A transcriptional activator, CBF1, from Arabidopsis thaliana, which has the AP2 domain for DNA binding and regulates the cold acclimation response, was over-expressed in Escherichia coli, purified, and characterized. Analyses of the interaction between CBF1 and the C-repeat/dehydration-responsive element by fluorescence measurement showed that CBF1 binds to C-repeat/dehydration-responsive element as a monomer irrespective of the temperature. CD spectra of the intact and truncated CBF1 proteins (1-213, 41-213, 41-157, and 41-146) were measured to examine the temperature-dependent changes of the secondary structure of CBF1. The results suggested that the CBF1 protein has regions exhibiting reversible cold denaturation in the range between 30 and -5 °C and also has a region exhibiting thermal denaturation between 40 and 60 °C. This cold denaturation occurred in both the N-terminal and acidic regions. The thermal denaturation occurred in the region encompassing the AP2 domain. The difference between the retention time of CBF1 at 4 °C and that at 25 °C in gel filtration, and the decrease of the sedimentation coefficient, $s_{20,w}$, caused by the temperature change from 25 to 3 °C, strongly suggested that the cold denaturation was accompanied by the extension of the molecule. The possible cold denaturation observed here might be a physiologically important structural response of CBF1 to cold stress.

Plants are exposed to various environmental stresses, such as low or high temperatures, dehydration, high salt, infection, injuries, etc. Many protective mechanisms have been evolved by plant cells to overcome the stress. Two major pathways have been suggested in the dehydration and low temperature-responsive processes (1). One is a nuclear factor (ABA)$^3$-dependent pathway, which involves an ABA-responsive element (2-4) and an ABA-responsive-element-binding protein with a bZIP motif (5), and the other is an ABA-independent pathway (6-8). The C-repeat/dehydration-responsive element (CRT/DRE) (9-11) and CRT/DRE-binding factor 1 (CBF1) (12) are thought to be important elements in the ABA-independent pathway. CRT has a 5-base pair core sequence of CCGAC and is present in the promoters of several cold-regulated plant genes, including COR15a (10) and COR78/RD29A (9) from Arabidopsis and BN115 from Brassica napus (11). DRE consists of the sequence TACCGACC in the RD29A promoter (9). Yamaguchi-Shinozaki and Shinozaki (9) showed that CRT/DRE was not responsive to the ABA level. Stockinger et al. (12) isolated an Arabidopsis thaliana cDNA encoding CBF1 by using the yeast one-hybrid method. Jaglo-Ottosen et al. (13) reported that over-expression of CBF1 in Arabidopsis induced cold-regulated (COR) gene expression and increased the freezing tolerance of the Arabidopsis plant. Recently, it has been reported that CBF1 belongs to a small family, which includes CBF2 and CBF3 (14, 15).

The CBF1 protein sequence deduced from the DNA sequence of CBF1 is composed of 213 amino acids, and can be divided into four regions (12), the N-terminal region (amino acid residues 1-32), the potential nuclear localization signal (33-44), the AP2 domain (48-106), and the acidic region (107-213), as shown in Fig. 1a. The AP2 domain, composed of about 60 amino acid residues, is a DNA-binding motif that has been found only in plant proteins thus far (16). Ohme-Takagi and Shinshi (17) reported that the AP2 domain bound to the cis-acting ethylene-responsive element designated the GCC-repeat. The AP2 domain is present in the APETALA2 (18), AINTEGUMENTA (19, 20), TINY (21), and AtEBP (22) proteins and in the RAP2 family of proteins (23) of Arabidopsis, ethylene-responsive element-binding proteins of tobacco (17), and the Glossy15 product of maize (24). The highly conserved core region capable of forming an amphipathic $\alpha$-helix is present in the latter half of the AP2 domain.

Stockinger et al. (12) concluded that CBF1 is a transcriptional activator, based on yeast transformation experiments. The acidic region of CBF1 is thought to play the leading role in transcriptional activation. Acidic activation domains are present, for example, in the yeast transcription factors GAL4 (25) and GCN4 (26), the herpes simplex virus-1 VP16 protein (27-29), the human p53 tumor suppresser gene product (30), and the RelA(p65) subunit of the cellular transcription factor NF-kB (31, 32). It has been reported that acidic activation domains interact with the general transcription factor TFIIIB (33, 34), TFIIID (35), the TATA box-binding protein (30), and the transcriptional adaptor ADA2 (36). These results led us to expect that the acidic region of CBF1 might also directly or indirectly interact with general transcription factor(s).

In this study, we report the characterization of the CBF1 protein purified from Escherichia coli transformants. Our purpose was to examine whether various responses of the CBF1 protein occurred when the temperature was lowered from normal to low, nonfreezing temperatures. CD spectra of the intact and truncated CBF1 proteins measured at various temperatures suggested that cold denaturation (37, 38) locally occurred...
in the CBF1 protein in aqueous solution without denaturant and that thermal denaturation occurred in the AP2 domain. This local cold denaturation is thought to be a characteristic property of the CBF1 protein. We discuss the possible role of this local cold denaturation of CBF1 in the cold acclimation response.

**EXPERIMENTAL PROCEDURES**

**Cells and Plasmids—**Plasmids pET28a and pET79b, and E. coli BL21(DE3) were from Novagen, Inc. Competent cells of E. coli JM109 (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, AlacproAB, F’proD, proAB, lacIq, lacZAM15) were from Takara Shuzo Co., Ltd. Cells were grown in Luria-Bertani medium (39) containing 50 mg/liter kanamycin.

**Materials—**Restriction and DNA-modifying enzymes were from Takara Shuzo Co., Ltd. The DNA ligation kit (ligation high) and KOD DNA polymerase for the polymerase chain reaction (PCR) were from Takara Shuzo Co., Ltd. Lysyl endopeptidase (LEP), isopropyl-β-thio-galactopyranoside (IPTG), and guanidine hydrochloride (GdnHCl) were from Wako Pure Chemical Industries, Ltd. Thrombin was from Sigma. The proteins used as standards for gel filtration were from Bio-Rad. DNA oligomers were synthesized by Sawady Technology Co., Ltd. Other chemicals were of reagent grade.

**Construction of the Expression Vector—**The CBF1 gene was cloned from an A. thalaina cDNA library by PCR using the synthetic DNA oligomers 5′-GTACCTCGACATATGAACTCATTTTCAGC-3′, 5′-GAGGATCCAAATATTAGTAACTCCAAAGCGACACG-3′, and 5′-TCGGACTCTGAGAATTCCTCAAAACGGCAGAAGC-3′ as primers. Recognition sites of restriction endonucleases NdeI, BamHI, and XhoI are shown by the three underlined areas, respectively. The sequences downstream of the NdeI, BamHI, and XhoI sites of the primers are complementary to the 5′- and 3′-sequences of CBF1, respectively. Thirty cycles of PCR were performed using a Trio-Thermoblock (Biometra) apparatus with KOD DNA polymerase using the procedures recommended by the supplier. The nucleotide sequences of the truncated KOD DNA polymerase, according to the procedures recommended by the supplier, were confirmed as described above.

**Overproduction of the CBF1 Protein—**Expression of the CBF1 protein was induced in E. coli BL21 (DE3) cells harboring plasmid pETCBF1 by the addition of IPTG. Cultivation of the E. coli transformants was carried out at 30 °C. When the absorbance of the culture at 600 nm reached 0.8, 1 ml IPTG was added to the culture medium on ice, and cultivation was then continued for an additional 5 h at 25 °C. Cells were harvested by centrifugation and subjected to the purification procedures described below. The production of the CBF1 protein in cells was examined by analyzing the whole-cell extract by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (40). The solubility of CBF1 in KD DNA polymerase, according to the procedures recommended by the supplier. The nucleotide sequences of the truncated CBF1 genes were confirmed as described above.

**Purification—**All purification procedures were carried out at 4 °C. Cells from a 250-ml culture were suspended in 25 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, sonicated on ice for 2 min, and centrifuged at 15,000 rpm (27,000 × g) for 20 min. The resulting supernatants and pellets were analyzed by SDS-PAGE.

**Preparation of Crude Extracts—**The crude extracts were prepared by adding a solution containing 10 mM Tris-HCl, 0.5 M NaCl, 0.1 M Na2SO4, 5% glycerol, and 50% 2-mercaptoethanol to cells. The solution was added to the cell suspensions and incubated with gentle mixing overnight. The resulting solution was applied to a Ni2+-charged Q-Sepharose FF column (Amersham Pharmacia Biotech) and eluted by a gradient from 0 to 0.8 M NaCl in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol. The purity of the protein was analyzed by SDS-PAGE. CBF1 with a histidine tag at the N terminus was used for gel shift assay, footprinting, and fluorescence measurements, and gel filtration analyses. CBF1 with a histidine tag at the C terminus was used as the substrate for the digestion with LEP to generate LEP peptides. The N-terminal sequence of CBF1 was confirmed by Procise™ protein sequencing system from Applied Biosystems.

**Gel Shift Assays and Footprinting—**A 21-base pair duplex that was chemically synthesized and contained the sequence of CRT/DRE was mixed with the CBF1 protein in 25 μl of a binding buffer containing 20 mM Heps, pH 7.5, 30 mM NaCl, 1 mM dithiothreitol, 2 mM EDTA, and 10% glycerol, and the mixture was incubated for 20 min at room temperature and then electrophoresed on a nondenaturing 5% polyacrylamide gel at 100 V for 3 h. The bands containing DNA were visualized by ethidium bromide staining and UV illumination. DNASe I footprinting was performed using a 74-base pair synthetic double-stranded DNA fragment that contained CRT/DRE and that had been labeled with 32P by end-filling with T7 sequence from Amersham Pharmacia Biotech. The binding reaction was performed as described above in the presence of 1 μg of poly(dI-dC)poly(dI-dC) (Amersham Pharmacia Biotech). DNASe I digestion and gel analysis were carried out as described (41). The G/A sequencing ladder was generated by performing Maxam-Gilbert chemical reaction (42) using the same 74-base pair DNA fragment.

**Circular Dichroism—**CD spectra (200–300 nm) were measured on a 720 automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). Spectra were obtained using solutions containing the proteins or the peptides at 0.3 mg/ml in 20 mM sodium phosphate buffer at pH 7.9 containing 0.5 mM dithiothreitol and 10% glycerol or 20 mM sodium acetate buffer at pH 5.5. The mean residue ellipticity [θ], expressed in units of degrees·dmol−1·cm−1, was calculated using an average amino acid molecular weight of 110.

**Digestion with Lysyl Endopeptidase—**S-Carbamoylation of CBF1 was carried out as follows: iodoacetic acid (10 mg) was added to a solution (4 ml) of CBF1 (1.3 mg) in 200 mM Tris-HCl, pH 8.5, saturated with N2. The tube containing the reaction mixture was sealed and shaken at room temperature for 1 h in the dark. The resultant solution was dialyzed against a buffer containing 50 mM Tris-HCl, pH 9.0, and 10 mM NaCl at 4 °C overnight. Carbamoylated CBF1 (1.3 mg) was digested with LEP (25 μg) at 37 °C for 30 min to generate five peptides, which corresponded to the amino acid residues 1–32, 40–58, 59–68, 70–119, and 120–213, shown in Fig. 1b. These peptides were separated by reverse-phase high performance liquid chromatography using a TSK gel ODS-120T column, 4.6 × 250 mm (Tosho, Tokyo, Japan) with a 60-min linear gradient from 5 to 75% acetonitrile, containing 0.05% trifluoroacetic acid at the flow rate of 1 ml/min. Identification of the peptide in each peak was performed by comparison between the measured and theoretical mass values summarized in Table I and by N-terminal amino acid sequencing by stepwise Edman degradation. The CBF1 protein has five cysteine residues. The measured mass values of 3585.62 for LEP1 and 5609.96 for LEP4 (Table I) were larger than the theoretical mass values by 116.87 and 104.66, respectively, which correspond to the mass values of threonine and tryptophan, respectively. The results are consistent with the fact that four cysteine residues, at positions 30, 30, 100, and 117, were carbamoylated. The mass spectrum of LEP5 could not be determined. However, a peak from a carbamoylated cysteine residue was detected at peak 14 of the Edman degradation of LEP5, indicating that the cysteine residue at position 137 was also carbamoylated. Based on these results, we concluded that the five

### Table I

| LEP peptide | Measured mass | Theoretical mass* | Location |
|-------------|---------------|------------------|----------|
| LEP1        | 3585.62       | 3488.75          | 1–32     |
| LEP2        | 3534.76       | 2307.64          | 40–58    |
| LEP3        | 1307.52       | 1371.52          | 59–69    |
| LEP3        | 1224.43       | 1243.35          | 59–68    |
| LEP4        | 5609.96       | 5505.30          | 70–119   |
| LEP5        | 10433.02      | 120–213          |          |

*Theoretical mass was calculated based on the amino acid sequence of the CBF1 protein reported by Stockinger et al. (12).

**Mass spectrum of LEP5 could not be determined.**
cysteine residues in CBF1 were in the reduced form and were carboxymethylated with iodoacetic acid.

**Gel Filtration Chromatography of CBF1**—The gel filtration of the intact or truncated CBF1 protein was performed using Superdex 75 PC 3.2/30 in a SMART system from Amersham Pharmacia Biotech with a buffer containing 20 mM sodium phosphate, pH 7.9, 0.5 mM dithiothreitol, and 100 mM NaCl as the eluent with a flow rate of 30 μl/min at 4 or 25 °C.

**Protein and Peptide Concentrations**—The protein concentration of CBF1 with a tag was determined from the UV absorption at 280 nm. The \( A_{280} \) value of 1.43 for CBF1(1–213) with a molecular weight of 25,914 was calculated by using 1.576 × \( 10^{-3} \) cm\(^2\)/mg for tyrosine (7) and 5.225 × \( 10^{-3} \) cm\(^2\)/mg for tryptophan (5) at 280 nm (43). \( A_{280} \) values of 1.57, 1.42, and 1.44 for CBF1(41–213), CBF1(41–157), and CBF1(41–146), respectively, were also calculated as described for CBF1(1–213). The concentrations of peptides were determined using the following formula: Concentration (μg/ml) = (\( A_{280} \) – \( A_{232} \)) × 210 (44).

**Secondary Structure Prediction**—The secondary structure of CBF1 was predicted using a neural network system that was offered as a service on the World Wide Web, the Predict Protein Server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html). The program was provided by Rost and Sander (45).

**Analytical Ultracentrifugation**—Analytical ultracentrifugation experiments were carried out in an Optima XL-A analytical ultracentrifuge (Beckman Instruments Inc.). In a velocity sedimentation experiments, CBF1 (0.12 mg/ml) in a buffer containing 100 mM NaCl, 20 mM phosphate buffer, pH 7.8, and 0.5 mM dithiothreitol was centrifuged at 50,000 rpm and 25 or 3 °C, and radial scans at 230 nm were taken at 5-min intervals for 4 h. The sedimentation coefficient, \( s_w \), and the diffusion coefficient, \( D_w \), were calculated by the second moment method with the Optima XL-A data analysis software supplied by Beckman. The values from the data at 25 and 3 °C were corrected to the standard conditions (water, 20 °C) to obtain the \( s_{20,w} \) values (46, 47). They are designated as \( s_{20,w} \) and \( s_{3,w} \), respectively. The value for the diffusion coefficient was corrected to standard conditions (water, 20 °C) to obtain the \( D_{20,w} \) values. The frictional coefficient, \( f_w \), of CBF1 was obtained by the Svedberg equation (47). The value of the partial specific volume, \( \tilde{v} \), of CBF1 was estimated from its amino acid composition (47) as 0.718 ml g\(^{-1}\). The degree of hydration, \( \delta \), was estimated using the method of Kuntz based on the amino acid composition (47). The frictional coefficient of the hydrated sphere, \( f_{\text{hydr}} \), was calculated by the equation from the Stokes-Einstein relationship (47). The hydrodynamic parameters were calculated according to the flow diagram by Waxman et al. (48).

**RESULTS**

**Overproduction and Purification**—The expression of the CBF1 gene in the plasmid pETCBF1 was induced in *E. coli* by the addition of IPTG. The cultivation of the transformants was performed at 25 or 37 °C to examine the dependence of the solubility of CBF1 in cells on the temperature. The production level of CBF1 was estimated to be ~4 mg/liter of culture at 25 °C and ~8 mg/liter of culture at 37 °C, from the intensities of Coomassie Brilliant Blue-stained bands in SDS-polyacrylamide gels. The corresponding bands in SDS-polyacrylamide gels of the supernatants and pellets obtained after centrifugation of the suspension of sonicated cells showed that about 80% of the protein was soluble when the cultivation was carried out at 25 °C, whereas about 90% of the protein was insoluble at 37 °C (data not shown). Based on these results, the cultivation of *E. coli* transformants was carried out at 25 °C after the addition of IPTG in order to obtain the CBF1 protein in soluble form.

The CBF1 protein was efficiently and selectively trapped by the chelating column when a histidine tag was attached to the N or C terminus of CBF1. Elution with 300 mM imidazole solution yielded 3.3 mg of protein product containing CBF1 with 80% purity from 1 liter of culture. Subsequent ion exchange chromatography using Q-Sepharose FF yielded homogeneous CBF1, which appeared as a single band in SDS-PAGE analysis (data not shown). The final yield was 1.7 mg of the pure CBF1 protein from 1 liter of culture.

**Construction of Truncated Mutants**—The CBF1 protein is composed of four regions, that is, the N-terminal region (1–32), the potential nuclear localization signal (NLS), the AP2 domain, and the acidic region of the intact and truncated CBF1 proteins CBF1(1–213), CBF1(41–213), CBF1(41–157), and CBF1(41–146). Numbers indicate amino acid residues. α, schematic drawing showing locations of two α-helices and six β-sheets in the secondary structure. The solid and gray regions correspond to the α-helices and β-sheets, respectively. d, schematic drawing showing locations of the regions estimated or suggested to exhibit cold denaturation and thermal denaturation by CD spectra of the intact and truncated CBF1 proteins.

**DNA Binding of the Intact and Truncated CBF1 Proteins**—The binding ability of the purified CBF1 protein to CRT/DRE was analyzed by gel-shift assays, DNase I footprinting, and measurement of the intrinsic fluorescence. Gel-shift experiments were carried out to show that the CBF1 proteins bound to CRT/DRE (data not shown). No shift was detected without CBF1. Mobility shifts resulting from complex formation between CRT/DRE and the CBF1 proteins were observed in the assays. The migration of the complex-containing band became faster with increasing truncation of the CBF1 protein.

DNase I footprinting was performed to study whether the purified CBF1 protein was able to bind specifically to CRT/DRE. A 75-base pair synthetic oligonucleotide probe containing CRT/DRE was digested with DNase I in the presence or absence of CBF1(1–213) and analyzed on a 12% sequencing gel. As shown in Fig. 2, regions on both strands surrounding CRT/
DRE were protected by CBF1(1–213) binding. On the bottom strand, 11 nucleotide residues including GTCGGC, were protected, whereas on the top strand, 20 nucleotide residues, including GCCGAC, were protected.

The intrinsic fluorescence spectra were measured to determine the binding constants between the intact and truncated CBF1 proteins and CRT/DRE. The intensity of the fluorescence peak at 338 nm was decreased to about 60% of the maximum level by the addition of a double-stranded synthetic DNA fragment containing CRT/DRE (data not shown). Titration of CBF1 with a double-stranded CRT/DRE-containing oligomer caused changes in the fluorescence anisotropy of CBF1 (Fig. 3). The binding curves (Fig. 3) showed a stoichiometry of one protein bound per CRT/DRE either at 30 °C or at 1 °C. The apparent binding constant of CBF1(1–213) was (4.6 ± 0.9) x 10^7 M^-1 at 1 °C, (4.8 ± 0.9) x 10^7 M^-1 at 10 °C, (6.2 ± 1.4) x 10^7 M^-1 at 20 °C, and (6.4 ± 0.7) x 10^7 M^-1 at 30 °C. The apparent binding constants of the intact protein, CBF1(1–213) and the three truncated proteins, CBF1(41–213), CBF1(41–157), and CBF1(41–146), are summarized in Table II. The apparent binding constant of CBF1(41–213), lacking 40 amino acid residues in the N-terminal region, was (1.8 ± 0.4) x 10^8 M^-1, suggesting that the N-terminal region is not necessary for the binding of CBF1 to CRT/DRE. The possible protein-protein interactions caused by the N-terminal region might reduce the apparent binding constant of the intact CBF1 protein. Two mutants, CBF1(41–157) and CBF1(41–146), lack 56 and 67 amino acid residues, respectively, in the acidic region. CBF1(41–146) bound to CRT/DRE less tightly than did CBF1(41–213), with a binding constant of (4.0 ± 0.8) x 10^7 M^-1 (Table II).

Secondary Structures of the LEP-Peptides—Stockinger et al. (12) reported that CBF1 had nine lysine residues at amino acid positions 32, 33, 38, 39, 58, 68, 69, 119, and 123. In order to study the structures of the peptides from CBF1, the CBF1 protein was digested with LEP. Prior to the digestion with LEP, the cysteine residues in the CBF1 protein were S-carboxymethylated, as described under “Experimental Procedures,” to avoid the formation of non-native disulfide bonds during the protease digestion. Carboxymethyl-CBF1 was then digested with LEP to generate five peptides, LEP1, LEP2, LEP3, LEP4, and LEP5, shown in Fig. 1b. The measurement of the far-UV CD spectra of the peptides LEP1, LEP2, LEP3, LEP4, and LEP5 from CBF1 in aqueous solution was carried out at intervals of 10 °C by raising and lowering the temperature in the range between 2 and 60 °C. Fig. 4, a–e, shows the far-UV CD spectra of the peptides at 60 °C and those in 3 M GdnHCl. Fig. 4f shows the temperature dependence of the [θ] value of each peptide at 220 nm. The CD spectra of LEP4,
which included the latter half of the AP2 domain, were indicative of $\alpha$-helicity (Fig. 4d). Its $\theta$ value, $-4300$ at 220 nm at 60 °C, was the lowest among those of the five peptides, and it was decreased to $-6400$ by lowering the temperature to 2 °C (Fig. 4, d and f). The helical content of LEP4 was about 9% at 40–60 °C, and it was increased to about 17% by lowering the temperature to 2 °C. The difference between the $\theta$ value at 220 nm in the spectrum of LEP4 at 60 °C and that in 3 M GdnHCl (Fig. 4d) suggested that LEP4 still had a considerable amount of thermally stable secondary structure at 60 °C but was almost completely denatured in 3 M GdnHCl.

In contrast, the CD spectra of the other four peptides, LEP1, LEP2, LEP3, and LEP5, were indicative of random coil, and the negative molar ellipticity at 220 nm at 60 °C was lower than that at 2 °C (Fig. 4, a–e). The molar ellipticity for the four peptides at 220 nm increased as the temperature was decreased from 60 to 2 °C (Fig. 4f). The spectrum of LEP1 at 2 °C was very similar to that of the same peptide denatured with 3 M GdnHCl (Fig. 4a). The similarity between the spectrum of the peptide at 2 °C and that of the peptide denatured in 3 M GdnHCl solution, are shown in Fig. 5, a–d, respectively. The helical content of the protein, which was calculated by the method of Wu et al. (49), increased with increasing truncation of both the N-terminal and acidic regions in the following order: CBF1(1–213) (13.8%), CBF1(41–213) (15.5%), CBF1(41–157) (22.3%), and CBF1(41–146) (23.0%). The temperature dependence of the $\theta$ value at 220 nm for the proteins is shown in Fig. 5, e–h. The negative molar ellipticity at 220 nm was minimal (−5600) at 30 °C, increased by about 500 (to −5090) as the temperature was decreased from 30 to −5 °C, and also increased by about 1300 (to −4300) as the temperature was raised from 30 to 70 °C (Fig. 5c). These results strongly suggest that reversible cold denaturation of the secondary structure occurred in the temperature range between −5 and 70 °C by lowering the temperature from 30 to −5 °C, raising it from −5 to 70 °C, and again lowering it from 70 to 2 °C, in steps of 10 °C (Fig. 5). The spectra of the intact CBF1 protein, (1–213) and of the truncated proteins, (41–213), (41–157), and (41–146), in the solution at pH 7.9 containing 10% glycerol at −5, 30, and 70 °C, and those in 3 M GdnHCl solution, are shown in Fig. 5, (a–d). The helical content of the protein, which was calculated by the method of Wu et al. (49), increased with increasing truncation of both the N-terminal and acidic regions in the following order: CBF1(1–213) (13.8%) < CBF1(41–213) (15.5%) < CBF1(41–157) (22.3%) < CBF1(41–146) (23.0%). The temperature dependence of the $\theta$ value at 220 nm for the proteins is shown in Fig. 5, e–h. The negative molar ellipticity at 220 nm was minimal (−5600) at 30 °C, increased by about 500 (to −5090) as the temperature was decreased from 30 to −5 °C, and also increased by about 1300 (to −4300) as the temperature was raised from 30 to 70 °C (Fig. 5c). Similar temperature dependence of the ellipticity was observed in the spectra of CBF1(41–213) and CBF1(41–157) (Fig. 5, b, c, f, and g). These results strongly suggest that reversible cold denaturation of the secondary structure occurred in the temperature range between 30 and −5 °C and that thermal denaturation also occurred in the range between 40 and 60 °C in CBF1(1–213), CBF1(41–213), and CBF1(41–157). The ratio of the change in the ellipticity at 220 nm resulting from possible cold denaturation to
that resulting from thermal denaturation was 500/1300 for CBF1(1–213), 500/1600 for CBF1(41–213), and 400/2700 for CBF1(41–157) (Fig. 5, e–g). The ratio decreased with increasing truncation of the protein in the following order: CBF1(1–213) > CBF1(41–213) > CBF1(41–157). An increase of the ellipticity as the temperature was decreased from 30 to −5 °C was not observed in CBF1(41–146), as shown in Fig. 5, d and h, suggesting that the region exhibiting the cold denaturation was removed from the protein by the truncation. The increase of the ellipticity between 40 and 60 °C was still observed in the spectra of CBF1(41–146) (Fig. 5, e and g), suggesting that the region exhibiting the cold denaturation remained despite the truncation. The change of ellipticity was reversible in the temperature range between −5 and 30 °C in the spectra of all CBF1 proteins (Fig. 5, e–h). These results strongly suggest that the region exhibiting the possible cold denaturation was diminished or removed by the truncation of the protein, whereas the region exhibiting the thermal denaturation remained despite the truncation. The difference for CBF1(41–146) was smallest and the difference between the retention volume for each protein at 4 °C and that at 25 °C decreased with increasing truncation of the protein. The difference for CBF1(41–146) was smallest and was almost the same as that for the standard proteins. The apparent molecular weights for the proteins are as follows: 38,900 at 4 °C and 33,100 at 25 °C for CBF1(1–213), 29,500 at 4 °C and 25,700 at 25 °C for CBF1(41–213), 15,800 at 4 °C and 14,500 at 25 °C for CBF1(41–157), and 9800 at 4 °C and 9800 for 25 °C for CBF1(41–146). These results suggest that extension of the CBF1 molecule was caused by cold denaturation. The molecule was extended by 18, 15, 3, and 0% for CBF1(1–213), CBF1(41–213), CBF1(41–157), and CBF1(41–146), respectively.

Secondary Structure Prediction of CBF1—The predicted secondary structure of CBF1 is summarized in Fig. 1c. The analysis suggested that residues 4–10 and 79–94 had the propensity to form α-helices, whereas residues 60–64, 70–75, 99–101, 136–140, 153–156, and 208–212 had the propensity to form β-sheet structures. The prediction of the formation of the α-helix designated as helix 2 for residues 79–94 (Fig. 1c) agreed with the conclusions about the secondary structure of CBF1 from the CD spectroscopy.

Gel Filtration of the Intact and Truncated CBF1 Proteins at 4 and 25 °C—Gel filtration of CBF1(1–213), CBF1(41–213), CBF1(41–157), and CBF1(41–146) was performed at 4 and 25 °C to examine the change in the molecular shape and volume of the CBF1 protein caused by lowering the temperature. The values of the relative retention volumes (Ve/Vo) for each protein at 4 and 25 °C are shown in Fig. 6. The retention volume for each protein was smaller at 4 °C than at 25 °C. The difference between the retention volume for each protein at 4 °C and that at 25 °C decreased with increasing truncation of the protein. The difference for CBF1(41–146) was smallest and was almost the same as that for the standard proteins. The apparent molecular weights for the proteins are as follows: 38,900 at 4 °C and 33,100 at 25 °C for CBF1(1–213), 29,500 at 4 °C and 25,700 at 25 °C for CBF1(41–213), 15,800 at 4 °C and 14,500 at 25 °C for CBF1(41–157), and 9800 at 4 °C and 9800 for 25 °C for CBF1(41–146). These results suggest that extension of the CBF1 molecule was caused by cold denaturation. The molecule was extended by 18, 15, 3, and 0% for CBF1(1–213), CBF1(41–213), CBF1(41–157), and CBF1(41–146), respectively.

Ultracentrifugation Studies—Sedimentation velocity experiments were carried out to determine the sedimentation and diffusion coefficients, $s_{20,w}$ and $D_{20,w}$, respectively. The sedimentation process of a 50,000 rpm centrifugation of CBF1 at 25
or 3 °C was analyzed by following the movement of the boundary. Table III summarizes the results from the sedimentation velocity analysis. The corrected sedimentation coefficient, $s_{20, w}$, obtained from the data at 25 °C was $(1.71 \pm 0.06) \times 10^{-13}$ s, and that obtained from the data at 3 °C $(s_{20, b})$ was $(1.42 \pm 0.07) \times 10^{-13}$ s (Table III). This sedimentation coefficient change reflects the conformational transition of the CBF1 protein caused by lowering the temperature from 25 to 3 °C. The increase of the friction ratio $f/s_{20, w}$ from 1.52 ± 0.05 to 1.83 ± 0.09 caused by lowering the temperature from 25 to 3 °C suggests a change of the axial ratio of the molecule. The decrease of the diffusion coefficient $D_{20, w}$ caused by lowering the temperature (Table III) may be correlated with the conformational change of CBF1 caused by lowering the temperature.

**DISCUSSION**

Possible local cold denaturation suggested by the CD measurement and gel filtration analyses is thought to be a characteristic property of the CBF1 protein. The regions that caused cold denaturation suggested by the CD spectra of the intact and truncated CBF1 proteins were present in residues 1–40 and 147–213, as shown in Fig. 1d. The retention times of the intact and truncated CBF1 proteins in gel filtration (Fig. 6) and the results from the sedimentation velocity analysis suggested the following: (i) the intact and truncated CBF1 proteins were monomeric; (ii) the shape of CBF1(1–213) was not spherical, but rather extended, whereas that of CBF1(41–146) was likely to be spherical; (iii) the volume of a molecule of CBF1(1–213) at 4 °C was larger than that at 25 °C; and (iv) the difference between the molecular volume at 4 °C and that at 25 °C decreased with increasing truncation of the protein, suggesting that the local cold denaturation was accompanied by the extension of the protein molecule.

CBF1 could bind to CRT/DRE both at normal and at near-zero temperatures in vitro (Fig. 3). However, CBF1 in Arabidopsis did not activate the transcription of the COR gene at normal temperatures (12). It seems to be necessary for CBF1 bound to CRT/DRE to be transformed from a repressor to an activator via the reduction of temperature in Arabidopsis. Thus, we propose two possible models to explain the transcriptional activation involving CBF1. The first model is that the local cold denaturation of CBF1 caused by the low temperature initiates the transcriptional activation of the COR gene, because extension of the molecule resulting from the cold denaturation facilitates interaction with the factor(s) that activate or initiate transcription of the genes. The second model is that other cofactor(s) associate with CBF1 for repression of the transcription at normal temperatures, but these factors are released from the locally denatured CBF1 protein, which results in the transcriptional activation, at the low temperature. Jago-Otto et al. (13) reported that CBF1 overexpression induced COR gene expression without a low temperature stimulus, probably suggesting the shortage of some repressor to suppress the transcription. This result seems to support the second model.

The content of secondary structure in the peptides LEP1, LEP2, LEP3, and LEP5 was largest at 60 °C, in the temperature range between 2 and 60 °C, as shown in Fig. 4. The intact and truncated CBF1 proteins also still had considerable amounts of secondary structure at 70 °C, after thermal denaturation between 40 and 60 °C as shown in Fig. 5, a–f. The residual structure was thermally stable but was denatured in 3 M GdnHCl, suggesting that it was maintained by hydrophobic interactions (50).

Cold denaturation is thought to be caused by the interaction between water and nonpolar groups exposed to the solvent (51). The nonpolar groups are usually buried in a protein molecule by hydrophobic interactions at normal temperatures. As the temperature is decreased, the hydrophobic forces are reduced, and the resultant disruption of the hydrophobic interactions results in the denaturation of the structure at low temperatures. Cold denaturation has been observed in multimeric enzymes, such as ATPases and fatty-acid synthetases, as well as in proteins under destabilizing conditions, such as moderate

**TABLE III**

| Parameter | Value |
|-----------|-------|
| $M_r$     | 24,895 |
| $R_b$     | 2.00 $\times 10^{-7}$ cm |
| $V_r$     | 3.20 $\times 10^{-20}$ ml |
| $\bar{v}$ | 0.718 ml/g |
| $\delta$  | 0.43 |
| $V_m$     | 5.10 $\times 10^{-20}$ ml |
| $R_{sphere}$ | 2.30 $\times 10^{-7}$ cm |
| $f_{sphere}$ | 4.40 $\times 10^{-6}$ g/s |
| $s_{25,b} \rightarrow 20,w$ | (1.71 ± 0.06) $\times 10^{-13}$ s |
| $s_{25,b} \rightarrow 20,w$ | (1.42 ± 0.07) $\times 10^{-13}$ s |
| $f_{25,b} \rightarrow 20,w$ | (6.86 ± 0.23) $\times 10^{-6}$ g/s |
| $D_{25,b} \rightarrow 20,w$ | (0.04 ± 0.40) $\times 10^{-9}$ g/s |
| $D_{25,b} \rightarrow 20,w$ | (1.52 ± 0.05) |

* $M_r$ Molecular weight from the components of the CBF1 protein with a His tag at the C terminus.
* Radius of an anhydrous sphere with the same $M_r$ as CBF1 estimated from 6.723 $\times 10^{-9}$ M.
* Volume of the hydrated sphere.
* Volume of the anhydrous sphere.
* Radius of the hydrated sphere.
denaturant concentrations, low pH, or high pressure (51), and in proteins destabilized by site-directed mutagenesis (52), as well as in the artificially designed peptides (53). The possible cold denaturation of CBF1 suggested by the CD spectra, gel filtration, and ultracentrifuge analyses is thought to be a rare case, in which a monomeric protein with a natural sequence possibly exhibits the cold denaturation under physiological conditions. It seems likely that this cold denaturation of CBF1 leads to initiation of the transcriptional activation, because cold denaturation is thought to be a direct physical response of the protein to the low temperature stimulus, and it seems reasonable that the plant might utilize the physical properties of its constituents to respond to this environmental change. Some physiological and biochemical properties conferring tolerance to cold temperatures have been reported, such as an increase of the degree of unsaturation of fatty acids in the membrane, and the accumulation of osmolytes. Murata et al. (54) reported that the sensitivity of plants to cold stimuli was correlated with the degree of unsaturation of fatty acids of the membrane and that the tolerance was improved by introducing an appropriate glycerol-3-phosphate acetyltransferase. Xin and Browse (55) reported a mutant of Arabidopsis with constitutive freezing tolerance conferred by the accumulation of proline.

The CD spectra of the intact CBF1 protein seemed to show that the secondary structure content of CBF1 was relatively low (Fig. 5a). Secondary structure prediction suggested that there were only two a-helices, one in the N-terminal region and one in the AP2 domain (Fig. 1c, helix1 and helix2). The formation of helix2 in the AP2 domain was confirmed by the CD spectrum of the peptide LEP4 (Fig. 4d). However, the CD spectra of the LEP peptides other than LEP4 were mainly consistent with random coil. In general, the structure of a peptide does not completely match the structure of the corresponding region in the protein, because of different environments or neighborhoods, but the structure of the peptide does reflect a tendency to form a particular structure based on a particular amino acid sequence. The CD spectra of the LEP peptides (Fig. 4) suggest that the peptide sequences in both the N-terminal and the acidic regions have a tendency to cause cold denaturation and that the CBF1 protein has at least one a-helix in the AP2 domain, which functions in DNA binding. The calculated helical content of the protein, based on the CD spectra in Fig. 5, a–d, increased with increasing truncation of both the N-terminal and acidic regions, possibly suggesting that the α-helix could form in the AP2 domain despite the truncation. It is very likely that the thermal denaturation between 40 and 60 °C, shown in Fig. 5, e–h, is due to the thermal destabilization of the AP2 domain. In addition, the spectra shown in Fig. 5d and the thermal denaturation plot shown in Fig. 5h suggest that the region around the AP2 domain does not participate in the cold denaturation. The fact that the CBF1 protein could bind to CRT/DRE with some stoichiometry at 1 °C and at 30 °C in vitro (Fig. 3) is consistent with the fact that the structure of the region around the AP2 domain was stable in the temperature range between −5 and 30 °C, as shown in Fig. 5, d and h. The amphipathic α-helix (Fig. 1c, helix2), which was previously predicted in the AP2 domain (16), is thought to be a core structure of the CBF1 protein.

On the other hand, the formation of helix1 that was predicted as shown in Fig. 1c was not clearly demonstrated. The plot of the [θ] values at 220 nm as a function of temperature showed that the values for LEP1 were lower than those for LEP2, LEP3, and LEP5 by about 1000, and higher than those for LEP4 (Fig. 4f). The spectrum of LEP1 at 60 °C (Fig. 4a) exhibited a slight hump around 220 nm. These results might suggest that LEP1, which corresponds to the amino acid residues 1–32 in CBF1, included a small amount of helicity (corresponding to helix1 in Fig. 1c). Limited proteolysis of the CBF1-CRT/DRE complex with trypsin selectively released the N-terminal fragment 1–41 (data not shown). The binding ability of CBF1 was improved by the removal of the region 1–40, as summarized in Table II. These results suggest that the region from positions 1–40 is not necessary for the binding of CBF1 to CRT/DRE. On the other hand, the results of the present study and its location close to the nuclear localization signal (Fig. 1z) might suggest some function for the N-terminal region. Finally, the creation of transgenic Arabidopsis plants that overexpress the intact or truncated CBF1 will be helpful for determining the molecular components of the cold stress-responsive pathway involving CBF1.

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