity were checked by Lowry's assay and SDS−PAGE, respectively. The yield of purification was about 15 mg of protein/L of cell culture. Unless indicated otherwise, all experiments were performed in 50 mM phosphate buffer at pH 4 and 100 mM KCl, at 25 °C. All reagents used were of an analytical grade of purity.

Size-Exclusion Chromatography. Size-exclusion chromatography of O2bZIP was performed in a Shimadzu HPLC system, with a Superdex-200 column equilibrated with 50 mM phosphate buffer at pH 4 and 100 mM KCl. The flow rate used was 0.7 mL/min. Molecular weight was confirmed using the elution volumes of standard proteins under the same conditions.

CD Experiments. CD experiments were performed in a JASCO J-715 spectropolarimeter using 2 nm bandwidth and 0.2 cm optical path cell. For unfolding experiments, a temperature ramp (1 °C/min) was built up with a Peltier unit. Ordinate scales employed for CD plots are in molar ellipticity, in deg cm^2 $dmol^{-1}$, given by

$$\theta = \theta_R/[10bC(n-1)] \quad (1)$$

where θ_R is the raw ellipticity, b is the cell path in centimeters, C is the molar concentration of the protein used, and n is the number of amino acid residues present in the protein primary sequence.

NMR Experiments. Isotopically ^{15}N-labeled O2bZIP samples for HMQC experiments were prepared as described above using M9 minimum medium containing $^{15}NH_4Cl$ as a unique nitrogen source. All of the purification steps used were the same as those used for the unlabeled samples. Sample preparation included the addition of 10% D_2O for lock. Diffusion-ordered NMR (*16*, *17*) and HMQC experiments were carried out in a Bruker Avance DRX 600 MHz spectrometer using a 5 mm triple-resonance inverse probe. Bruker XWinNMR software was used for data acquisition and processing. The pulse sequence used was a stimulated echo (*16*, *17*). Plots of the natural logarithm of the intensity of select peaks versus the square of the strength of the

A. O2bZIP:

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1    MVRLATSSSS   RDPSPSDEDM    DGEVEILGFK   MPTEERVRKR   40
          <----------------------DNA Binding Domain-----------
41   KESNRESARR   SRYRKAAHLK    ELEDQVEQLK   AENSCLLRRL   80
     ------------------>  <--------------------Leucine Zipper
81   AALNQKYNEA   NVDNRVLRAD    METLRAKVKM   GEDSLKRVMD   120
     Domain------------------------------>
121  PAANKARKEA   ELAAATAEQ                               139
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B. O2Z:

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55                    MAAHLK    ELEDQVEQLK   AENSCLLRRL   80
                           <--------------------Leucine Zipper
81   AALNQKYNEA   NVDNRVLRAD    METLRAKVKM   GEDSLKRVMD   120
     Domain------------------------------>
121  PAANKARKEA   ELAAATAEQ                               139
```

FIGURE 1: Primary sequence of O2bZIP (A) and O2Z (B). Amino acid residues are represented by a one-letter code. Regions containing the basic DNA-binding and leucine-zipper domains are indicated. Residues in positions *a* and *d* of the heptads are in bold.

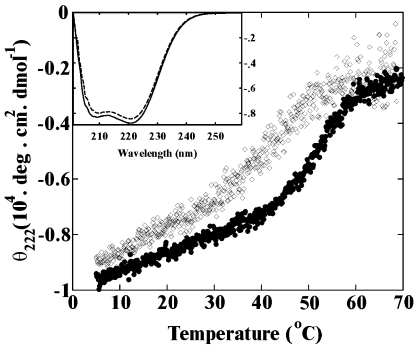

FIGURE 2: Effect of pH on the thermal denaturation of O2bZIP. CD ellipticity of 55 μM O2bZIP was recorded at 222 nm as a function of the temperature at pH 4 (●) and 7 (◇). The inset shows CD spectra at 25 °C at pH 4 (—) and 7 (- - -).

gradient pulse, used to calculate the apparent translational diffusion, at each protein concentration, in diffusion-ordered NMR experiments are available in the Supporting Information.

RESULTS

The plant transcription factor O2 has been characterized as a bZIP protein based on the homology of amino acid sequence with other known bZIP transcription factors (*14*, *18*). O2 shares sequence similarity with the "leucine zipper" DNA-binding domain of several mammalian oncogenes and fungal transcription activation factors (*14*). Despite the biological importance of O2, there is no experimental information available about its structure, subunit association, and thermodynamics. Submission of the primary sequence of O2bZIP (shown in Figure 1) to the COILS2 server (*19*) predicted the existence of a coiled coil in the leucine repeat region of O2, compatible with the prediction of a secondary structure made by the PROSITE server (*20*), which showed a long region with high helical propensity and a leucine-zipper region. CD spectra of O2bZIP showed a typical helix band, with negative minima at 222 and 208 nm (inset of Figure 2). The molar ellipticity of O2bZIP at 222 nm was about -9000 deg cm^2 $dmol^{-1}$. The helical content, calculated from CD spectra, was ~30%, which corresponds to ~40 amino acid residues. Because the region bearing the leucine repeats of O2 contains 49 residues (total of 138 amino acids in O2bZIP), these results suggest that the DNA-binding domain of O2bZIP is unstructured, as found in other bZIP transcription factors (*21*−*23*).

Thermostability. The estimated isoelectric point of O2bZIP was about 9.1 according to the EMBL Gateway to Isoelectric Point Service. This is in agreement with the observation that O2bZIP was more soluble at pH 4 than at pH 7 (data not shown). The stability of O2bZIP was determined by following the decrease of the negative CD band at 222 nm, as a function of the temperature, from 5 to 70 °C. Figure 2 shows the thermal denaturation curve of O2bZIP at pH 4.0 or 7.0. Although CD spectra of O2 recorded at pH 4.0 and 7.0 showed similar patterns (inset of Figure 2), the thermal stability of O2bZIP changed significantly within this pH range. As shown in Figure 2, O2bZIP is more stable to thermal denaturation at pH 4 ($T_{1/2}$ = 50 °C) than at pH 7 ($T_{1/2}$ = 40 °C). The thermal denaturation curves shown in Figure 2 display an initial linear decrease of negative ellipticity, from 5 to 35 °C. This linear component in the thermal denaturation curve has been described for other coiled coils and was associated with the fraying of the extremities of the zipper (*9*, *24*−*28*). Above 35 °C, the ellipticity band decreased in a cooperative fashion, characterizing the loss of the secondary structure of the protein. Thermal denaturation of O2bZIP was completely reversible with a reduction in temperature, and similar denaturation curves were obtained if several heating cycles were performed with the same sample (data not shown).

The thermal denaturation of O2bZIP was found to be strongly dependent on the protein concentration (Figure 3). At pH 4, increasing protein concentration shifts the $T_{1/2}$ of denaturation to higher temperatures, as expected by the law of mass action. The initial linear component of the curve, observed at lower temperatures, shows no dependence on protein concentration, corroborating the idea of the fraying of the zipper before subunit dissociation. The inset of Figure 3 displays CD spectra of O2bZIP at pH 4 and at 25 and 70 °C, where an almost complete loss of secondary structure of the protein upon thermal denaturation can be observed.

Thermal denaturation of the isolated leucine-zipper domain of O2 (O2Z) was also followed at pH 4 by the decrease in the CD band at 222 nm (Figure 4). Isolated O2Z displayed a raw ellipticity signal as intense as that of O2bZIP. However, when the molar ellipticities were calculated (see the Experimental Procedures), O2Z displayed about twice the that of the O2bZIP absolute ellipticity signal. This can be explained by the fact that O2Z has 85 amino acid residues (*n*), almost half of the number of residues in O2bZIP (Figure

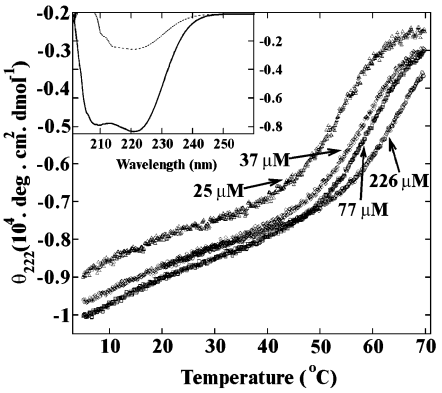

FIGURE 3: Thermal denaturation of O2bZIP as a function of the protein concentration at pH 4. CD ellipticity of O2bZIP was recorded at 222 nm, as a function of the temperature, at four different protein concentrations (226, 77, 37, and 25 μM). Protein concentrations are indicated by arrows. The inset shows CD spectra of O2bZIP at 25 (—) and 70 °C (- - -).

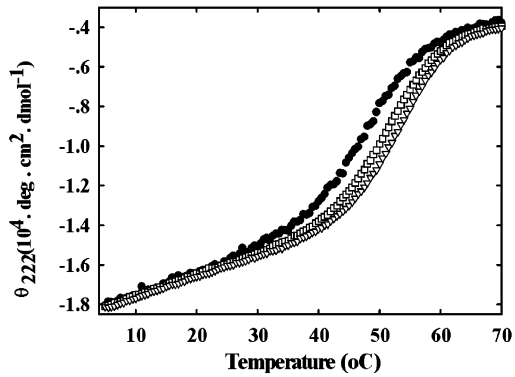

FIGURE 4: Thermal denaturation of O2Z at pH 4. CD ellipticity of O2Z was recorded at 222 nm, as a function of the temperature, at three protein concentrations: 20 (●), 100 (□), and 200 μM (▽).

1). These results suggested that all the ellipticity signal of O2bZIP is due to the structured leucine-zipper domain. Thermal denaturation curves of O2Z also displayed a cooperative transition that was dependent on the protein concentration (Figure 4), suggesting that the isolated leucine-zipper domain was able to form folded homodimers in solution. However, the dependence on the protein concentration observed for the thermal denaturation of O2Z was not so large as the one observed for the O2bZIP form, suggesting that some role may be played in the process by the DNA-binding domain of the O2bZIP molecule. Additionally, a linear change in ellipticity was observed at temperatures below 35 °C, suggesting that O2Z underwent zipper fraying with the increasing temperature.

Dissociation upon Dilution. To address the oligomerization behavior of O2bZIP upon dilution of the protein, we performed diffusion-ordered NMR and CD experiments at different concentrations of the protein. Diffusion-ordered NMR has been extensively used to determine particle size and protein self-association states (*16*, *17*). As shown in Figure 5, at high protein concentrations (>100 μM), the diffusion coefficient of O2bZIP increased linearly because dilution of the protein leads to an increase of the translational mobility of the particles in solution, because of the smaller molecular impairment to the translational diffusion at lower protein concentrations. At protein concentrations lower than ~100 μM, the diffusion coefficient of O2bZIP increased in

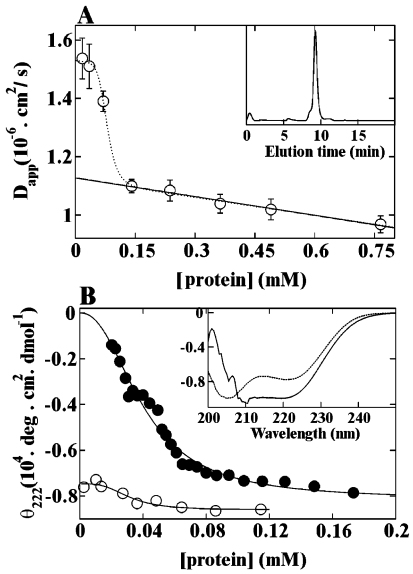

FIGURE 5: Dissociation of O2bZIP by dilution of the protein. (A) NMR diffusion coefficients of O2bZIP as a function of the protein concentration at 25 °C and pH 4. The bars correspond to standard errors from the three measurements (see the Supporting Information). Solid and dashed lines are fits to the data with linear and linear plus sigmoidal equations, respectively. Additional information on the diffusion-ordered NMR experiment is available in the Supporting Information. The inset shows size-exclusion chromatography of O2bZIP in a Superdex-200 column at room temperature and pH 4, with the elution volume corresponding to a ~35 kDa protein. (B) CD ellipticity of O2bZIP at 222 nm as a function of protein concentration at 25 °C and pH 4 (○) or 7 (●). The inset shows the CD spectra of O2bZIP at pH 4 and 200 (—) or 17 μM (- - -).

a cooperative fashion, characterizing the dissociation of the protein (Figure 5A). Diffusion coefficients of associated and dissociated forms could be calculated from the intercepts of the linear (solid line) and sigmoidal (dotted line) components of the curve. The values found were 1.13×10^{-6} cm^2 s^{-1} for the associated form and 1.56×10^{-6} cm^2 s^{-1} for the dissociated form. Stokes−Einstein analysis of the diffusion coefficients (*32*) showed that they correspond to protein particles with molecular radii characteristic of O2bZIP monomers and dimers, respectively (data not shown). Additionally, O2bZIP was confirmed to be dimeric by size-exclusion chromatography in a Superdex-200 column (inset of Figure 5A), because the protein eluted at the retention time compatible with an ~35 kDa protein, relative to a molecular weight calibration curve with standard proteins (not shown).

The dissociation of O2bZIP upon dilution was additionally followed by the decrease in the CD ellipticity band at 222 nm. At pH 7, O2bZIP monomers underwent complete unfolding upon dissociation by dilution (Figure 5B). In contrast, at pH 4, O2bZIP monomers kept ~80% of the secondary structure originally present in the native dimers, indicating that the dissociated monomers remain mostly folded at this pH. O2bZIP dissociation constant ($K_d \cong 79$ μM) could be calculated from the dilution curve only for pH 7 (closed circles of Figure 5A) because the dissociation curve followed by diffusion-ordered NMR has few data points because of the time-consuming experimental procedures. The inset of Figure 5B shows the CD spectra of O2bZIP at 200 and 17 μM. Furthermore, HMQC spectra

recorded at pH 4 and at the two protein concentrations in which diffusion-ordered NMR experiments indicated that O2bZIP was completely dimeric (2 mM) or monomeric (17 μM) displayed very similar peak distributions and chemical shift dispersions (see the Supporting Information). Chemical shift dispersion alone is not a sufficiently strong argument to ensure that O2bZIP retains its helical structure upon subunit dissociation; however, these results add support to the findings, shown above, that O2bZIP remains partially structured at pH 4 when it dissociates.

DISCUSSION

It has long been recognized that numerous proteins lack a stable globular folding or contain long disordered regions under physiological conditions. These proteins usually adopt a specific structure only when bound to their targets and are frequently involved in regulatory functions in the cell (*34*−*36*). There are many examples of transcription factors that acquire ordered structure only when bound to DNA or other proteins in the cell (*37*, *38*). Several mechanistic advantages for the existence of disordered protein domains have been proposed, including the ability to bind different targets in the cell and the increased binding specificity at the expense of thermodynamic stability (*38*). In particular, leucine zippers are known to have conformational fluctuations that are important for the specificity of the formation of heterodimers (*7*). GCN4, the most studied leucine-zipper transcription factor, possesses an N-terminal basic DNA-binding domain that only achieves a stable structure when bound to DNA and a C-terminal leucine zipper that presents 100% of its amino acid residues in a helical conformation at low temperature. At 5 °C, the leucine-zipper domain of GCN4 displays a molar ellipticity of about −33 000 deg cm^2 dmol^{-1} at 222 nm (*7*, *21*).

The plant transcription factor O2 has a seven-repeat leucine zipper, which corresponds to 49 amino acid residues. CD spectra of the bZIP region of O2 (O2bZIP) and of its isolated leucine-zipper domain (O2Z) displayed essentially the same raw ellipticity at 222 nm, suggesting that all the CD ellipticity signal present in O2bZIP comes from the leucine-zipper region. These findings are supported by the fact that the isolated DNA-binding domain of O2 displayed a characteristic random coil CD spectrum (data not shown), suggesting that the DNA-binding domain remains unstructured, as previously reported for other bZIP proteins (*7*, *21*). According to the CD spectra, ∼30% of O2bZIP and ∼58% of O2Z are in the helical conformation (when compared with GCN4), corresponding to about 40 helical-structured residues, which is considerably smaller than the predicted number of residues for a seven-repeat leucine zipper (49 residues). This suggests that, even forming stable homodimers in solution, the O2 leucine-zipper domain is not completely structured. It has been suggested that the presence of an asparagine residue in position *a* of the c-Jun leucine zipper leads to a destabilization of the homodimer (*39*). In fact, the presence of an asparagine residue in the *a* position of the heptad has been shown to confer less stability to the subunit association of leucine zippers when compared with hydrophobic residues, such as valine, leucine, and isoleucine (*5*). O2bZIP has two asparagine residues at positions *a* of its heptads (N73 and N94, Figure 1). Thus, these residues may be partially responsible for the reduced helical content of O2bZIP compared to that of GCN4.

O2bZIP was found to be more stable to thermal denaturation at pH 4 than at pH 7. Studies with mutants of vittelogenin binding protein (*1*) showed that positions *e* and *g* can make important contributions to the stability of the dimer. Depending on the *e*−*g*′ pairs, the stability changed with the pH, with no changes in the amount of secondary structure; when amino acids with opposite charges occupy positions *e* and *g*′, there is a maximum stability around neutral pH (*1*). For O2, the *e*−*g*′ pairs are Q65−K70, E72−L77, R79−N84, K86−N91, D93−R98, D100−R105, and K107−E109 (Figure 1) and a histidine residue corresponding to position *g* before the first heptad (H58, Figure 1), which may be involved in a salt bridge with the glutamate residue in position *e* of the first heptad (E63, Figure 1). Although some of these pairs are able to form electrostatic bridges, it is not clear which of them could be responsible for the stabilization of the O2 dimer at low pH (pH 4). The lower stability at the physiological pH probably may be important for the heterodimerization process observed for O2 in vivo. In this sense, the high-resolution structure of the O2 leucine zipper will bring some clues regarding the dimer stability and specificity. Also, site-directed mutagenesis is going to be performed in order to evaluate the contribution of each amino acid residue in the dimer−monomer equilibrium of the O2 leucine zipper.

Diffusion-ordered NMR experiments were performed to follow the subunit dissociation of O2bZIP upon dilution of the protein. Because the translational diffusion depends upon the volume and shape of the particles, transitions from associated to dissociated species can be followed using this approach (*16*, *17*). We monitored changes in the translational diffusion coefficient with the dilution of the protein at pH 4. The curve presented in Figure 5A clearly displays two components: the first linear component associated with the dilution of the dimer and its intercept gives the absolute diffusion coefficient of the associated particle, while the second sigmoid component was associated with a change in the particle size because of the subunit dissociation of the protein. The intercept of this second component gives the diffusion coefficient of the dissociated particle. Stokes−Einstein analysis (*32*) of these two diffusion coefficients resulted in hydrodynamic radii corresponding to O2bZIP dimers and monomers, respectively. Size-exclusion chromatography was used to confirm the oligomeric state of O2bZIP (inset of Figure 5A). O2bZIP eluted at the retention time compatible with a dimer (∼35 kDa), relative to a molecular weight calibration curve with standard proteins (not shown).

There was a good agreement between the diffusion coefficient and the ellipticity at 222 nm of O2bZIP as a function of the protein concentration (Figure 5B). This suggested that the loss of the secondary structure of O2bZIP may be due to the subunit dissociation of the protein. It is worth mentioning that even dissociated, the monomers of O2bZIP remained ∼80% helical at pH 4. In contrast, at pH 7.0, the monomers lost almost all of the helical content upon dissociation by dilution (Figure 5B). At this time, we have no clues about the chemical contacts that stabilize the partially folded monomer in solution; because the presence of partially folded monomers is dependent on the pH, it is possible that the monomers could be stabilized by intra-

molecular salt bridges formed by the residues with opposite charges filling *e* and *g* positions of neighboring heptads. On the other hand, amino acid residues of the DNA-binding domain could be involved in the existence of meta-stable partially folded monomers. Palmer and co-workers (*7*) have suggested that the apparently unfolded DNA-binding domain of bZIP proteins may present residual secondary structure in order to decrease the entropic penalty upon subunit association and DNA binding. We believe that the partially structured monomers observed in O2bZIP must play an important biological role by reducing this entropic penalty associated with the subunit exchange (dissociation/association) of bZIP proteins, which would facilitate the formation of heterodimers in the nuclear microenvironment, where the pH may be slightly acidic.

Another interesting feature present in the O2 leucine zipper is the existence of an alanine residue filling position *d* of one of the heptads (A90). From our knowledge, O2 is the only leucine zipper described to date in which a *d* position is not filled by a leucine residue (excluding OHP proteins known to heterodimerize with O2). Junius and co-workers (*39*) reported that an alanine in position *a* of c-Jun causes a "packing defect" in the leucine-zipper domain, because the alanine side chain is too short to make significant side-by-side van der Waals contacts. Because this alanine residue in position *d* of the O2 leucine zipper must occupy a smaller volume than a leucine, it should be reasonable to expect a "packing defect" in the interface of O2 dimers. Although position *d* is filled by an alanine, position *a* of the same heptad is filled by a tyrosine (Y87), a residue that has a high-volume side chain. Tyrosine residues in position *a* of the heptad of the leucine zippers have never been reported before as well, and it may be able to minimize the packing defect caused by the alanine residue at position *d*, compensating for the possible destabilizing effect of the absence of a leucine residue in this position of the heptad. Although we actually do not know whether these features are important for the stability of O2bZIP, we speculate that the presence of the Ala-Tyr pair making a $d-a'$ interaction may be involved within the specificity of heterodimerization of O2 with other plant bZIP transcription factors, because OHP1 and OHP2, known to heterodimerize with O2, have an alanine residue at position *d* and high-volume side-chain (phenylalanine and tyrosine, respectively) residues filling position *a* of the same heptad (*12*). Because high-affinity binding of bZIPs to target DNA, and consequently, the gene transcriptional response, has been proposed to be mediated by the specificity of the heterodimerization of the leucine-zipper domain (*40*, *41*), further investigation on the specific formation of the O2−OHP heterodimer needs to be carried out to address these features.

Most aspects of subunit association of O2 to form homodimers and/or heterodimers with OHP, as well as how these associations regulate its function as a transcription factor, are still unknown. This is the first systematic work carried out using thermodynamic approaches to study the subunit association of a plant bZIP transcription factor. O2 can become a good model for comprehension of the role played by leucine-zipper transcription factors in eukaryotic cells. This is true especially for plants, because no other plant leucine zipper has been studied at this level before. Solving the solution structure of the O2 leucine zipper will be a great advance in the understanding of the structure−function relationship of such kinds of transcription regulatory proteins.

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SUPPORTING INFORMATION AVAILABLE

Dissociation of O2bZIP, by dilution of the protein, measured by diffusion-ordered spectroscopy (DOSY) and $^{1}H-^{15}N$ HMQC spectra of dimeric and monomeric O2bZIP. This material is available free of charge via the Internet at http://pubs.acs.org.

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