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Purification, Characterization, and Structural Analysis of a Plant Low-Temperature-Induced Protein[1]

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We have purified to near homogeneity a recombinant form of the protein BN28 (rBN28), expressed in response to low temperature in *Brassica napus* plants, and we have determined its solution structure. Antibodies raised against rBN28 were used to characterize the recombinant and native proteins. Similar to many other low-temperature-induced proteins, BN28 is extremely hydrophilic, such that it remains soluble following boiling. Immunoblot analysis of subcellular fractions indicated that BN28 was not strongly associated with cellular membranes and was localized exclusively within the soluble fraction of the cell. Contrary to predicted secondary structure that suggested significant helical content, circular dichroism analysis revealed that rBN28 existed in aqueous solution largely as a random coil. However, the helical propensity of the protein could be demonstrated in the presence of trifluoroethanol. Nuclear magnetic resonance analysis further showed that rBN28 was in fact completely unstructured (100% coil) in aqueous solution. Although it had earlier been speculated that BN28-like proteins from *Arabidopsis thaliana* might possess antifreeze protein activity (S. Kurkela and M. Franck [1990] Plant Mol Biol 15: 137–144), no such activity could be detected in ice recrystallization assays with rBN28.

The phenomena of cold acclimation and freezing tolerance in plants have been reviewed extensively (Steponkus, 1984; Singh and Laroche, 1988; Guy, 1990). Among the many morphological and biochemical changes that occur with low-temperature acclimation is the synthesis of a number of new proteins (Mezza-Basso et al., 1986; Guy and Haskell, 1987; Gilmour et al., 1988). Recently, genes encoding low-temperature-induced proteins have been isolated from a variety of different species (Cattivelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Monroy et al., 1993; Neven et al., 1993; Weretilnyk et al., 1993). Most of the work to date has focused on characterizing the regulation and expression of these genes (Baker et al., 1994; Dunn et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Monroy and Dhindsa, 1995; Wang et al., 1995). In some studies antibodies have been used to examine the expression (Lin and Thomashow, 1992a; Neven et al., 1993; Kazuoka and Oeda, 1994; Boothe et al., 1995; Houde et al., 1995) and to determine the cytological location (Lin and Thomashow, 1992a; Neven et al., 1993; Houde et al., 1995) of low-temperature-induced proteins. In only a few cases have low-temperature-induced proteins been purified (Kazuoka and Oeda, 1994; Houde et al., 1995; Gilmour et al., 1996) and no detailed structural analyses of these proteins have as yet been reported to our knowledge.

A common feature among many low-temperature-induced proteins is their tendency to remain soluble following boiling (Lin et al., 1990; Lin and Thomashow, 1992a; Neven et al., 1993). This property is a reflection of the fact that these proteins are extremely hydrophilic, which is also shared by many dehydration-induced proteins. In fact, several of these proteins are induced in response to both low temperature and dehydration. Some low-temperature-induced proteins also show sequence homology with the group 2 Lea proteins in that they possess repeated stretches of residues rich in Lys (Gilmour et al., 1992; Neven et al., 1993; Kazuoka and Oeda, 1994; Houde et al., 1995). As with other members of the Lea family, the proteins in group 2 are highly hydrophilic and are expressed late in embryogenesis, during the period of seed desiccation (Dure et al., 1989). This common response to both low temperature and dehydration is probably due to the fact that cellular dehydration occurs as a result of extracellular ice formation and hence represents a significant component of freezing stress (Steponkus, 1984). Thus, similarities in the conditions under which Lea, dehydration-induced, and low-temperature-induced proteins are expressed, together with their hydrophilic character, may underlie a common function for at least a subset of these proteins, possibly in ameliorating the injurious effects of cellular dehydration.

Various forms of cryoprotection have been reported for low-temperature-induced proteins from plants. It has been demonstrated that protein fractions isolated from cold-acclimated leaves of cabbage are able to protect isolated thylakoid membranes from freeze-thaw-induced damage by decreasing their permeability to solutes during freezing

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Abbreviations: AFP, antifreeze protein; CD, circular dichroism; Lea, late embryogenesis abundant; MBP, maltose-binding protein; ppm, parts per million; TFE, trifluoroethanol.

and increasing their ability to expand upon thawing (Hincha et al., 1990). Other studies (Lin and Thomashow, 1992b; Kazuoka and Oeda, 1994; Houde et al., 1995) have shown that certain boiling-soluble proteins afford protection to lactate dehydrogenase against freeze-induced inactivation in vitro. However, the precise mechanism through which this protection was achieved is unknown. AFP-like activity has also been detected in plants. Proteins isolated from the apoplast of acclimated leaves of winter rye (Hon et al., 1994, 1995) exhibit AFP-like activity, causing a noncolligative depression of the freezing point and inhibition of ice recrystallization in in vitro assays. The presence of these proteins in the apoplastic space may slow or prevent the formation of extracellular ice and thus also prevent the accompanying cellular dehydration.

Purification and structural analysis are often crucial to elucidating the function of a protein and understanding its mechanism of action. Previously, we reported the expression of BN28, a low-temperature-induced protein in *Brassica napus*, and determined its concentration in vivo with respect to total protein. BN28 belongs to a small family of low-temperature-induced proteins so far only reported in *Brassica* spp. (Orr et al., 1992) and Arabidopsis (Kurkela and Franck, 1990; Kurkela and Borg-Franck, 1992; Gilmour et al., 1992). Based on similarity in amino acid composition and predicted secondary structure to the type I AFP from winter flounder, it had been suggested that these proteins may possess AFP activity. In the present study we purified a recombinant form of BN28 to near homogeneity, determined its structure in solution, and used antibodies to examine the subcellular fractionation of the native protein. We also used ice recrystallization inhibition assays to test for possible AFP activity associated with rBN28.

MATERIALS AND METHODS

Brassica napus cv Cascade plants were grown in controlled-environment chambers programmed for a photoperiod and temperature regime of 16 h of light at 22°C and 8 h of dark at 14°C. For low-temperature treatment, plants were transferred to a 4°C chamber set for the same photoperiod for 14 to 28 d. Protein samples were obtained from the fourth (youngest) leaf collected from either control or low-temperature-treated plants.

Sequence Analysis

Predictions of protein secondary structure were based on the deduced amino acid sequence for BN28 using the algorithms of Chou and Fasman (1978) and Garnier et al. (1978). Hydrophilicity was predicted using the algorithm of Kyte and Doolittle (1987).

Subcloning and Expression of *bn28*

The *bn28* cDNA in pUC 119 (Orr et al., 1992) was modified to remove the 5′ untranslated region and the codon for the N-terminal Met. The original clone was infected with helper phage M13KO7 and single-stranded DNA was prepared using standard protocols (Sambrook et al., 1989). A primer specific for the 5′ end of the *bn28*-coding sequence but lacking the codon for the N-terminal Met was used to prepare double-stranded DNA. The modified sequence was subcloned into the bacterial expression vector pMAL-c (New England Biolabs) as an in-frame, C-terminal translational fusion to the coding sequence for the bacterial MBP. This vector is under the control of the synthetic *tac* promoter and also contains the sequence for a factor Xa protease recognition site situated at the 3′ end of the MBP-coding region to enable cleavage of the resulting fusion protein.

Transformed *Escherichia coli* containing the recombinant fusion protein plasmid was grown in Luria Bertani medium at 37°C to an A_{600} of 0.5. Expression of the fusion protein was induced with the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.3 mM and incubation continued for a further 3 h at 37°C. Cells were subsequently harvested through centrifugation at 6000*g* for 20 min and washed with ice-cold 50 mM Tris-HCl, pH 8.0.

Purification of Recombinant BN28

Induced *E. coli* were resuspended in 10 mL of ice-cold 50 mM Tris-HCl, pH 8.0, per liter of original culture and disrupted through sonication. The suspension was then centrifuged at 10,000*g* for 20 min and the supernatant containing soluble protein was recovered. This crude soluble protein fraction was then applied in-batch to 1 to 2 volumes of a slurry of DEAE-cellulose previously equilibrated with 50 mM Tris-HCl, pH 8.0. The slurry was drained through filtration and washed thoroughly with the equilibration buffer to remove any unbound protein and then completely drained again through vacuum filtration. An equal volume of 50 mM Tris-HCl, pH 8.0, containing factor Xa (New England Biolabs) at a concentration of 0.2 unit mg^{-1} of crude soluble protein was added and the slurry was incubated for approximately 12 h at 4°C. Proteolytic digestion of the fusion protein resulted in the release of rBN28, which was not bound by the DEAE-cellulose at pH 8.0. All other proteins, including factor Xa, remained bound to the resin under these conditions. Following cleavage the DEAE-cellulose was drained through vacuum filtration and purified rBN28 was recovered in the eluate. The purity of the eluted fraction was assessed by silver-staining following electrophoresis on Tricine-SDS gels.

Antibody Production

To increase its antigenicity, the recombinant protein was conjugated to a larger carrier protein prior to its use in antibody production. Purified rBN28 was mixed with an equal mass of keyhole limpet hemocyanin in PBS and the two proteins were cross-linked with the addition of an equal volume of 0.2% glutaraldehyde. Following incubation for approximately 12 h at room temperature, the glutaraldehyde was removed by passage through a PD-10 column (Pharmacia) and pre-equilibrated with PBS. Polyclonal antibodies to the cross-linked protein conjugate were

raised in rabbits using established procedures (Harlowe and Lane, 1988).

Protein Extraction

Extracts of both soluble and SDS-soluble proteins were prepared from leaves and *E. coli*. Leaves were frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. For extraction of soluble proteins, 5 volumes of 50 mM Tris-HCl, pH 8.0, 1 mM PMSF were added to the frozen powder and grinding continued until the tissue was thoroughly homogenized. Following extraction the samples were centrifuged at 10,000*g* to remove insoluble debris. If the samples were not to be used immediately for electrophoresis or for the preparation of boiling-soluble proteins (as described below), SDS was added to a final concentration of 2% (w/v) and the samples were heated in a boiling water bath for 5 min prior to storage at −20°C. SDS-soluble proteins were extracted from leaves in a similar manner, except that SDS (2%, w/v) was added to the tissue extraction buffer. After centrifugation to remove insoluble debris, these samples were heated in a boiling water bath for 5 min and then stored at −20°C until required.

Aliquots of *E. coli* cells grown to an A_{600} of at least 0.5 were pelleted through centrifugation and the supernatant was discarded. For soluble protein extraction, cells were resuspended in 50 mM Tris-HCl, pH 8.0, 1 mM PMSF and sonicated until the suspension became viscous. The samples were then centrifuged at 10,000*g* to remove insoluble debris and either used directly or made up to 2% (w/v) SDS and heated in a boiling water bath as described above for leaf proteins. SDS-soluble proteins were prepared by adding extraction buffer containing 2% (w/v) SDS to cell pellets and heating in a boiling water bath for 5 min before centrifuging and storing the samples at −20°C.

Preparation of Boiling-Soluble Protein

Soluble proteins prepared as described above from either leaves or *E. coli* were heated in a boiling water bath for 10 min and then incubated on ice for a further 15 min. Denatured, aggregated proteins were removed by centrifugation at 10,000*g* for 20 min. The boiling-soluble proteins were recovered in the supernatant and stored at −20°C until required.

Subcellular Fractionation

Leaves from low-temperature-treated plants were ground on ice with a mortar and pestle in 50 mM Tris-HCl, pH 8.0, 1 mM PMSF containing either 50 mM NaCl (low salt) or 500 mM NaCl (high salt). The extracts were centrifuged at 9,000*g* for 20 min and the pellets saved. An aliquot of the supernatant was removed and saved to represent the 9,000*g* fraction and the remainder was recentrifuged at 100,000*g* for 1 h. The supernatant from this centrifugation was saved as the 100,000*g* fraction. The pellets from both centrifugations were solubilized in 50 mM Tris-HCl, pH 8.0, 1 mM PMSF, 2% (w/v) SDS. Similarly, both the 9,000*g* and 100,000*g* supernatants were made up to 2% (w/v) SDS. All samples were heated in a boiling water bath for approximately 5 min and stored at −20°C until required.

Protein Determination, Electrophoresis, and Immunoblotting

Protein concentrations for samples of leaf and bacterial extracts were determined using the bicinchoninic acid assay (Smith et al., 1985). The concentrations of purified rBN28 samples were determined by quantitative acid hydrolysis. Prior to electrophoresis, samples were made up to 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 0.012% (w/v) bromphenol blue. Aliquots containing 30 to 50 μg of protein were separated on 12% SDS-polyacrylamide gels (Laemmli, 1970) or, alternatively, on 16.5% Tricine-SDS gels containing 3% bis-acrylamide cross-linker (Schägger and von Jagow, 1987). Electroblotting of SDS gels to nitrocellulose membranes and immunological detection of BN28 were performed as previously described (Boothe et al., 1995).

Recrystallization Inhibition Assays

rBN28 was tested for the ability to inhibit ice recrystallization as previously described (Knight et al., 1988; Mueller et al., 1991). SpaAfa5, a synthetic type I winter flounder AFP fused to the C terminus of staphylococcal protein A (Spa) (Mueller et al., 1991), was used as a positive control. Briefly, samples of rBN28 and SpaAfa5 were prepared at concentrations from 0.01 to 1.0 mg/mL in deionized, distilled water. A 10-μL aliquot was dropped from a height of approximately 2.6 m onto a polished aluminum plate that had been precooled to −78°C. The frozen "splat" was transferred with a knife to a microscope slide, which was then placed onto the stage of a compound microscope. Ice crystal formation was followed for 60 min and inhibition of recrystallization was assessed by visual inspection. All instruments used during the transfer and observation operations were precooled and maintained at −7°C.

Mass Spectroscopy

Mass spectra were recorded on a desorption mass spectrometer (BioIon Nordic 20, Applied Biosystems). Samples (100–250 pmol) were applied to a nitrocellulose-coated Mylar disc in a 50:50 mixture of 0.05% aqueous trifluoroacetic acid and ethanol, respectively. The sample solutions were allowed to absorb to the discs for 20 min and then spin-dried. The spectra were collected for 3 h at 16 kV and were calibrated using H^+ and NO^+ as standards.

Amino Acid Composition Analysis

For amino acid analysis, purified rBN28 was hydrolyzed in 6 N HCl containing 0.1% phenol at 110°C for 24 h or 1 h at 160°C in evacuated sealed tubes with added norleucine as an internal standard. The analysis was performed on an amino acid analyzer (model 6300, Beckman).

CD

CD spectra were recorded on a spectropolarimeter (J-500 C, Jasco, Easton, MD) equipped with a Jasco IF500II interface, an IBM PS/2 running the Jasco DP-500/PS-2 system, version 1.33a, software, and a Lauda (model RMS) water bath (Brinkman) used to control the temperature of the cell. Constant N_2 flushing was used. The instrument was routinely calibrated with an aqueous solution of recrystallized d-10-(+) camphosulfonic acid at 290 nm. Measurements were made with protein solutions ranging from 0.08 to 1.0 mg/mL in 20 mM sodium phosphate buffer and KCl ranging from 0 to 100 mM. Molar ellipticity is reported as mean residue molar ellipticity ($[\theta]$, deg cm^2 $dmol^{-1}$) and calculated from the equation:

$$[\theta] = [\theta]_{obs}/10\ lc\ (\text{no. of amino acid residues})$$

where $[\theta]_{obs}$ is the observed ellipticity measured in degrees, l is the optical path length of the cell in centimeters, and c is the molar concentration of the peptide.

Deconvolution of the CD spectra was performed using the RBOcon algorithm, a modified version of the algorithm described by Chang et al. (1978). RBOcon is incorporated as part of the CDPENCE deconvolution program (R. Boyco, D.S. Wishart, and B.D. Sykes, University of Alberta, Edmonton, Alberta, Canada).

NMR

One-dimensional and two-dimensional ^{1}NMR experiments were carried out on a Unity 500 spectrometer (Varian, Sunnyvale, CA) at a ^{1}H frequency of 500 MHz. Purified rBN28 was concentrated and ^2H_2O added to yield a 0.5 mM (approximately 3 mg/mL) protein solution in 90% H_2O and 10% ^2H_2O. Spectra were obtained at 5°C in 20 mM sodium phosphate, pH 6.9 (not corrected for ^{2}H isotope effects). One-dimensional spectra were acquired using 1024 scans with a spectral width of 6500 Hz, 25984 data points, a 90° pulse width of 10.5 μs, and a 2.0-s presaturation delay. The spectra were processed with zero filling to 64K and an exponential line broadening of 0.5 Hz. Chemical shifts are referenced to the trimethylsilyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate at 0 ppm, which was added as an internal standard. 2D-DQF-COSY spectra (Rance et al., 1983) were acquired in a phase-sensitive mode (Wüthrich, 1986) using a spectral width of 6500 Hz in both dimensions, a 2.0-s presaturation delay, 32 scans of 1k complex data points in t_2, and 256 complex points in t_1. The data were processed with zero filling to 2k × 2k complex points using shifted sinebell weighting functions. All processing was performed with VNMR software on a Sun IPC work station (Varian).

RESULTS

Sequence Analysis

The *bn28* gene was isolated previously from a cDNA library prepared from cold-acclimated leaves of *B. napus* cv Jet Neuf (Orr et al., 1992). The gene encodes a polypeptide with a predicted molecular mass of approximately 6.6 kD and a pI of 9.0. Searches of protein and nucleic acid databases uncovered four genes with significant homology to *bn28*: *cor6.6* (Gilmour et al., 1992), *kin1* (Kurkela and Franck, 1990), and *kin2* (Kurkela and Borg-Franck, 1992) in *Arabidopsis thaliana* and *brkin1* in *Brassica rapa* (Protein Information Resource accession no. S34660). All of these genes appear to code for similar proteins differing in a few amino acids, with most substitutions being conservative in nature (Fig. 1, top). The *B. rapa* protein, BRKIN1, exhibits 95% amino acid sequence identity with BN28, whereas the three Arabidopsis proteins, COR6.6, KIN1, and KIN2, show slightly lower levels of 69, 66, and 43% identity, respectively. Hydrophilicity and secondary structure predictions based on the deduced amino acid sequence (Fig. 1, bottom) suggest that the BN28 protein is highly hydrophilic and contains a significant stretch of α-helical structure. A consensus region of α-helical structure is predicted to exist in

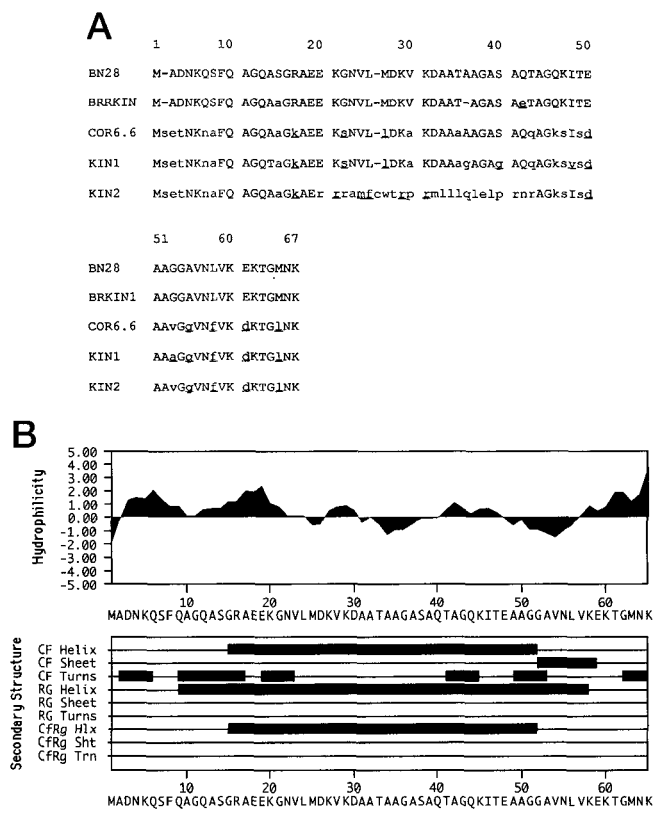

Figure 1. Analysis of deduced amino acid sequence from BN28. A, Multiple sequence alignment of the deduced amino acid sequence for BN28 with those of homologous proteins BRKIN1 (Protein Information Resource accession no. S34660), COR6.6 (Gilmour et al., 1992), KIN1 (Kurkela and Franck, 1990), and KIN2 (Kurkela and Borg-Franck, 1992). Uppercase letters indicate identical amino acids; conserved residues are underlined and gaps are represented as dashes. B, The top panel shows the hydrophilicity profile for BN28 determined according to the method of Kyte and Doolittle (1987), with positive values indicating regions of hydrophilicity. The lower panel shows the secondary structure predictions for BN28 based on the algorithms of Chou and Fasman (CF) (1978) and Garnier et al. (RG) (1978), together with the consensus prediction (CfRg). The hydrophilicity profile was determined using a moving seven-residue window. Hlx, Helix; Sht, sheet; Trn, turn.

the central portion of the protein spanning from approximately residue 15 to residue 52. The distribution of charged/polar and nonpolar amino acids does not reveal an amphipathic α helix. Both the N- and C-terminal ends of the protein are predicted to consist largely of turns or random coil.

Purification of Recombinant BN28

The *bn28* cDNA was modified to remove the N-terminal Met codon and was subcloned into the pMAL-c prokaryotic expression vector downstream of a gene encoding a bacterial MBP. The coding regions of the two proteins were separated by a sequence encoding a factor Xa protease cleavage site. Synthesis of the fusion protein in transformed *E. coli* could be induced with the addition of isopropyl β-D-thiogalactopyranoside, with maximum levels obtained at a concentration of 0.3 mM and incubation for 3 h. Following harvest and disruption of cells, the MBP-rBN28 fusion was recovered in the soluble fraction, where it appeared to make up at least 20% of the total protein (Fig. 2). Initial attempts to purify the fusion protein from this fraction through affinity binding to amylose resin proved unsuccessful. This was probably due either to steric hindrance or to a conformational change in the maltose-binding site of MBP, as a result of its fusion with rBN28.

As shown by comparison of lanes I and IC in Figure 2, efficient cleavage of the MBP-rBN28 fusion protein was achieved following digestion with factor Xa. Incubation of the crude soluble protein fraction from induced cells with protease resulted in the apparent complete cleavage of the fusion protein as evidenced by the disappearance of the band corresponding to this protein and the appearance of a band with the same molecular mass of MBP. A band corresponding to rBN28 was not observed on these gels because of poor resolution of low-molecular-mass proteins with the Tris/Gly buffer system. However, rBN28 could be detected in factor Xa-cleaved samples following electrophoresis on Tricine-polyacrylamide gels (see below).

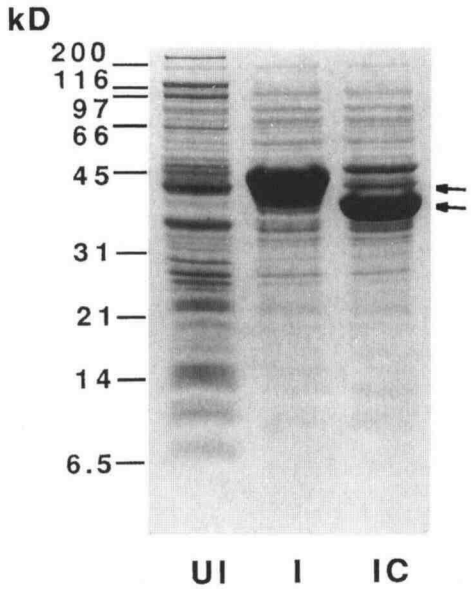

Figure 2. Expression and cleavage of the MBP-rBN28 fusion protein. Coomassie blue-stained Tris/Gly-SDS polyacrylamide gel of total soluble protein from recombinant *E. coli* containing the MBP-BN28 plasmid. Each lane contained 30 μg of protein. Lanes UI and I show protein from uninduced and induced cells, respectively. Lane IC shows protein from induced cells following cleavage with factor Xa. Arrows indicate the position of bands corresponding to the fusion protein (top) and MBP cleavage product (bottom).

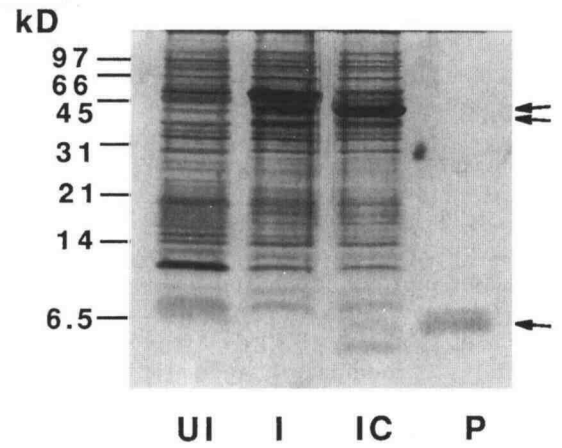

Figure 3. Purification of rBN28. The silver-stained Tricine-SDS polyacrylamide gel shows the purification of rBN28 following binding to DEAE-cellulose and factor Xa cleavage as described in the text. The lanes show protein from uninduced (UI, 5 μg) and induced *E. coli* before (I, 5 μg) and after (IC, 5 μg) cleavage with factor Xa and purified rBN28 (P, 0.4 μg). Arrows indicate the position of bands corresponding to the fusion protein (top), MBP cleavage product (middle), and rBN28 (bottom).

The inability of the fusion protein to bind amylose required that an alternative strategy for purification be developed. The broad difference in the predicted pI values of the fusion protein (pI 5.0) and BN28 suggested that anion-exchange chromatography might be successfully used for this purpose. DEAE-cellulose equilibrated at pH 8.0 was found to bind most of the protein present in the soluble fraction from induced cells. Thorough washing of the resin ensured that any unbound protein was removed before proceeding to cleave the fusion protein. Cleavage was accomplished by adding factor Xa present in approximately 1 volume of the equilibration buffer directly to the anion-exchange resin loaded with bound protein. Digestion did not appear to be significantly impaired, despite the fact that both the fusion protein and protease were bound to the resin. Since rBN28 is positively charged at pH 8.0, it was not bound by the resin. Thus, once released from the fusion protein, it could be recovered in the eluate essentially free from contaminating bacterial proteins and also from the factor Xa. Although it appears that cleavage of some bacterial proteins may also have taken place (Fig. 3, lane IC), these products must have remained bound to the resin, since they are not present in the purified fraction.

As shown in Figure 3, this procedure enabled purification of rBN28 to near homogeneity in a single chromatographic step. Although a doublet is observed in Figure 3, lane P (showing the purified protein), we believe that the

upper band is most likely due to aberrant migration of a small portion of the rBN28 rather than a contaminating bacterial protein. This conclusion is supported by the examination of several different gels loaded with the same sample of purified rBN28, in which the fainter upper band was observed to vary in intensity. Furthermore, a similar faint upper band was observed on some immunoblots of protein extracted from cold-acclimated leaves, whereas no corresponding band could be detected in extracts of either uninduced or untransformed *E. coli* cells digested with factor Xa (not shown). Using the above procedure we were able to obtain 5 to 10 mg rBN28 L^{-1} induced culture.

The identity of the purified product was confirmed by amino acid composition analysis and the molecular mass was determined by MS. The value of 6422.8 D obtained for rBN28 was similar to the mass of 6420.5 D predicted from the deduced amino acid sequence (without the N-terminal Met).

Immunological Characterization

Polyclonal antibodies raised against rBN28 were used to compare properties of the recombinant protein with that of the native protein produced in plants (Fig. 4). Samples of total and boiling-soluble protein fractions from purified rBN28 and from control and low-temperature-treated leaves of *B. napus* were separated on Tricine-SDS gels and electroblotted to nitrocellulose membranes. Preimmune serum did not cross-react significantly with either the recombinant or native proteins. However, anti-rBN28 serum detected both rBN28 and the native plant protein, with the latter present only in extracts from low-temperature-treated leaves. Both proteins appeared to have identical relative mobilities with an apparent molecular mass of slightly less than 6.5 kD and remained soluble following treatment in a boiling water bath.

Since membrane stabilization is an important factor in plant freezing tolerance, and some low-temperature proteins have been found to be associated with membrane fractions (Mohapatra et al., 1988), we wished to determine whether BN28 fractionated with cellular membranes. This was accomplished by separating leaf extracts into soluble and microsomal pellet fractions. Leaf proteins were extracted in either low-salt or high-salt buffers (the latter to disrupt any weak ionic associations that might exist) and then centrifuged to obtain 9,000*g* and 100,000*g* pellets and supernatants. Aliquots from each fraction were separated on Tricine-SDS gels. The volume loaded for each sample was determined to preserve the relative distribution of protein in the original extract. Immunoblot analysis (Fig. 5) revealed that BN28 was found exclusively in the supernatants of both high- and low-salt extracts, indicating that BN28 is not strongly associated with any cellular membranes.

Structural Analysis

Structural analysis of rBN28 was undertaken using CD and NMR spectroscopy. Contrary to predictions based on the deduced amino acid sequence, CD analysis revealed that rBN28 existed largely in a random coil configuration. Similar results were obtained under various conditions of

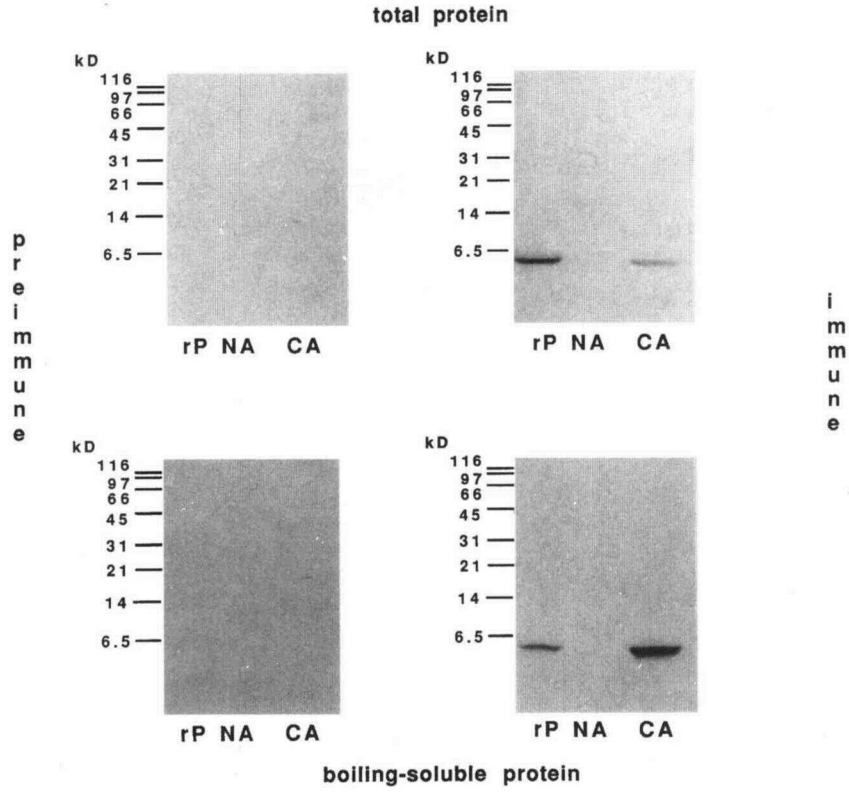

Figure 4. Immunoblot analysis comparing native and recombinant BN28. Immunoblots of total and boiling-soluble protein probed with anti-rBN28 polyclonal antibodies. Lanes rP show purified rBN28 (50 ng) protein and lanes NA and CA show protein (50 μg) from nonacclimated and cold-acclimated leaves of *B. napus*, respectively.

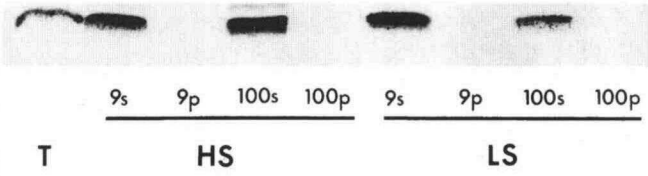

Figure 5. Subcellular fractionation of leaf protein. Immunoblot of protein from cold-acclimated *B. napus* leaves extracted in either high-salt (HS) or low-salt (LS) buffer and probed with anti-rBN28 polyclonal antibodies. Lanes show total protein (T) or protein obtained from either the supernatant (s) or pellet (p) following centrifugation at 9,000*g* (9) and 100,000*g* (100).

protein concentrations (0.08–1 mg/mL), pH (6.0–8.0), and KCl concentration (0, 50, and 100 mM). The conformation also appeared to be independent of the temperature at which the analysis was performed, since nearly identical spectra were obtained at both 5 and 25°C (not shown). Deconvolution of the CD data indicated that rBN28 was made up of approximately 13.5% α helix, 0% β sheet, 22.5% turns, and 64% coil. Since a much larger proportion (approximately 58%) of the protein was expected to be helical, the structure was re-examined in the presence of increasing concentrations (10–50%) of the helix-promoting solvent TFE (Nelson and Kallenbach, 1986; Sönnichsen et al., 1992). As shown in Figure 6B, the addition of TFE had a dramatic effect on the conformation of rBN28, causing an increase in the negative ellipticity at 220 nm, which is indicative of a shift toward greater helical content. Maximum helicity was attained at a TFE concentration of 40 to 50%, yielding a structure made up of approximately 74.5% α helix, 0% β sheet, 0% turns, and 25.5% coil. However, when the solvent was removed from a preparation of rBN28 in 50% TFE, the protein returned to its original conformation, indicating that the helical structure adopted in the presence of TFE is unstable in its absence (Fig. 6C).

The protein was further analyzed by one-dimensional and two-dimensional NMR spectroscopy at 5 and 25°C to corroborate the results from CD spectroscopy and possibly to obtain local structural information. Spin-type assignments were performed using standard procedures (Würthrich, 1986), but the complete sequential assignment could not be accomplished due to resonance overlap. The chemical shift degeneracy is immediately obvious in a representative one-dimensional ^{1}H-NMR spectrum acquired at 25°C with a protein concentration of 0.5 mM (approximately 3 mg/mL; Fig. 7). All side chain resonances of a similar kind are clustered, with little dispersion in between, leading to distinct groups of overlapped resonance lines separated by baseline regions. As an example, all methyl-group protons resonate at approximately 1.4, 1.2, and 0.9 ppm, corresponding to methyl groups in Ala, Thr, and Leu, Ile, or Val residues, respectively. These resonance frequencies are all also basically identical with the respective random coil chemical shifts. Identical observations were made for all side chain protons in rBN28 under all conditions. Thus, the lack of sequence-dependent frequency dispersion between amino acids or absence of secondary shifts (deviation from random coil shifts) for all side chain protons strongly indicates that the protein is unstructured in solution. This is supported by the obtained proton resonance line width, which is smaller than expected for a folded protein of this molecular weight, and the reduced intensity for amide proton resonances (Fig. 7, 8.6–8.1 ppm) due to chemical exchange and presaturation transfer, being a reflection of the lack of stable intramolecular hydrogen bonds. Similarly, a statistical analysis of αCH and HN-proton frequencies (Wishart et al., 1991), obtained from DQF-COSY data at 5°C, reveals the absence of any secondary backbone proton shifts at lower temperature and, consequently, the absence of any regular secondary structure in this protein (100% coil).

Ice Recrystallization Inhibition Studies

A recrystallization inhibition assay was used to test for possible AFP-like activity associated with rBN28. Solutions of 0.01, 0.1, and 1.0 mg/mL rBN28 were prepared in deionized, distilled water and dropped onto a precooled aluminum plate as described in "Materials and Methods." At the same time, samples of water and a protein A-synthetic AFP fusion, SpaAfa5, were assayed as negative and positive controls of recrystallization inhibition, respectively. SpaAfa5 was tested at the same concentrations as rBN28 and had previously been shown to inhibit recrystallization within this concentration range (Mueller et al., 1991). Recrystallization was followed with a compound microscope for 1 h. Within 15 min significant inhibition of recrystallization could be observed at all concentrations of SpaAfa5, as evidenced by the appearance of numerous tiny crystals. In contrast, large ice crystals were observed with all concentrations of rBN28 and the samples appeared to be identical to the water control with respect to both the size of the

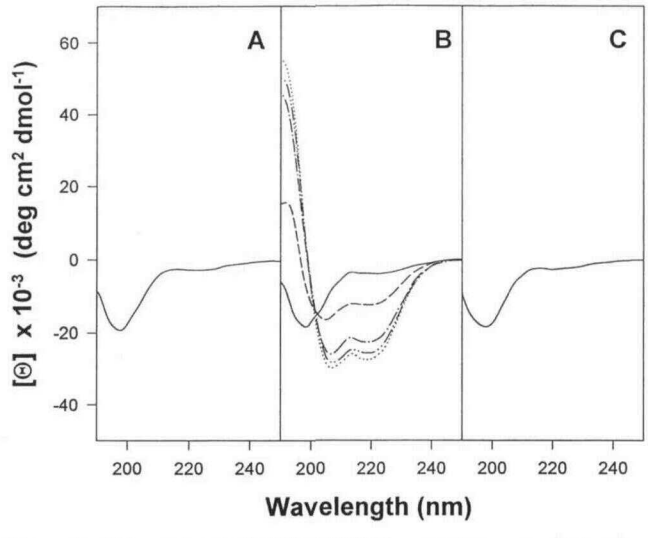

Figure 6. CD spectra of rBN28 at 5°C. The spectra were obtained as described in "Materials and Methods." Measurements were made in 20 mM sodium phosphate buffer, pH 6.9, 50 mM KCl at a protein concentration of 1 mg/mL rBN28. A, Spectrum of freshly purified rBN28. B, Spectral changes at various concentrations of TFE (v/v) (solid line = 10%, dashed line = 20%, dash-dotted line = 30%, dash-double-dotted line = 40%, and dotted line = 50%). C, Previous sample after removing TFE by dialysis.

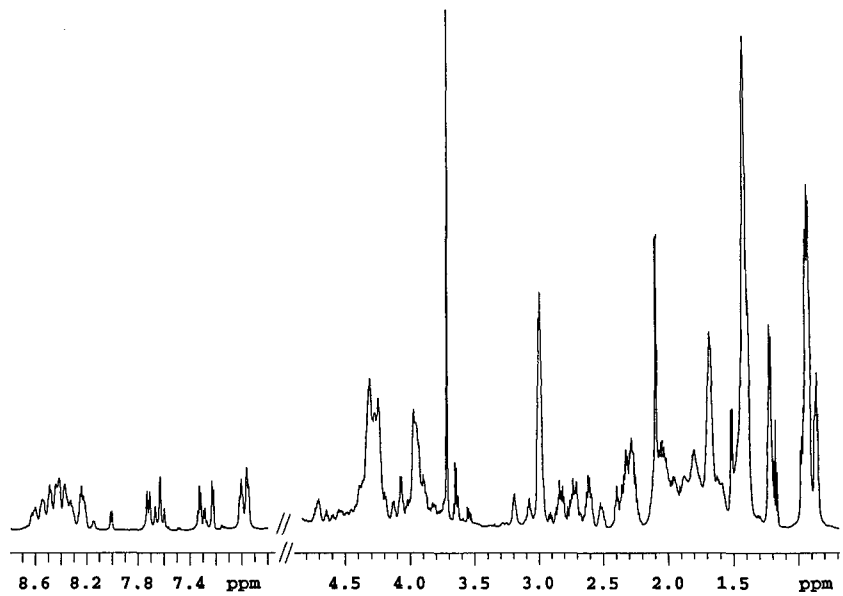

Figure 7. One-dimensional ^{1}H-NMR spectrum of rBN28 at 25°C. Measurements were made in 20 mM sodium phosphate buffer, pH 6.9, with a protein concentration of 0.5 mM (approximately 3 mg/mL) rBN28. The regions containing all amide proton and aromatic proton resonances (left) and aliphatic protons (right) are shown. The solvent peak was omitted (H_2O, 4.8 ppm). Chemical shifts were referenced to the methyl resonances of 2,2-dimethyl-2-silapentane-5-sulfonate, which was added as an internal standard (0 ppm, not shown).

ice crystals and their time of formation (results not shown). These results indicate that rBN28 lacks any appreciable recrystallization inhibition activity.

DISCUSSION

Although correlative studies have identified proteins associated with low-temperature acclimation in plants and implied their involvement in the development of freezing tolerance (Guy and Haskel, 1987; Gilmour et al., 1988), the precise role of these proteins is as yet unknown. Purification, physical characterization, and, where possible, in vitro assays of function are often essential in establishing the role of a specific protein. Toward this end, we have purified a recombinant form of a protein, BN28, expressed during low-temperature acclimation in leaves of *B. napus*. The solution structure of rBN28 was determined and the protein was assayed for previously proposed AFP-like properties.

Antibodies raised against rBN28 were used to characterize both the recombinant protein and the native protein synthesized in cold-acclimated plants. In common with many other plant stress proteins (Lin and Thomashow, 1992a; Neven et al., 1993; Houde et al., 1995), our results indicate that BN28 is highly hydrophilic, to the extent that it remains soluble following boiling, and is localized within the soluble fraction of the cell. In the latter case, this is supported by the results of both subcellular fractionation reported here and immunolocalization studies that show BN28 present throughout the cytosol in cells from cold-acclimated leaves (de Beus et al., 1997). Despite these similarities, BN28 appears to differ from other boiling-soluble, stress-induced proteins in sequence and also immunogenically. Database searches revealed only a small group of proteins with homology to BN28, all of which are found in closely related species or genera, and antibodies raised against rBN28 did not appear to cross-react with other low-temperature-induced proteins in *B. napus* leaves. In contrast, a number of boiling-soluble proteins, varying widely in molecular weight and identified in divergent species including Arabidopsis (Gilmour et al., 1992), spinach (Neven et al., 1993; Kazuoka and Oeda, 1994), and wheat (Houde et al., 1995), show both sequence and immunological relatedness. These proteins possess repeated stretches of amino acids that are also found in the group 2 Lea proteins and thus most likely also contain common structural elements. Structural analysis may be useful in further clarifying the relationship among the various types of hydrophilic plant stress proteins identified so far, supporting or refuting speculation regarding their functional similarity.

Whereas a large proportion (58%) of the BN28 protein was predicted to reside in an α helical conformation, one-dimensional and two-dimensional NMR studies found that rBN28 in fact existed as a 100% random coil in aqueous solution. Although no change in conformation was observed within the ranges examined for pH, KCl concentration, or temperature, the helical character of the protein was clearly evident in the presence of TFE. This shift to secondary structure observed at TFE concentrations of 40 to 50% would seem to confirm that the helical propensity of rBN28 is relatively high (Lehrman et al., 1990). Thus, BN28 may exist in a helical conformation in vivo given the proper cellular environment. High ionic concentrations can stabilize helices in peptides that are unstructured in low-ionic-strength environments (Ramalingam et al., 1992). It is believed that this stabilization is accomplished through either charge screening or enhancement of weak hydrophobic interactions. Since increases in intracellular salt concentration would be expected to occur as a result of osmotic, drought, or freeze-induced dehydrative stress, these conditions might induce such a conformational change in BN28.

The failure to detect inhibition of recrystallization in assays with rBN28 indicates that it does not possess AFP-

like properties. Such a result might also have been inferred from our structural analysis. Evidence has suggested that type I AFPs exert their effect through binding to the ice lattice and thereby inhibit growth of the ice crystal (Wen and Laursen, 1992). These AFPs form an amphipathic α helix and binding is believed to occur via an interaction between the hydrophilic face of the helix and one axis of the growing ice crystal. The hydrophobic face of the helix is presented outward, blocking the addition of neighboring water molecules. This dependence on a very specific structure would seem to preclude rBN28 from exhibiting type I AFP-like activity, since it was determined to be completely unstructured in aqueous solution. In contrast, the recombinant synthetic AFP used as a positive control in these assays possessed significant activity and was, therefore, presumably able to adopt an active conformation in aqueous solution. Accordingly, any similarities between BN28 and the winter flounder type I AFP would seem to be superficial in nature.

A number of other functions have been proposed for low-temperature- and dehydration-induced proteins, including membrane stabilization (Hincha et al., 1990), water binding (McCubbin et al., 1985; Roberts et al., 1993), and cryoprotection of proteins (Kazuoka and Oeda, 1994; Houde et al., 1995). With respect to BN28, no evidence has been found that would strongly support any of these functions. We did not detect any association with cellular membranes from our subcellular fractionation studies. Also, recent experiments with a recombinant form of the homologous COR6.6 (rCOR6.6) protein from Arabidopsis did not reveal any changes in the dehydration-induced phase behavior (Webb et al., 1996) or cryoprotective activity (Uemura et al., 1996) following addition of the protein to artificial membranes in vitro. The same rCOR6.6 protein examined over a range of osmotic pressures also showed only intermediate levels of hydration compared with other proteins (Webb et al., 1996). Finally, our preliminary results obtained with rBN28 indicate that it does not possess any extraordinary cryoprotective behavior toward soluble enzymes in vitro (on a molar basis, it was less effective than BSA) (M.D. de Beus, unpublished results). However, it should be noted that all of the above studies with BN28 and COR6.6 have utilized recombinant forms of the protein. It is possible that different cellular environments, protein modifications, or protein-protein interactions might alter both the structure and activity of these proteins in vivo. In fact, isoforms of native COR6.6 have been observed that differ in charge from rCOR6.6, suggesting that at least a portion of the protein is modified in vivo (Gilmour et al., 1996). Further characterization of the native protein and its microenvironment in vivo may be necessary to provide information for future structural and functional studies.

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