Plant Physiol. (1982) 70, 418–423
0032-0889/82/70/0418/06/$00.50/0

Structural Changes in Thylakoid Proteins during Cold Acclimation and Freezing of Winter Rye (*Secale cereale* L. cv. Puma)[1]

Received for publication October 26, 1981 and in revised form March 30, 1982

MARILYN GRIFFITH[2], GREGORY N. BROWN, AND NORMAN P. A. HUNER
Department of Forest Resources, University of Minnesota, St. Paul, Minnesota 55108 (M. G., G. N. B.); and Department of Plant Sciences, University of Western Ontario, London, Ontario N6A 5B7 Canada (N. P. A. H.)

ABSTRACT

Thylakoids were isolated from nonhardened and cold-hardened winter rye (*Secale cereale* L. cv. Puma), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence and absence of sulfhydryl reagents. Electrophoresis of cold-hardened rye thylakoid proteins revealed the presence of a 35 kilodalton polypeptide and the absence of a 51 kilodalton polypeptide found in nonhardened rye thylakoid proteins. The 35 kilodalton band could be induced by adding β-mercaptoethanol to nonhardened rye thylakoid proteins, whereas the 51 kilodalton band could be formed by adding cupric phenanthroline to these same proteins. Sulfhydryl group titration showed that cold-hardened rye thylakoid proteins contained more free sulfhydryls than nonhardened rye proteins. Although amino acid analysis of thylakoid proteins revealed quantitative differences in several amino acid residues, the polarity of thylakoid proteins did not change during cold acclimation. No significant changes in sodium dodecyl sulfate-polyacrylamide gels of thylakoid proteins appeared when either nonhardened or cold-hardened plants were frozen *in vivo* or *in vitro*. However, thylakoid proteins did aggregate when frozen in the presence of β-mercaptoethanol. Although thylakoid proteins isolated from cold-hardened rye contained more reduced thiols, a general state of reduction did not act as a cryoprotectant. It is hypothesized that conformational changes of specific proteins may be important for low temperature growth of rye.

Functional studies have shown that thylakoid membranes are subject to freezing injury. Heber and Santarius (10, 12) demonstrated that freezing uncoupled photophosphorylation from electron transport, inactivated the light-dependent ATPase, and increased proton permeability of washed spinach thylakoids.

Several investigators have searched for a compositional or structural alteration in frozen thylakoid membranes which could account for these functional changes. Using negative staining techniques for the electron microscope, Garber and Steponkus (7) observed that CF1[3] particles were released from spinach thylakoids frozen *in vitro*. However, Heber (10) found that the addition of EDTA-solubilized CF1 to freeze-thawed spinach thylakoids did not reconstitute photophosphorylation. Volger and coworkers (34) supported this conclusion when they reported that inactivation of cyclic photophosphorylation preceded release of CF1 subunits during freezing of spinach thylakoid suspensions. After showing that the three high mol wt subunits of CF1 are released from pyrophosphate-washed spinach thylakoids by freezing, Lineberger and Steponkus (21) also encountered difficulties in reconstituting photophosphorylation and in accounting for increased proton permeability of frozen thylakoids. Apparently, freezing thylakoids leads to uncoupling of photophosphorylation through both inactivation of CF1 and loss of the proton gradient across these membranes.

Studies of thylakoids isolated from cold-acclimated plants have shown them to be more resistant to the effects of freezing. Senser and Beck (29), for example, reported that thylakoid membranes isolated from winter hardy spruce needles exhibited an increased capacity for cyclic photophosphorylation. Garber and Steponkus (8) also observed that thylakoids isolated from cold-hardened spinach required less sucrose than nonhardened spinach thylakoids to recover light-dependent proton uptake after freezing. Compositional or structural alterations of thylakoid membranes would seem to be prerequisities for maintenance of photophosphorylation at low temperatures. Yet surveys of the protein complement of thylakoid membranes have failed to show gross compositional changes during cold acclimation of the plants (6, 13). Heber (11), though, has isolated two soluble cryoprotective proteins from hardened rye leaves. These proteins have been characterized as hydrophilic, 10 to 20 kd in mol wt, and more effective than sugars in protecting the photophosphorylating capacity of thylakoids after freezing (33). Using freeze-fracture techniques for the electron microscope, Garber and Steponkus (8) observed that particles on the inner fracture face of spinach thylakoids changed from a heterogeneous population of two size groups to a uniform intermediate particle size upon cold acclimation. However, the role of either the cryoprotective proteins or the freeze-fracture particles in freezing resistance has not been well defined.

Recently, Huner and co-workers (14, 16) have shown that ribulose bisphosphate carboxylase undergoes conformational changes upon cold acclimation in winter rye. These changes in the tertiary structure of the enzyme lead to a more stable protein at low temperatures and can be detected by electrophoresing the enzyme in the presence and absence of reducing agent. The purpose of the experiments described in this report was to investigate the possibility that conformational rather than compositonal changes in thylakoid proteins are important for cold acclimation of winter rye.

[1] This paper is a contribution from the Minnesota Agricultural Experiment Station. Journal Series Article No. 11,991.

[2] Present address: Department of Botany, University of British Columbia, Vancouver, B.C. V6T 2B1 Canada.

[3] Abbreviations: CF1, coupling factor 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; D/N, day/night; RNH, nonhardened rye; RH, cold-hardened rye; RNHF, nonhardened frozen rye; RHF, cold-hardened frozen rye; CuP, cupric phenanthroline; DTNB, 5-5′-dithiobis (2-nitro-benzoic acid); LHC, light-harvesting complex; βSH, β-mercaptoethanol; SH, sulfhydryl group.

MATERIALS AND METHODS

Plant Materials. Winter rye (*Secale cereale* L. cv. Puma) plants were grown in coarse vermiculite, watered with modified Hoagland solution, and provided with an illumination of 450 μE m^{-2} s^{-1} during a 16-h photoperiod (15). All plants were grown at 25/20°C D/N temperatures for 1 week. Control plants then remained at 25/20°C D/N for 3 week (RNH), whereas plants to be cold hardened (RH) were transferred to 5/2°C D/N temperatures for 12 weeks (13).

The ability of winter rye leaves to tolerate freezing was determined by changes in electrical conductivity (5). Wetted leaf sections (15 mm) were inoculated with ice at 0°C, cooled at 3°C/h, and allowed to thaw at 2°C overnight. After a 24-h equilibration period in deionized H_2O, conductivity of the leaf sections was measured before and after boiling. Freezing injury was defined as a loss of 50% of total solutes. Thus, the temperature of freezing injury was −9°C for RNH leaves, and −18°C for RH leaves.

Freezing. For some experiments, pots of nonhardened and cold-hardened rye plants were frozen *in vivo* prior to thylakoid isolation. RNHF plants were frozen to −20°C, and RHF plants to −70°C, by placing pots in an appropriate freezer for 16 h. Plants were thawed quickly at room temperature. All plants died, as determined by regrowth. *In vitro* freezing was accomplished be resuspending thylakoid pellets in 0.05 M triethanolamine (pH 8.5), and placing them in a freezer at −20°C for 16 h.

Thylakoid Isolation. Chloroplasts were isolated from fully expanded leaves of RNH and RH plants which had been destarched by placing them in a darkened cabinet for 25 h. Rye leaves were chilled, cut, and briefly ground with a mortar and pestle in a continuous flow of isolation medium (13) containing sorbitol, 2 mM EDTA, 2 mM ascorbic acid, and 25 mM Hepes-NaOH at pH 7.6 (1). The isolation medium contained 0.4 M sorbitol for RNH leaves, and 0.7 M sorbitol for RH leaves (15). The brei was filtered through 16 layers of cheesecloth, and the filtrate was centrifuged at 300*g* for 1 min. The pellet was discarded, and the supernatant was recentrifuged at 1,500*g* for 5 min to obtain intact chloroplasts. Phase contrast microscopy (30) revealed that preparations from both RNH and RH contained about 70% class I chloroplasts.

Chloroplasts were resuspended in 5 ml of bursting medium containing 50 mM Hepes-NaOH (pH 7.6), 25 mM KCl, and 3.5 mM $MgCl_2$, for 0.5 h. Approximately 80% of the chloroplasts now were judged to be class II. Following the addition of 15 ml 30% (w/v) sucrose in resuspension buffer (66 mM Hepes-NaOH [pH 7.6], 50 mM KCl, 3.3 mM $MgCl_2$), the thylakoids were pelleted by centrifugation at 8,000*g* for 15 min. The thylakoid pellet was resuspended in 23% sucrose in resuspension buffer, layered on a discontinuous gradient of 60% and 42% sucrose in resuspension buffer, and centrifuged at 73,000*g* for 1.5 h. The 42% sucrose band containing thylakoids was removed from the gradient, diluted with resuspension buffer, and pelleted at 73,000*g* for 0.5 h. Washed thylakoid pellets were used immediately for protein separations.

Protein and Chl Determinations. Protein contents were determined by the method of Lowry *et al.* (22). Chl was determined according to Bruinsma (2).

Sulfhydryl Reagents. Where noted, β-mercaptoethanol was added to thylakoid preparations in a final concentration of 5% (v/v). Cupric phenanthroline was prepared by mixing equal volumes of 13.6 mM *O*-phenanthroline and 6.8 mM cupric sulfate. Thylakoids suspended in triethanolamine buffer were incubated with CuP for 0.5 h at 22°C. The reaction was stopped by adding 10 mM EDTA (27).

SDS-PAGE. Samples were prepared for electrophoresis by resuspending thylakoids in 0.05 M triethanolamine buffer (pH 8.5) (27). SDS (4%, w/v) was added just before electrophoresis. Slab gels (0.75 mm) were prepared in duplicate according to Studier (31), using the SDS buffer system described by Laemmli (19). Separating gels contained 15% (w/v) polyacrylamide (30:0.8 ratio of acrylamide:*N*,*N*′-methylenebisacrylamide) and 1% (w/v) SDS. Electrophoresis was performed at 16 mamp at 22°C for 4.5 h. Proteins were fixed in 10% (w/v) TCA for 30 min, stained with 0.1% (w/v) Coomassie Brilliant Blue R for 15 min at 60°C, and destained in 10% (v/v) acetic acid for 1 to 2 h at 60°C. Molecular masses were estimated by comparing mobilities of thylakoid proteins with those of proteins of known molecular mass according to the method of Weber and Osborn (35). Marker proteins included albumin (66 kd), ovalbumin (45 kd), pepsin (34.7 kd), trypsinogen (24 kd), β-lactoglobulin (18.4 kd), and lysozyme (14.3 kd). The entire procedure from isolation of thylakoids to electrophoresis of polypeptides was repeated three times for each of the treatments (RNH, RH, RNHF, RHF).

Isolation and SDS-PAGE of CF1. CF1 was extracted from RNH thylakoids using the chloroform method followed by purification on a sucrose gradient as described by Moase and Green (26). CF1, RNH thylakoids, and markers were subjected to SDS-PAGE as described above, except that 1.5-mm slab gels containing 10% polyacrylamide were used.

Amino Acid Analyses. Thylakoid pellets were extracted twice with 80% (v/v) acetone, followed by two extractions with chloroform:methanol (1:2). The remaining proteins were dried, then hydrolyzed with boiling HCl at 110°C for 25 h in sealed, evacuated ampoules. After removing HCl under vacuum, residues were resuspended in 0.2 M citrate buffer (pH 2.2), and analyzed for amino acid compositions using a Beckman 119 Automatic Amino Acid Analyzer.

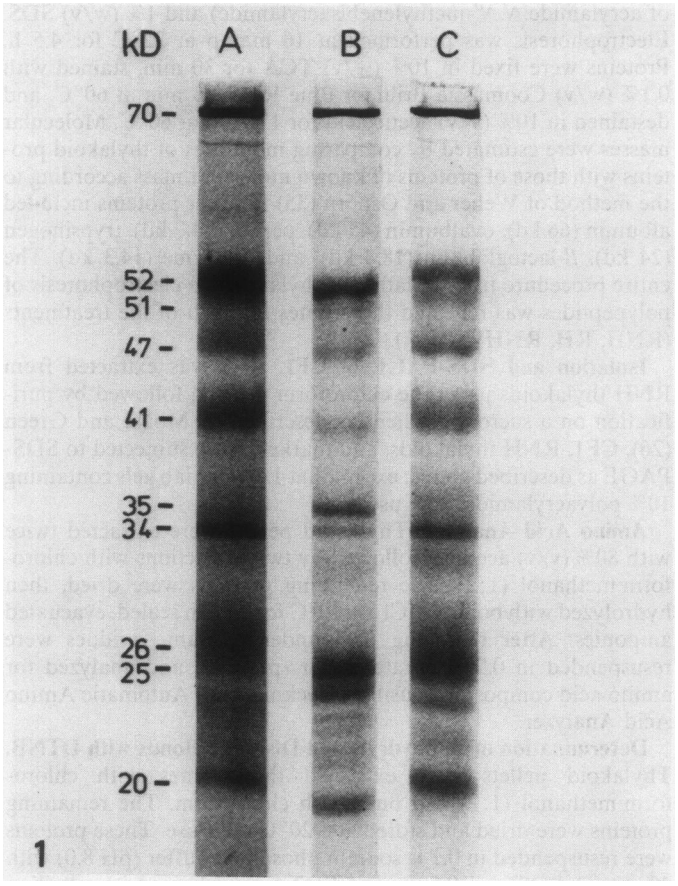
Determination of Sulfhydryls and Disulfide Bonds with DTNB. Thylakoid pellets were extracted three times with chloroform:methanol (1:2) and once with chloroform. The remaining proteins were dried and stored at −20°C until use. These proteins were resuspended in 0.1 M sodium phosphate buffer (pH 8.0) with 2% (w/v) SDS and 0.5 mg/ml EDTA, and heated in a boiling water bath for 5 min. To determine protein sulfhydryl content, 0.95 ml protein solution and 0.05 ml 10 mM DTNB in 0.1 M sodium phosphate buffer (pH 8.0) were mixed. The color was allowed to develop for 30 min before being read at 410 nm against a protein solution blank. Sulfhydryl content was calculated using a molar absorptivity value of 13,600 M^{-1} cm^{-1} (9). To determine protein disulfide bonds, 1.5 ml protein solution, 1 ml 2.5% $NaBH_4$, and 0.5 ml H_2O were mixed, incubated at 38°C for 30 min, and then mixed with 0.5 ml 1 M KH_2PO_4 containing 0.2 N HCl. After 5 min, 2 ml acetone were added, then the mixture was flushed with N_2 for 5 min. Following the addition of 0.5 ml 0.01 M DTNB and 0.5 ml H_2O, the mixture was again flushed with N_2 for 2 min, allowed to stand for 15 min, and read at 412 nm in a spectrophotometer. A molar absorptivity of 12,000 M^{-1} cm^{-1} was used for calculating the number of −SH groups after reduction (9).

RESULTS

Thylakoid Membranes. Thylakoids from both hardened and nonhardened rye were characterized by protein:Chl ratios and by Chl *a*:*b* ratios. No significant change in either the protein:Chl ratios (RNH, 6.8 ± 2.9; RH, 7.1 ± 1.2) or the Chl *a*:*b* ratios (RNH, 3.3 ± 0.1; RH, 3.3 ± 0.3) occurred upon cold acclimation of winter rye.

Thylakoid Polypeptides during Cold Acclimation. SDS-PAGE resolved 15 polypeptide bands (Fig. 1) from winter rye thylakoids. Although these polypeptides were only partially denatured, as evidenced by the presence of three Chl-protein complexes, the banding patterns were reproducible in all six gels associated with each treatment.

Cold acclimation of winter rye resulted in several qualitative changes in the banding patterns of SDS-polyacrylamide gels. A 51-kd band apparent in RNH thylakoid proteins was not visible in RH thylakoid proteins (Fig. 1 and 2). Furthermore, a 35-kd band apparent in RH thylakoid proteins could not be discerned



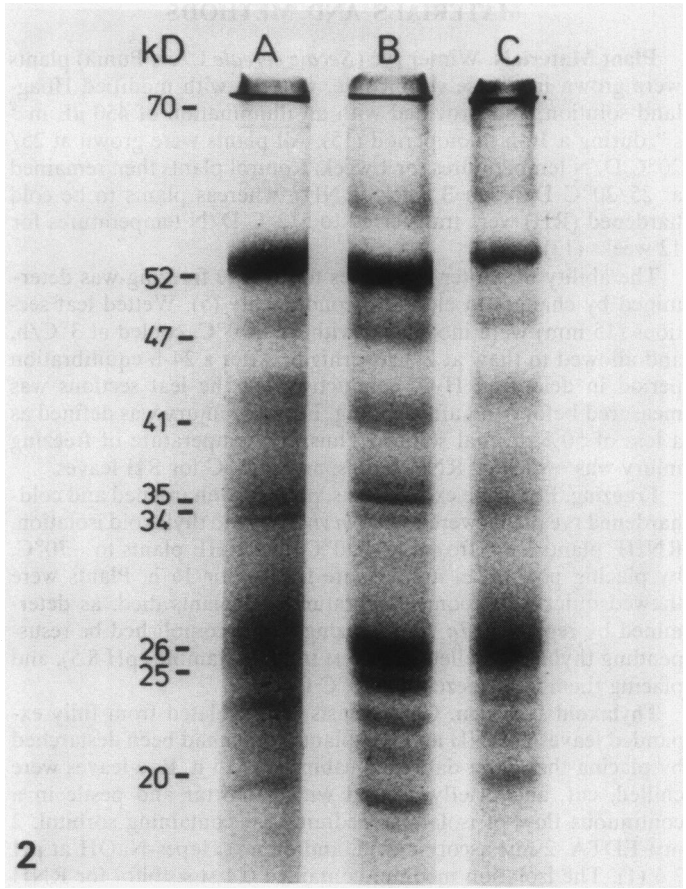


FIG. 1. Effects of sulfhydryl reagents on thylakoid proteins isolated from nonhardened winter rye. Proteins were subjected to electrophoresis on 15% polyacrylamide slab gels containing 1% SDS and stained with Coomassie Brilliant Blue. Bands are labeled by apparent molecular mass. A, RNH thylakoids; B, RNH thylakoids + β-mercaptoethanol; C, RNH thylakoids + β-mercaptoethanol + cupric phenanthroline.

FIG. 2. Effects of sulfhydryl reagents on thylakoid proteins isolated from cold-hardened winter rye. Proteins were subjected to electrophoresis on 15% polyacrylamide slab gels containing 1% SDS and stained with Coomassie Brilliant Blue. A, RH thylakoids; B, RH thylakoids + β-mercaptoethanol; C, RH thylakoids + β-mercaptoethanol + cupric phenanthroline.

in gels of RNH thylakoid proteins.

Sulfhydryl effects were observed in both RNH and RH thylakoid proteins. Addition of β-mercaptoethanol to both RNH and RH thylakoids prior to electrophoresis resulted in an increase in free Chl and resolution of the 26- and 25-kd bands. Two new bands were observed after electrophoresis in the presence of β-mercaptoethanol; the 35-kd band in RNH thylakoid proteins and the 36-kd band in RH thylakoid proteins.

Effects caused by β-mercaptoethanol could be reversed using CuP. When CuP was added in excess of φ-mercaptoethanol, the 26- and 25-kd bands merged into one large band in both RNH and RH thylakoid proteins. The 35-kd band in RNH and RH thylakoid proteins and the 36-kd band in RH thylakoid proteins disappeared upon addition of CuP. An increase in staining intensity of the 51-kd band from RNH thylakoid proteins was observed after addition of β-mercaptoethanol and an excess of CuP.

Thylakoid Polypeptides after Freezing. When RNH and RH thylakoids were frozen and thawed *in vivo*, thylakoid proteins separated by SDS-PAGE exhibited no differences other than those associated with cold acclimation (data not presented). Addition of β-mercaptoethanol to preparations of RH and RNH thylakoids after *in vivo* freezing resulted in a decrease in the amount of 34-kd polypeptide with a corresponding increase in the amount of 35-kd polypeptide. This effect is similar to that described for unfrozen RNH and RH thylakoids in the presence of reducing agent.

In contrast, when RNH and RH thylakoids were frozen and thawed *in vitro*, there was an increase in the 35-kd polypeptide in the absence of β-mercaptoethanol (Fig. 3). In addition, a new, minor protein band which migrated with an apparent mol wt of 36 kd appeared in RH thylakoid preparations, but not in RNH preparations (data not presented). Upon addition of β-mercaptoethanol to these frozen and thawed thylakoid samples, SDS-PAGE showed similar results to those obtained for unfrozen RNH and RH thylakoids in the presence of reducing agent.

However, when RNH and RH thylakoids were frozen and thawed *in vitro* in the presence of β-mercaptoethanol, drastic changes were noticed in the polypeptide patterns. Chl was lost from the 70- and 41-kd polypeptides, which resulted in the presence of their apoproteins migrating as 56 and 141 kd, respectively, in both RNH and RH thylakoid preparations. In addition, large mol wt aggregates were apparent, the amount of the 34-kd polypeptide decreased with a corresponding increase in the 35-kd polypeptide, the amount of the 47-kd band was reduced, and the 25- and 26-kd protein bands were resolved in both RNH and RH preparations.

Amino Acid Composition. The amino acid composition of winter rye thylakoid proteins (Table I) was similar to those of thylakoid proteins isolated from *Vicia* (25) and spinach (17). During the cold acclimation, however, the amino acid composition of rye thylakoid proteins changed; leucine, alanine, and glutamate residues all increased, while methionine and proline decreased.

The polarity of thylakoid proteins was calculated using the method of Capaldi and Venderkooi (3). Thylakoid proteins iso-

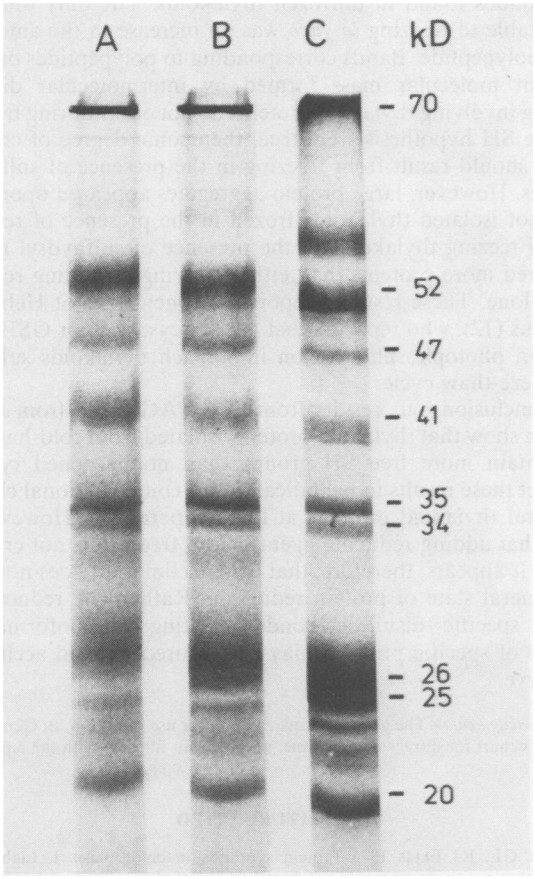


FIG. 3. Effects of freezing *in vitro* on thylakoid proteins isolated from nonhardened winter rye. Proteins were subjected to electrophoresis on 15% polyacrylamide slab gels containing 1% SDS and stained with Coomassie Brilliant Blue. A, RNH thylakoids frozen *in vitro*; B, RNH thylakoids frozen *in vitro*, then β-mercaptoethanol was added; C, RNH thylakoids frozen *in vitro* in the presence of β-mercaptoethanol.

Table I. *Amino Acid Composition of Thylakoid Proteins Isolated from Nonhardened and Cold-Hardened Winter Rye*

Relative molar ratios of phenylalanine.

Amino Acid	RNH		RH	
	Mean[a]	SD	Mean	SD
Met[b]	0.26	0.02	0.21	0.00
Leu[b]	1.68	0.05	1.84	0.05
Ala[b]	1.59	0.06	1.76	0.06
Glu[b]	1.49	0.05	1.62	0.03
Pro[c]	0.99	0.06	0.64	0.03
Gly	1.62	0.02	1.63	0.05
Ilu	0.79	0.04	0.77	0.02
Val	0.61	0.02	0.70	0.05
Asp	1.30	0.05	1.33	0.03
Thr	0.69	0.04	0.66	0.06
Ser	0.60	0.01	0.58	0.02
Tyr	0.48	0.01	0.48	0.01
Phe	1.00		1.00	
Lys	0.59	0.01	0.62	0.03
His	0.20	0.03	0.26	0.03
Arg	0.60	0.02	0.64	0.02

[a] $n = 3$.
[b] Significantly different at $p = 0.05$.
[c] Significantly different at $p = 0.01$.

Table II. *Sulfhydryl Content of Thylakoid Proteins*

Source	—SH before Reduction of Disulfide Bonds		—SH after Reduction of Disulfide Bonds	
	Mean[a]	SE	Mean	SE
	nmol —SH/mg protein			
RNH	9.2[b]	1.9	11.3	4.9
RH	15.3[b]	0.3	20.2	1.4

[a] $n = 3$.
[b] Significantly different at $p = 0.01$.

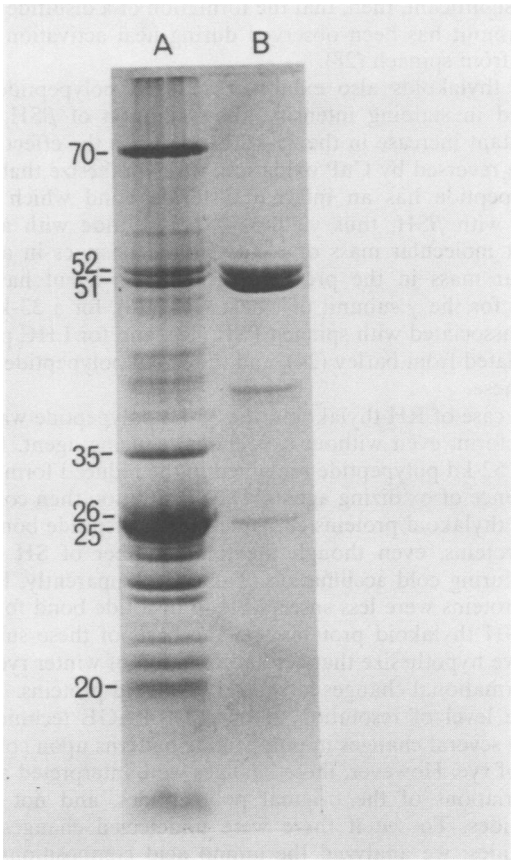


FIG. 4. Identification of CF1 subunits. Proteins were electrophoresed on 10% polyacrylamide slab gels containing 1% SDS and stained with Coomassie Brilliant Blue. A, RNH thylakoids + β-mercaptoethanol; B, CF1 + β-mercaptoethanol.

lated from RNH were calculated to contain 37.7 ± 0.3% polar amino acid residues. Hardened rye thylakoid proteins exhibited a polarity value of 38.8 ± 0.6%.

Sulfhydryl Content. DTNB titration revealed an increase in sufhydryl content of thylakoid proteins as winter rye acclimated to colder temperatures (Table II). The sulfhydryl content of RNH and RH thylakoid proteins was not significantly different following reduction of disulfide bonds by $NaBH_4$.

DISCUSSION

Fifteen polypeptides were resolved from rye thylakoid proteins by SDS-PAGE and characterized by apparent molecular mass. Three Chl-protein bands, detected at 70, 41, and 25 kd, were cut out and scanned using a Unicam Sp 1750 spectrophotometer (data not presented). The 70-kd band was a polypeptide complexed with Chl *a* and associated with PSI, while the 56-kd band represented its apoprotein (23). A polypeptide binding both Chl *a* and *b*

constituted the 41-kd band. Two of the polypeptides comprising the light harvesting Chl *a/b*-protein complex were thought to correspond to bands at 26 and 25 kd (32).

By electrophoresing purified CF1 and RNH thylakoids on adjoining lanes, the 52- and 51-kd polypeptides were identified as the *a* and *b* subunits of CF1 (Fig. 4). The 51-kd polypeptide was never resolved as a separate band in RH thylakoid preparations. However, the 51-kd band became more prominent in RNH thylakoids subjected to oxidation by CuP. This suggests that an additional disulfide bond can be formed in the β subunit of CF1 from RNH thylakoids, resulting in a more compact polypeptide that migrates with a lower apparent molecular mass of 51 kd. It may be significant, then, that the formation of a disulfide bond in the β subunit has been observed during heat activation of CF1 isolated from spinach (28).

RNH thylakoids also exhibited a 34-kd polypeptide which decreased in staining intensity upon addition of βSH, with a concomitant increase in the 35-kd band. Since the effect of βSH could be reversed by CuP oxidation, we hypothesize that the 34-kd polypeptide has an internal disulfide bond which can be reduced with βSH, thus yielding a polypeptide with a higher apparent molecular mass of 35kd. Similar changes in apparent molecular mass in the presence of reducing agent have been reported for the γ subunit of wheat CF1 (26), for a 33-kd polypeptide associated with spinach PSII (18), and for LHC polypeptides isolated from barley (24), and the 34-kd polypeptide may be one of these.

In the case of RH thylakoids, the 35-kd polypeptide was in the reduced form, even without exogenous reducing agent. In addition, the 52-kd polypeptide remained in the reduced form even in the presence of oxidizing agents. DTNB titration then confirmed that RH thylakoid proteins contained fewer disulfide bonds than RNH proteins, even though the total number of SH did not change during cold acclimation (Table II). Apparently, RH thylakoid proteins were less susceptible to disulfide bond formation than RNH thylakoid proteins. On the basis of these sulfhydryl effects, we hypothesize that cold acclimation of winter rye results in conformational changes in several thylakoid proteins.

At the level of resolution of our SDS-PAGE technique, we observed several changes in polypeptide patterns upon cold acclimation of rye. However, these changes were interpreted as structural variations of the original polypeptides, and not as new polypeptides. To see if there were undetected changes in the polypeptides, we analyzed the amino acid compositions of rye thylakoid proteins. Increases in leucine, alanine, and glutamate residues, along with decreases in methionine and proline, were observed in RH thylakoid proteins. Although it was difficult to interpret this data in terms of individual polypeptides, we could calculate protein polarity. No significant differences appeared between RNH and RH thylakoid proteins. Apparently the increase in glutamate, a polar residue, was equivalent to increases in the nonpolar residues leucine and alanine. These results agree with Chou and Levitt (4), who found no change in hydrophobicity of either soluble and membrane proteins upon cold acclimation.

We examined the effect of thiol reagents on the polypeptides of RNH and RH thylakoids during freezing. Levitt (20) had hypothesized that freezing injury resulted from intermolecular disulfide bonding of cellular proteins. At low temperatures, proteins would unfold reversibly, exposing free SH groups. As extracellular freezing concentrated the protoplasm, proteins would undergo SH oxidation or SH $\rightleftarrows$ SS or SS $\rightleftarrows$ SS interchange. When the cell thawed, its proteins would be irreversibly aggregated, thus killing the cell.

This hypothesis was tested by freezing thylakoids *in vivo* in the presence of stromal proteins, or *in vitro* in the absence of soluble proteins (Fig. 3). When RNH and RH thylakoids were frozen *in vivo*, SDS-PAGE revealed the same complement of thylakoid polypeptides found in unfrozen thylakoids. The only difference attributable to freezing *in vitro* was an increase in the amount of 35-kd polypeptide. Bands corresponding to polypeptides of larger apparent molecular mass formed by intermolecular disulfide bonding involving thylakoid proteins do not occur during freezing.

If the SH hypothesis were true, then some degree of cryoprotection should result from freezing in the presence of sulfhydryl reagents. However, large protein aggregates appeared upon SDS-PAGE of isolated thylakoids frozen in the presence of reducing agent. Freezing thylakoids in the presence of sulfhydryl reagent denatured more proteins than either freezing or adding reducing agent alone. These results support the conclusions of Heber and Santarius (12), who reported that neither cysteine nor GSH could maintain photophosphorylation in spinach thylakoids subjected to a freeze-thaw cycle.

In conclusion, our results from SDS-PAGE and from DTNB titration show that thylakoid proteins isolated from cold-hardened rye contain more free SH groups than nonhardened rye. We interpret these results to be indications of conformational changes in several thylakoid proteins at low temperatures. However, we found that adding reducing agent before freezing is not cryoprotective. It appears, therefore, that cold acclimation does not result in a general state of protein reduction. Rather, the reduction of several specific disulfide bonds resulting in conformational changes of specific proteins may be required by cold acclimated winter rye.

Acknowledgments—The authors thank P. H. Li for use of his lab, B. Gengenbach and B. Lockhart for ultracentrifuge time, and C. Chin for help with the amino acid analyses.

LITERATURE CITED

1. BLAIR GE, RJ ELLIS 1973 Protein synthesis in chloroplasts I. Light-driven synthesis of the large subunit of fraction 1 protein by isolated pea chloroplasts. Biochim Biophys Acta 319: 223–234
2. BRUINSMA J 1961 A comment on the spectrophotometric determination of chlorophyll. Biochim Biophys Acta 52: 576–578
3. CAPALDI RA, G VANDERKOOI 1972 The low polarity of many membrane proteins. Proc Natl Acad Sci USA 69: 930–932
4. CHOU JC, J LEVITT 1972 The hydrophobicity of proteins from hardy (freezing-resistant) and nonhardy species of grains. Cryobiology 9: 268–272
5. DEXTER ST, WE TOTTINGHAM, LF GRABER 1932 Investigations of hardiness of plants by measurement of electrical conductivity. Plant Physiol 7: 63–78
6. DEYOE DR 1977 Adaptive changes in Eastern white pine during winter. PhD Thesis. University of Missouri, Columbia
7. GARBER MP, PL STEPONKUS 1976 Alterations in chloroplast thylakoids during an *in vitro* freeze-thaw cycle. Plant Physiol 57: 673–680
8. GARBER MP, PL STEPONKUS 1976 Alterations in chloroplast thylakoids during cold acclimation. Plant Physiol 57: 681–686
9. HABEEB AFSA 1972 Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol 25B: 457–464
10. HEBER U 1967 Freezing injury and uncoupling of phosphorylation from electron transport in chloroplasts. Plant Physiol 42: 1343–1350
11. HEBER U 1970 Proteins capable of protecting chloroplast membranes against freezing. *In* GEW Wolstenholm, M O'Conner, eds, The Frozen Cell. J and A Churchill, London, pp 175–188
12. HEBER UW, KA SANTARIUS 1964 Loss of adenosine triphosphate synthesis caused by freezing and its relationship to frost hardiness problems. Plant Physiol 39: 712–719
13. HUNER NPA, FDH MACDOWALL 1976 Chloroplastic proteins of wheat and rye grown at warm and cold-hardening temperatures. Can J Biochem 54: 848–853
14. HUNER NPA, FDH MACDOWALL 1978 Evidence of an *in vivo* conformational change in ribulose bisphosphate carboxylase-oxygenase from Puma rye during cold adaptation. Can J Biochem 56: 1154–1161
15. HUNER NPA, JP PALTA, PH LI, JV CARTER 1981 Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Bot Gaz 142: 103–109
16. HUNER NPA, JV CARTER 1981 Reversible structural variation associated with the isolated subunits of RuBP carboxylase from cold-hardened and unhardened rye. Z Pflanzenphysiol In press
17. KIRK JTO, RAE TILNEY-BASSETT 1978 The Plastids. Elsevier/North-Holland Biochemical Press, Amsterdam, pp 19–35
18. KUWABARA T, N MURATA 1979 Purification and characterization of 33 kD protein of spinach chloroplasts. Biochim Biophys Acta 581: 228–236
19. LAEMMLI V 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685
20. LEVITT J 1962 A sulfhydryl-disulfide hypothesis of frost injury and resistance in

plants. J Theor Biol 3: 355–391
21. LINEBERGER RD, PL STEPONKUS 1980 Effects of freezing on the release and inactivation of chloroplast coupling factor 1. Cryobiology 17: 486–494
22. LOWRY OH, NJ ROSEBROUGH, A FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
23. MACHOLD O, DJ SIMPSON, BL MOLLER 1979 Chlorophyll-proteins of thylakoids from wild-type and mutants (*Hordeum vulgare* L.). Carlsberg Res Commun 44: 235–254
24. MACHOLD O 1982 Chlorophyll a/b-proteins and light-harvesting complex of *Vicia faba* and *Hordeum vulgare*. Biochem Physiol Pflanzen In press
25. MACKENDER RO 1978 The amino acid composition of the chloroplast envelope membranes of *Vicia faba* L. Plant Sci Lett 12: 279–285
26. MOASE EH, BR GREEN 1981 Isolation and properties of chloroplast coupling factor from wheat. Eur J Biochem 119: 145–150
27. NOVAK-HOFER I, PA SIEGENTHALER 1978 Chemical cross-linking of neighboring thylakoid membrane polypeptides. Plant Physiol 62: 368–372
28. RAVIZZINI RA, CS ANDREO, RH VALLEJOS 1980 Sulfhydryl groups in photosynthetic energy conservation. Biochim Biophys Acta 591: 135–141
29. SENSER M, E BECK 1977 On the mechanism of frost injury and frost hardening of spruce chloroplasts. Planta 137: 195–201
30. SPENCER D, H UNT 1965 Biochemical and structural correlations in isolated spinach chloroplasts under isotonic and hypotonic conditions. Aust J Biol Sci 18: 197–210
31. STUDIER W 1973 Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J Mol Biol 79: 237–248
32. SUSS KH 1980 Identification of chloroplast thylakoid membrane polypeptides. FEBS Lett 112: 255–259
33. Volger HG, U Heber 1975 Cryoprotective leaf proteins. Biochim Biophys Acta 412: 335–349
34. Volger H, U Heber, RJ Berzborn 1978 Loss of function of biomembranes and solubilization of membrane proteins during freezing. Biochim Biophys Acta 511: 455–469
35. WEBER K, M OSBURN 1969 The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244: 4406–4412