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Purification and Characterization of Glutathione Reductase Isozymes Specific for the State of Cold Hardiness of Red Spruce[1]

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Isozymes of glutathione reductase (GR) have been purified from red spruce (*Picea rubens* Sarg.) needles. Two isozymes could be separated by anion-exchange chromatography from both nonhardened or cold-hardened tissue. Based on chromatographic elution profiles, the isozymes were designated GR-1NH and GR-2NH in preparations from nonhardened needles, and GR-1H and GR-2H in preparations from hardened needles. N-terminal sequencing and immunological data with antisera obtained against GR-1H and GR-2H established that the isozymes from hardened needles are different gene products and show significant structural differences from each other. Chromatographic, electrophoretic, and immunological data revealed only minor differences between GR-2NH and GR-2H, and it is concluded that these isozymes are very similar or identical. Anion-exchange chromatography and native polyacrylamide gel electrophoresis also established that GR-1NH and GR-1H are different proteins. From these data we conclude that GR-1H is a distinct gene product, present only in hardened needles. Therefore, GR-1H can be considered to be a cold-hardiness-specific GR isozyme, and GR-1NH can be considered to be specific for nonhardened needles. It is proposed that GR-1H is a cold-acclimation protein.

Conifers are relatively susceptible to freezing temperatures during the growing season. Temperatures of −10 to −5°C can kill unhardened needle tissue (Senser and Beck, 1984). After the hardening period during fall, however, conifers are able to withstand temperatures below −40°C (Senser and Beck, 1984). As frost tolerance is acquired, conifers also enter a dormant state, characterized by a cessation of growth (Christersson, 1978), decreased rates of photosynthesis (Öquist et al., 1980; Doulis et al., 1993), and reorganization of chloroplast ultrastructure (Senser and Beck, 1984). Cold hardening leads to specific changes in gene expression (Guy et al., 1985; Guy and Haskell, 1987). Those proteins that are expressed specifically during cold acclimation and are correlated with cold tolerance have been termed CAPs. CAPs have been proposed to function in altering protein turnover (Schaffer and Fischer, 1990) or as cryoprotectants (Hincha et al., 1989). However, in no case could a specific physiological function of a CAP be demonstrated.

Many environmental conditions or xenobiotics favor the reduction or activation of molecular oxygen in the chloroplast. Exposure to herbicides (Richmond and Halliwell, 1982) and air pollutants (Mehlhorn et al., 1990) has been shown to lead to the formation of toxic oxygen species. Similarly, oxyradicals have been implicated as one mechanism of injury in plants exposed to low environmental temperatures. As the temperature decreases, enzymic reactions of the reductive pentose phosphate cycle will slow down, whereas temperature-independent light harvesting by photosynthetic pigments remains relatively high (Steffen and Palta, 1987). Using wheat crown tissue, Wise and Naylor (1987a, 1987b) demonstrated that lipid peroxidation induced by chilling is dependent on light and can be prevented by inhibitors of photosynthetic electron transport. Combined treatment with light and low temperatures also depleted or oxidized cellular antioxidants, such as ascorbate, GSH, α-tocopherol, and violaxanthin, indicative of the presence of activated oxygen species. It has been shown that freeze-thaw stress of wheat protoplasts leads to increased production of superoxide and membrane degradation (Kendall and McKersie, 1989).

Hodgson and Raison (1991) have directly measured the production of superoxide by electron-spin-resonance spectroscopy in isolated illuminated thylakoids. They showed that $O_2^{-}\cdot$ production at 25°C occurs even when Fd and $NADP^+$ are present in saturating concentrations. However, illumination of thylakoids from chilling-sensitive plants at 5°C increased the rate of $O_2^{-}\cdot$ production to the same degree as incubation at 25°C when only Fd was present. On the other hand, when chilling-insensitive plants were used, no increase in $O_2^{-}\cdot$ production was observed at low temperatures. The authors concluded that one mechanism of low-temperature-induced photooxidation is a greater decrease of $NADP^+$ reduction with temperature in chilling-sensitive plants than in chilling-resistant plants.

Several small molecules or enzymes scavenge free radicals

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Abbreviations: CAP, cold-acclimation protein; GR, glutathione reductase; GR-1H, GR isozyme 1 from hardened needles; GR-2H, GR isozyme 2 from hardened needles; GR-1NH, GR isozyme 1 from nonhardened needles; GR-2NH, GR isozyme 2 from nonhardened needles.

in cells. Superoxide in the chloroplast is scavenged by superoxide dismutase, and the resulting H_2O_2 is reduced to H_2O in subsequent reactions with ascorbate and GSH. In this cycle of oxidation and rereduction of the antioxidants ascorbate and GSH, GR catalyzes the NADPH-dependent reduction of GSSG to GSH, thereby linking the removal of toxic oxidants to reducing power provided by photosynthetic electron transport. In vivo studies have shown that oxidative stress can lead to an increase of components of the antioxidative system. Activities of superoxide dismutase, ascorbate peroxidase, and GR increased after exposure of plants to oxidative conditions, such as herbicide treatment and air pollutants (Mehlhorn et al., 1986, 1987; Schmidt and Kunert, 1986). Leaf contents of GSH (Smith, 1985; Schmidt and Kunert, 1986; Hausladen et al., 1990) also increased markedly on exposure to oxidative stress. GSH content (De Kok and Oosterhuis, 1983; Guy et al., 1984) and GR activity (Guy and Carter, 1984) have been shown to increase during cold acclimation in several plant species. In conifers, GSH levels and GR activity are low in summer and increase during the hardening period (Esterbauer and Grill, 1978; Hausladen et al., 1990; Anderson et al., 1992). In red spruce (*Picea rubens* Sarg.), GSSG was undetectable in unhardened needles, but substantial amounts of GSSG accumulated during winter. By early spring, up to 30% of the total GSH pool was oxidized (Hausladen et al., 1990).

In our study of seasonal changes in antioxidants of red spruce we have found that in addition to an increase in GSH content in spruce needles during the hardening season, GR activities increase severalfold and remain high during winter (Hausladen et al., 1990; Madamanchi et al., 1991; Doulis et al., 1993). The increase in GR activity was correlated with the acquisition of frost tolerance and the onset of dormancy (Doulis et al., 1993). The increased activity of GR during winter suggests that the enzyme is functioning in dormant tissue at low temperatures.

Recently, the occurrence of multiple forms of GR has been demonstrated in pea (Edwards et al., 1990), mustard (Drumm-Herrel et al., 1989), and pine (Anderson et al., 1990). Individual isozymes may differ with respect to subcellular localization and their respective responses to stress. Therefore, we have studied the presence of GR isozymes in nonhardened and hardened needle tissue. Our results demonstrate the occurrence of hardiness-specific GR isozymes in red spruce. In an accompanying paper, we describe the temperature dependence of kinetic parameters, substrate specificities, and possible regulation of these isozymes (Hausladen and Alscher, 1994).

MATERIALS AND METHODS

Plant Material

Red spruce (*Picea rubens* Sarg.) needles were collected at Whitetop Mountain, VA, at an altitude of 1628 m above sea level from approximately 20-year-old saplings as described by Doulis et al. (1993). Twigs with previous year's needles were collected in batches of approximately 2 kg and stored on ice for transport to the laboratory. Needles were removed by hand after briefly immersing the twigs in liquid nitrogen and stored at −80°C until they were used for protein purification. Harvest dates were August 12, 1990, for nonhardened needles, and January 8, 1991, for hardened needles.

Reagents

Unless otherwise indicated, all chemicals were from Sigma. Antisera were produced by Cocalico Biologicals (Reamstown, PA). The band corresponding to GR-1H in SDS gels was identified as described below and excised from an SDS gel. The gel slice was macerated, mixed with complete Freund's adjuvant, and used for antiserum production. GR-2H (500 μg) was used for immunization without further purification. The rabbits were given booster injections after 14, 21, and 49 d, and the serum was collected after 59 d.

Assays and Data Analysis

GR was assayed at 25 ± 0.1°C in 0.1 M Tris-HCl, with the pH adjusted to 7.5. Standard assays contained 1 mM EDTA, 1 mM GSSG, and 0.05 mM NADPH (Foyer and Halliwell, 1976) and were performed in a Beckman DU65 spectrophotometer equipped with a Peltier temperature controller by following the decrease in A_{340}. One unit of GR activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min.

Protein was measured by the Bradford (1976) method using the Bio-Rad protein determination kit. In purified preparations, protein was estimated by the A_{280}, assuming 1 A unit = 1 mg/mL protein.

Electrophoresis

SDS-PAGE was carried out according to Laemmli (1970); native gels were prepared according to the same protocol excluding SDS and 2-mercaptoethanol from all solutions. Gels were stained with silver using the Sigma or Bio-Rad silver stain kits according to the manufacturer's instructions. Activity staining of native gels was in 3.4 mM GSSG, 0.4 mM NADPH, 1.2 mM 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 0.04 mM 2,6-dichlorophenol-indophenol (Kaplan, 1968; modified by Anderson et al., 1990). Electroelution was performed in a Bio-Rad model 422 electroeluter according to the manufacturer's instructions, using gel slices cut from activity-stained native PAGE.

Immunoblotting was carried out by transferring protein from SDS gels to nitrocellulose (Bio-Rad or Schleicher & Schuell) in a semidry blotting apparatus (Millipore) according to the manufacturer's instructions. Detection of GR protein on the membrane was performed according to Ausubel et al. (1988) using a 1:500 dilution of antiserum and 1:1000 dilution of horseradish peroxidase conjugated goat-anti-rabbit IgG (Bio-Rad). Color development was with 4-chloro-1-naphthol (Bio-Rad).

Purification of GR

Batches of 100 g of needles were homogenized in 2 L of 50 mM Pipes-NaOH, pH 6.5, containing 1 mM EDTA, 0.1 mM DTT, 0.5% Triton X-100, and 2% polyvinylpolypyrrolidone using a Tekmar Tissuemizer, and the homogenates were

strained through eight layers of cheesecloth. The solution was centrifuged at 17,000*g* for 20 min and the supernatant was brought to 40% saturation with solid ammonium sulfate. After stirring for 1 h, the solution was centrifuged and the supernatant was strained through cheesecloth to remove the green layer that had formed on top of the solution. The solution was then brought to 80% saturation with ammonium sulfate, stirred for 2 h, and centrifuged for 1 h at 17,000*g*. The pellet was redissolved in buffer A (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.1 mM DTT), and the ammonium sulfate precipitation (40–80% saturation) was repeated. The solutions were stored at −20°C until they were used for further purification.

After thawing, several preparations were combined, dialyzed against buffer A, and centrifuged for 30 min at 20,000*g*. The solution was applied to a column of 2′,5′-ADP Sepharose (Pharmacia) as described by Anderson et al. (1990). Fractions containing GR activity were pooled, brought to 1 M $(NH_4)_2SO_4$, filtered through a 45-μm syringe filter (Acrodisc), and applied to an HR 5/5 Phenyl-Superose column (Pharmacia) equilibrated in buffer A containing 1 M $(NH_4)_2SO_4$. The column was washed in 20 mL of the same buffer, and elution was performed with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer A. Fractions containing GR activity were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.1 mM DTT). The enzyme was applied to an HR 5/5 MonoQ column (Pharmacia) equilibrated in buffer B. The column was washed in 10 mL of buffer B, and elution was carried out with a linear gradient from 0 to 500 mM NaCl in buffer B. Peaks of activity were pooled separately. The solutions were concentrated and the buffer was exchanged for buffer C (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, 10 mM NaCl) using Centricon 30 microconcentrators (Amicon). The final volume of each enzyme preparation was about 100 μL. The solutions were then applied to a Superose 12 column (Pharmacia), equilibrated in buffer A containing 10 mM NaCl, and eluted at a flow rate of 0.25 mL/min.

N-Terminal Sequencing

Purified enzyme (isozyme GR-2H) from hardened needles (100 pmol) was subjected to Edman degradation after extensive dialysis against H_2O. To prepare isozyme GR-1H for Edman degradation, the partially purified enzyme was concentrated 10-fold in a Centricon 30 microconcentrator (Amicon) and a total of 1 mg of protein was applied to a 10% SDS-polyacrylamide gel. Protein in the gel was electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer, pH 9.0, containing 20% methanol as anode buffer in a semidry blotting apparatus (Millipore) at 2.5 mA cm^{-2}. To identify protein, the membrane was stained in 0.1% Coomassie brilliant blue R-250, dissolved in 50% methanol, and destained with several changes of 50% methanol. After destaining, the membrane was air dried (Matsudaira, 1990). The GR band, previously identified by electroelution from activity-stained native gels (see "Results"), was cut from the membrane with a razor blade and stored at −80°C until analysis. Sequencing was performed using an automated gas-phase amino acid sequencer (Applied Biosystems) by the Virginia Polytechnic Institute and State University Biotechnology Center.

RESULTS

Red Spruce GRs Can Be Separated into Several Forms

The purification of GR from hardened and nonhardened needles collected at Whitetop Mountain is shown in Table I. Several ammonium sulfate preparations have been pooled, and since the yield and purification factor in these preparations have been variable, the $(NH_4)_2SO_4$ preparation in Table I has been arbitrarily set to 100% and 1-fold, respectively. Affinity chromatography and hydrophobic interaction chromatography resolved one peak of GR activity. Two peaks of GR activity were eluted by subsequent anion-exchange chromatography using preparations from either hardened or nonhardened needles. GR activity eluted at 220 and 360 mM NaCl in preparations from hardened needles, and at 285 and 370 mM NaCl in preparations from nonhardened needles (Fig. 1).

The proteins that eluted at 220 and 285 mM NaCl from nonhardened and hardened needles were designated GR-1NH and GR-1H, respectively, and those that eluted at 360 and 370 mM were designated GR-2NH and GR-2H, respectively. After gel filtration, GR-2NH appeared as a single band of 55 kD on SDS-PAGE and GR-2H appeared as a double band of 57/58 kD. Two bands, with molecular masses of 52 and 30 kD, were found for GR-1NH, and one band each of 54 and 60 kD, along with three proteins of about 30 kD, were visible in preparations of GR-1H (Fig. 2). Native gels stained for protein showed three bands for GR-1NH and GR-1H, and one band each for GR-2NH or GR-2H (Fig. 3A). One band was seen on GR activity-stained native gels for each preparation (GR-1NH, GR-2NH, GR-1H, GR-2H). GR-1H showed the slowest migration, followed by GR-1NH. GR-2NH and GR-2H had the same mobility but migrated faster than either GR-1H or GR-1NH (Fig. 3B).

Identity of GR-1H on SDS Gels

Further purification of GR-1NH and GR-1H by electroelution from native or SDS gels resulted in very low yields. Therefore, electroelution was not used for preparative purposes. This method could, however, be used to positively identify the GR band in SDS gels of the partially purified preparation of GR-1H. When the GR-1H band from an activity-stained native gel was electroeluted and the eluate was subjected to SDS-PAGE and stained for protein, only one band was found (Fig. 4). The gel in Figure 2 has been stained less intensely than the gel shown in Figure 4 to visualize the double bands in the preparation of GR-2H. Figure 4 (lane 2) shows that several more proteins are present in the preparation of GR-1H than can be seen in Figure 2.

N-Terminal Sequencing

The N-terminal amino acids of GR-2H (sequenced in solution without further purification) and of GR-1H (sequenced bound to a polyvinylidene difluoride membrane after further

Table I. *Purification of GR from 300 g of hardened needles and 500 g of nonhardened needles*

After MonoQ anion-exchange chromatography, both peaks were collected separately and applied individually to the Superose 12 column.

Purification Step	Units	Mass	Volume	Specific Activity	Purification	Yield
		mg	*mL*	*units/mg*	*-fold*	%
Hardened needles						
2 × 40–80% ammonium sulfate	900	580	74	1.6	1.0	100
ADP Sepharose	680	29	32	24	15	75
Phenyl Superose	460	14	8	32	21	51
MonoQ						
GR-1H	95	4.9	6	19	12	
GR-2H	430	3.2	7	140	87	
Total						58
Superose 12						
GR-1H	46	2.6	6.5	18	11	
GR-2H	350	1.9	3	190	120	
Total						44
Nonhardened needles						
2 × 40–80% ammonium sulfate	440	480	180	0.92	1.0	100
ADP Sepharose	300	9.1	3.2	33	36	68
Phenyl Superose	120	3.8	12	32	35	27
MonoQ						
GR-1NH	17	1.2	5	15	16	
GR-2NH	80	0.85	7	94	101	
Total						22
Superose 12						
1NH	7.2	0.32	6	22	24	
2NH	39	0.44	8	89	96	
Total						10

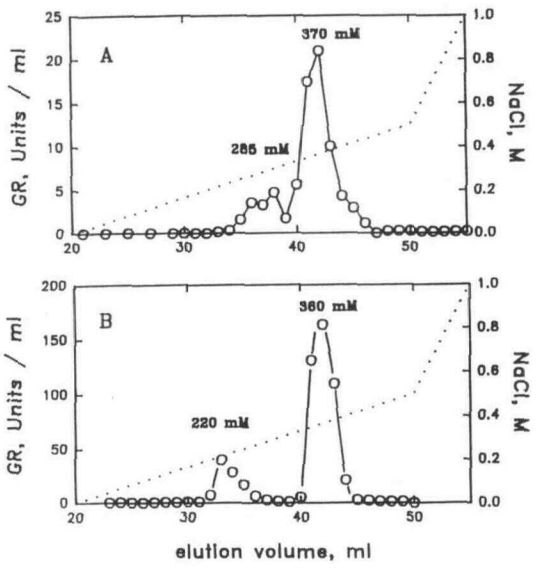

Figure 1. Elution profiles of red spruce GR activity after MonoQ anion-exchange chromatography during the purification procedure as described in the text. A, Nonhardened needles; B, hardened needles.

purification by SDS gel electrophoresis) are shown in Figure 5 along with sequences from other organisms. For GR-2H, 25 amino acids could be identified, and in each cycle, the amino acid assigned to the sequence was present in at least 2-fold higher amounts than other residues. For GR-1H, some residues could not be assigned to the sequence unequivocally. Residue 1 could be either Lys or Met, and residue 16 could be either Lys or Ile. No assignment could be made for cycle 4. Amino acids 11 to 25 of GR-2H were identical to amino acids 2 to 16 of Scots pine GR (Wingsle, 1991). Six residues

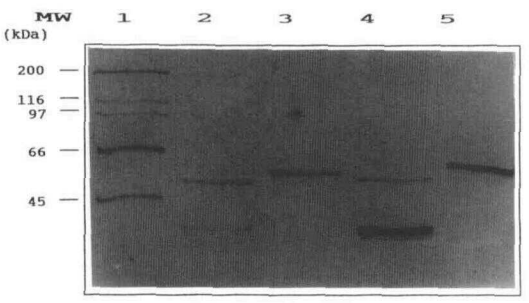

Figure 2. Electrophoretic separation of purified red spruce GR isozymes by SDS-PAGE on a 10% gel. Lane 1, Molecular mass standards as indicated on the left of the figure; lane 2, 500 ng of GR-1NH; lane 3, 100 ng of GR-2NH; lane 4, 800 ng of GR-1H; lane 5, 100 ng of GR-2H. The gel is stained with silver.

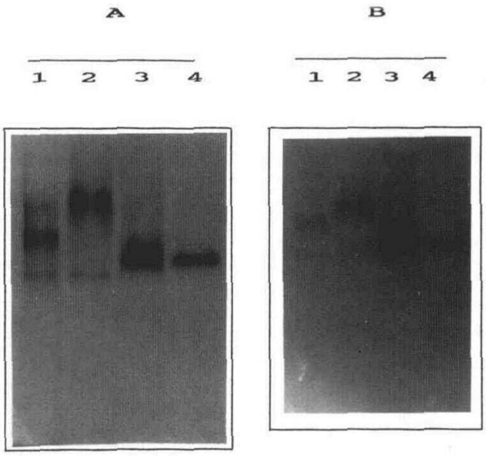

Figure 3. Electrophoretic separation of purified red spruce GR isozymes on 7.5% native gels. A, Silver stain; B, GR activity stain of a duplicate gel. Lanes 1, GR-1NH; lanes 2, GR-1H; lanes 3, GR-2NH; lanes 4, GR-2H. In each lane, 200 ng of protein was applied.

of GR-2H were identical to those of GR-1H, eight residues were identical to GRs from *Escherichia coli* and human, and nine residues identical to GR-2H were found in pea and *Pseudomonas aeruginosa* GRs (Creissen et al., 1992).

Immunological Properties

Antiserum raised against GR-2H recognized the native and the SDS-denatured forms of GR-2NH and GR-2H on immunoblots, but not those of GR-1NH or GR-1H (Fig. 6). The higher molecular mass protein seen on the immunoblots from native gels are probably due to residual GR in these lanes, or are caused by higher molecular mass aggregates of GR (Guy and Carter, 1984). Using SDS-denatured protein, no band was found at the expected molecular mass of 52 to 54 kD for GR-1NH and GR-1H with antiserum against GR-2H. However, the antiserum against GR-2H did recognize a peptide of about 30 kD in partially purified GR-1H. Antiserum raised against GR-1H was used only to test cross-reactivity with

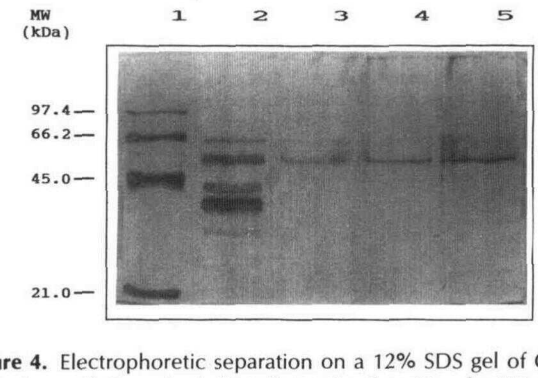

Figure 4. Electrophoretic separation on a 12% SDS gel of GR-1H after electroelution of gel slices that stained positive for GR activity on native gels. Lane 1, Molecular mass standards as indicated on the left of the figure; lane 2, 400 ng of GR-1H prior to electroelution; lanes 3 to 5, 10, 20, and 40 μL of eluate, respectively. The gel is stained with silver.

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GR-1H                         K L Q X S V E Q Y V Q Y L F X I P A I
GR-2H             E V D N G A A L L G K S E E F D Y D L F T I G A G
Pine                                S K S E E F D Y D L F T I G A G
Pea     H R T F A V R A E S Q N G A D P A R Q Y D F D L F T I G A G
P.a.                                      M S F D F D L F V I G A G
E.c.                                      M T K H Y D Y I A I G G G
human   M A C R Q E P Q P Q G P P P A A G A V A S Y D Y L V I G G G
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Figure 5. N-terminal amino acid sequences of GRs. X, Unidentified residue. Residues 1 and 16 in GR-1H could not be identified unequivocally, and might be M and L, respectively. P.a., *P. aeriginosa*; E.c., *E. coli*. Homologies to red spruce GR-2 are in boldface, homologies to GR-1H not found in GR-2 are underlined. The pea sequence starts at residue 63, after the presumed cleavage site for a chloroplastic transit peptide. The Scots pine sequence is taken from Wingsle (1990); the other sequences are part of full-length sequences taken from Creissen et al. (1992).

GR-2H. The antiserum recognized the SDS-denatured form of GR-1H but not of GR-2H (Fig. 7).

DISCUSSION

Purification of GR Isozymes from Red Spruce Needles

GR has been purified from a variety of plant tissues (see Smith et al., 1989), including conifers (Wingsle, 1989; Anderson et al., 1990). In several cases, GR was found to exist in two forms (Drumm-Herrel et al., 1989; Anderson et al., 1990; Edwards et al., 1990). In this instance a correlation has been made between the state of cold hardiness of conifer needles and their GR population. GR-2NH and GR-2H were purified to homogeneity by ammonium sulfate precipitation and three chromatography steps. Taking into account that ammonium sulfate fractionation presents a 2-fold purification, the purification factor for GR-2H is 400-fold, which is in agreement with the purification of the major GR isozyme achieved for eastern white pine (Anderson et al., 1990). The

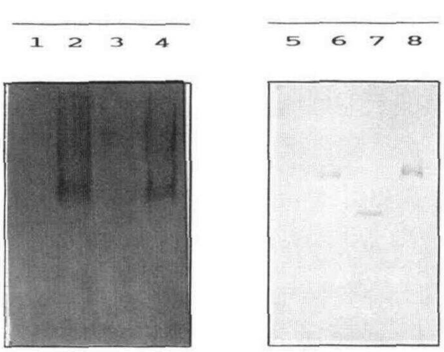

Figure 6. Immunoblots of purified red spruce GR isozymes after electrophoretic separation by native (A) or SDS (B) PAGE on 7.5 and 9% gels, respectively. Lane 1, 700 ng of GR-1NH; lane 2, 140 ng of GR-2NH; lane 3, 1000 ng of GR-1H; lane 4, 150 ng of GR-2H; lane 5, GR-1NH; lane 6, GR-2NH; lane 7, GR-1H; lane 8, GR-2H. In B, 0.02 milliunits of GR activity were loaded in each lane. The blots were probed with anti-GR-2H (polyclonal).

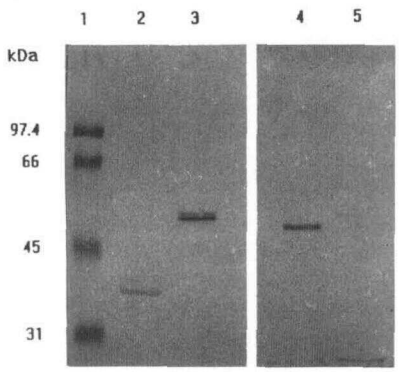

Figure 7. Immunoblots of purified red spruce GR isozymes after electrophoretic separation by SDS-PAGE (10% acrylamide). Lane 1, Molecular mass standards (prestained); lanes 2 and 4, 1000 ng of partially purified GR-1H; lanes 3 and 5, 157 ng of purified GR-2H. Lanes 1 through 3 were probed with anti-GR-2H (polyclonal); lanes 4 and 5 were probed with anti-GR-1H (polyclonal).

purification of both GR-2NH and GR-2H to homogeneity is consistent with many reports on the purification of plant GRs (Halliwell and Foyer, 1978; Wingsle, 1989; Anderson et al., 1990). However, some authors report contaminating proteins after similar purification procedures (Kalt-Torres et al., 1984).

Our results show evidence for some contaminating proteins after these procedures in the case of GR-1NH and GR-1H. As was the case for GR-1NH and GR-1H in this study, Connell and Mullet (1986) found a 32-kD protein that co-purified with pea chloroplast GR. Since GR-1NH and GR-1H were available only in limited quantities, no attempts were made to further purify these isozymes.

Subunit Composition of GR-1H

Mahan and Burke (1987) found 32/34-kD proteins in equimolar amounts to 63/65-kD proteins in GR purified from corn mesophyll chloroplasts and concluded that GR is a tetramer of four dissimilar polypeptides. The proteins of approximately 30 kD found in GR-1H preparations from red spruce do not seem to be associated with GR activity, however, since SDS electrophoresis of GR eluted from native gels produced only one band. Therefore, it can be concluded that GR-1H is composed of identical subunits and that the lower molecular mass proteins are contaminants.

Subunit Composition of GR-2H

Kalt-Torres et al. (1984) and Mahan and Burke (1987) report the occurrence of double bands on SDS gels, as has been found for GR-2H in this study. These authors suggested that GR is composed of dissimilar subunits, but did not address the possibility of multiple isoforms. The separation of GR-2H on two-dimensional IEF/SDS gels showed all charge isomers to be composed of proteins with molecular masses of 56 and 57 kD. Therefore, it seems unlikely that the double bands of GR-2H found on one-dimensional SDS gels result from the presence of isoforms with slightly different molecular masses. The double bands are of equal intensity (cf. Kalt-Torres et al., 1984; Mahan and Burke, 1987), which makes proteolytic cleavage of part of the enzyme preparation during purification unlikely and supports the hypothesis that different subunits are present. Mahan and Burke (1987) found no change in the banding pattern on SDS gels upon inclusion of protease inhibitors in the extraction medium, and Schirmer and Krauth-Siegel (1989) point out that human GR is extremely resistant to proteolytic cleavage.

A 30-kD Protein Immunologically Related to GR-2H

On immunoblots of GR-1H, a peptide of about 30 kD was found that cross-reacts with the antiserum raised against purified GR-2H. Since the preparations of GR-1H and GR-1NH have not been purified to homogeneity, this peptide may be a contaminant with a closely related amino acid sequence. This could be due either to a breakdown product of GR itself or to other flavoprotein-disulfide oxidoreductases, which show considerable sequence homology to GR (Perry et al., 1991). This 30-kD peptide seems to be related only to GR-2H, since it was not recognized by the antiserum against GR-1H. Although Connell and Mullet (1986) reported a 32-kD peptide that co-purified with GR and did not cross-react with antiserum raised against homogeneous GR, Mahan and Burke (1987) showed proteins of about 30-kD in pea leaves that cross-reacted with antiserum raised against spinach GR. Aono et al. (1991) presented western blots in which antiserum raised against *E. coli* GR cross-reacted with a 30-kD peptide in tobacco leaves but not with tobacco GR. Several hypotheses have been presented by these authors concerning the nature of this 30-kD peptide, but to date no definite conclusion seems to be warranted. The fact that this protein is not present in the preparation of GR-1NH suggests that there may also be seasonal changes in this 30-kD protein. The purification procedures for hardened and nonhardened needles were identical; therefore, it is unlikely that the protein was removed during purification from nonhardened needles.

N-Terminal Sequences of GR-1H and GR-2H

GRs from human erythrocytes and from *E. coli* have been extensively studied by x-ray crystallography (Karplus and Schulz, 1987; Ermler and Schulz, 1991). It has been shown for the human enzyme that the 17 N-terminal residues are not involved in either catalytic function or protein structure (Karplus and Schulz, 1987). Therefore, it may be speculated that homologies in this region of the protein express evolutionary distance more accurately than those within other regions, where functional or structural constraints restrict an evolutionary divergence of the sequences. The homologies in the N-terminal sequences of GRs are between 30 and 40%, whereas comparison of the complete sequences shows about 60% sequence homology (Perry et al., 1991; Creissen et al., 1992). The high degree of homology between N-terminal sequences of GR-2H from red spruce and Scots pine GR (Wingsle, 1990) suggests that these proteins are more closely related than are GR-1H and GR-2H, whose homology is within the range of 30 to 40% found between plant, prokaryotic, and human GRs. The N-terminal sequence of *Pseudomonas aeruginosa* GR seems to be more closely related to

those of the plant enzymes than to those of *E. coli* or human GR, with the exception of red spruce GR-1H. GR-1H is also the exception when compared with *E. coli* or human GRs, and it must therefore be seen as the GR with the greatest divergence from other GR sequences. Residues 16 to 19 of GR-1H and 22 to 25 of GR-2H extend into the flavin adenine dinucleotide-binding domain of the enzyme (Karplus and Schulz, 1987), which is highly conserved among organisms (Creissen et al., 1992). GR-2H shows the consensus sequence Ile-Gly-Ala-Gly in this region, but two nonconservative substitutions are found in GR-1H, where this sequence is changed to Ile-Pro-Ala-Ile. Although this change may be expected to have some influence on binding of the prosthetic group, an interpretation of these substitutions will have to await further biochemical and sequencing data.

For residue 1 of GR-1H, Lys and Met eluted in about equal amounts. If Met were the first amino acid of GR-1H, it would represent the transcription start site and indicate that the protein is cytosolic, since organellar proteins lose this residue after cleavage of the transit peptide. The possibility of residue 16 being either Leu or Ile has less importance for the interpretation of the sequencing data, since both amino acids have similar properties and no difference in the homology of GR-1H to the other sequences would result. There is some indication that residue 15 of GR-1H, for which no assignment was made, is Val. Val is also found in *P. aeruginosa* and human GRs in this position.

Cold-Hardiness-Specific GR Isozymes

Only one GR isoform appears to be present in animals (Schirmer and Krauth-Siegel, 1989), but it is now well established that GR in plants occurs in several distinct isoforms (Guy and Carter, 1984; Drumm-Herrel et al., 1989; Anderson et al., 1990; Edwards et al., 1990). A first indication of changes in GR isozyme composition in relation to cold tolerance has been presented by Guy and Carter (1984), who found different banding patterns on GR activity-stained gels when cold-hardened or nonhardened spinach tissue was used. In a previous publication we showed that the change in GR isoform composition, as judged by anion-exchange chromatography, correlates with the state of cold hardiness and dormancy/active growth in red spruce (Doulis et al., 1993). In this study we provide further evidence for hardiness-specific GRs after purification and characterization of the isozymes. Kinetic (Hausladen and Alscher, 1994) and immunological properties, as well as chromatographic and electrophoretic behavior of the red spruce GRs, support the conclusion that GR-1NH or GR-1H are distinct from each other and from both GR-2NH and GR-2H. However, since none of the methods used here showed differences between GR-2NH and GR-2H, it remains to be established whether these isozymes are distinct from each other or identical. The fact that antiserum raised against GR-2H does not cross-react with GR-1H, and vice versa, suggests that considerable structural differences exist between the isozymes, and from the N-terminal sequencing data it is clear that GR-1H and GR-2H are different gene products.

It is not yet clear if this cold-hardiness-specific change in isozyme composition is specific to spruce. Anderson et al. (1990) have found seasonal changes in GR activities in pine but no corresponding change in GR isoform composition.

The GR isoforms found in mustard have been proposed to be plastidic and cytosolic, based on the sensitivity of one form to photooxidation (Drumm-Herrel et al., 1989). Subcellular fractionation of pea tissue showed distinct isoforms of GR to be present in mitochondria, chloroplasts, and cytosol (Edwards et al., 1990). No attempts have been made to address the subcellular localization of the GRs in spruce needles because both approaches seemed impractical for this tissue. No indirect evidence for subcellular localization could be obtained from the properties of the isozymes. For chloroplastic enzymes, a higher pH optimum would be indicative of their localization, but all isozymes showed maximum activity around pH 7.5 (data not shown), which is consistent with pine but somewhat lower than for GRs from other plants (Anderson et al., 1990). The specificity for pyridine nucleotide could be another indicator of subcellular localization, but no isozyme showed activity with NADH as substrate. The absence of Met as the first residue in the N-terminal amino acid sequence of GR-2H is an indication that this isozyme has transversed an organellar membrane, because amino-terminal transit peptides are cleaved from proteins during transport across membranes.

Increases in total GR activity during cold hardening in conifers and broadleaf plants are well documented (Esterbauer and Grill, 1978; De Kok and Oosterhuis, 1983; Doulis et al., 1993). Along with increases in total GSH content (Hausladen et al., 1990; Madamanchi et al., 1991), this may ensure adequate protection against oxidative conditions encountered at low temperatures. The appearance of GR-1H coincides with the onset of cold hardening (Doulis et al., 1993). GR-1H can therefore be seen as a CAP in spruce. A number of proteins are specifically expressed during cold hardening in spinach (Guy et al., 1985; Guy and Haskell, 1987, 1989), and although the functional differences from the other GR isozymes remain unclear at this point, GR-1H is the CAP of known physiological function. Analogous to the CAP GR-1H, GR-1NH may be a GR that provides an advantage at high temperatures.

Clearly, further biochemical study is necessary to characterize functional differences among the hardiness-specific GR isozymes. The development of gene-specific probes will provide further information concerning timing and induction of GR gene expression. In light of the wealth of structural information available on GRs, sequences of the red spruce GR isozymes may allow some predictions concerning their properties. The seasonal changes of GR isozymes in red spruce offer a system that allows the study of the expression of these isozymes during hardening and dehardening, and in response to a range of environmental stimuli.

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