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STRUCTURAL AND FUNCTIONAL CHANGES IN RIBULOSE
BISPHOSPHATE CARBOXYLASE-OXYGENASE DURING THE COLD-
HARDENING OF PUMA RYE

by

NORMAN PETER ANDREW HUNER, B.Sc., M.Sc.

A thesis submitted to the Faculty of Graduate Studies
in partial fulfilment of the requirements for the
degree of Doctor of Philosophy

Carleton University
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September, 1978.

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FACULTY APPROVAL

The undersigned hereby recommend to the Faculty of
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Norman Peter Andrew Huner, B.Sc., M.Sc., in partial
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To Suzanne and Erin

ABSTRACT

Soluble proteins and membrane polypeptides were separated from chloroplasts isolated intact from a cultivar each of spring wheat, winter wheat and more freeze-resistant rye, and changes in them associated with cold-hardening were detected by means of polyacrylamide gel electrophoresis. No drastic changes in chloroplast membrane polypeptides occurred during growth at low temperatures in the three cultivars. However, subtle changes were evident in the soluble chloroplast protein fraction. A band of soluble protein which was present only after cold-hardening of Kharkov wheat and Puma rye was not present in extracts from cold-grown spring wheat. All unhardened material displayed two peaks in the region of Fraction 1 protein band with a concomitant decrease in ribulose biphosphate carboxylase activity whereas all cold-hardened material displayed one peak with no loss in enzyme activity.

Ribulose biphosphate carboxylase-oxygenase was purified to homogeneity from leaves of cold-hardened and unhardened Puma rye through gel filtration and ion exchange chromatography. The specific activity of the hardened form was consistently double that of the unhardened form. Both forms were separable on the

basis of charge with the pI values of 6.4 and 6.3 for the enzyme from hardened and unhardened rye respectively. Although the amino acid compositions were similar, the results of SH titration with 5,5-dithiobis (2-nitrobenzoic acid) showed that one half as many sulfhydryl groups were available in the cold-hardened form as in the unhardened form. Furthermore, the carboxylase activity and native structure of the enzyme from cold-hardened rye was less sensitive to sodium dodecyl sulfate than that from unhardened rye. SDS polyacrylamide gel electrophoresis showed that the quaternary structures of both enzymes consisted of large subunits (55,000 mol. wt.) and small subunits (14,000 mol. wt.). However, a dimer of the large subunit (110,000 mol. wt.) was consistently evident in enzyme samples from unhardened rye but never present in samples from hardened rye. Enzyme activity and large subunit structure of both enzymes were sensitive to NaCl which gave rise to an anomalous polypeptide of 47,000 mol. wt. In contrast to the enzyme from unhardened rye, the large subunit of the enzyme from hardened rye was not sensitive to pH, and was more stable in the absence of reducing agent and during freeze-thaw stress.

The kinetics of ribulose biphosphate carboxylase

indicated no change in the ~~enthalpy~~ of activation. The effects of pH on K_m CO_2 for the enzyme from hardened and unhardened rye showed that SH groups were probably involved in stabilizing the tertiary structure of the active site and that the ionization state of this chemical group ($SH \rightleftharpoons S^-$) was different. Furthermore, the enzyme from hardened rye had greater affinity for CO_2 below $10^\circ C$ whereas the form from unhardened rye had greater affinity for CO_2 at all temperatures above $10^\circ C$. Thus the temperature to which the rye plants were adapted had a profound effect on the efficiency of the carboxylation reaction. It is concluded that ribulose biphosphate carboxylase-oxygenase undergoes a conformational change which is stable to purification, most likely occurs in the large subunit and imparts significant structural and functional advantages to the enzyme and thus to the plant as a whole during the cold-hardening process in Puma rye.

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LIST OF ABBREVIATIONS

RH	cold-hardened Puma rye
RNH	unhardened Puma rye
KH	cold-hardened Kharkov winter wheat
KNH	unhardened Kharkov winter wheat
MH	cold-hardened Marquis spring wheat
MNH	unhardened Marquis spring wheat
RUBP	ribulose,1,5-bisphosphate
RUBPCase	ribulose-1,5-bisphosphate carboxylase-oxygenase
SDS	sodium dodecyl sulfate
DTNB	5,5-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
PGA	phosphoglyceric acid
Km	substrate concentration which gives half maximal velocity
Vmax	maximum velocity
LS	large subunit
SS	small subunit
X	anomalous polypeptide
P	polymeric forms of the large subunit
Å	angstroms
S _{20,w}	sedimentation coefficient at 20°C
Ea	activation energy

INTRODUCTION

I. Ribulose Bisphosphate Carboxylase-Oxygenase

(1.1) Purification

Wildman and Bonner (204) observed a major protein in green leaves and called it Fraction 1 protein. Subsequently, ribulose bisphosphate carboxylase-oxygenase (RUBPCase) (EC. 4.1.1.3) was purified for the first time by Weissbach et al (202) and found to be similar to Fraction 1 protein. Purification methods subsequently employed included ammonium sulfate fractionation with RUBPCase being precipitated between 25 and 50% saturation. This was followed by gel filtration on Sephadex G200 or ion exchange chromatography or both. RUBPCase has been successfully purified to homogeneity, as indicated by sedimentation profiles in the analytical ultracentrifuge and electrophoresis, from several sources such as spinach (137), French bean (60) and soybean (133) by means of these techniques. The enzyme from tobacco appears to be unique compared to RUBPCase from other higher plants with respect to purification. Chan et al (32) were able to exploit the fact that RUBPCase from tobacco was extremely soluble in the presence of ribulose bisphosphate (>100 mg/ml) but not in the presence of Mg^{2+} and HCO_3^- (<1.0mg/ml) to induce crystallization of the enzyme from crude homogenates. One milligram of crystals was obtained for

each gram (fresh weight) of leaf tissue. Recently, Gray et al (61) raised antibodies against pure RUBPCase from N. tabacum and covalently linked them to a Sepharose 4B matrix. RUBPCase present in crude extracts of green leaves of higher plants was absorbed on a column of the immobilized antibody and eluted with 8M urea. RUBPCase eluted was in the form of dissociated subunits uncontaminated with other proteins. This represents a rapid technique for the isolation of the subunits of RUBPCase from tobacco and other higher plants (61).

(1.2) Physical Properties

RUBPCase can constitute up to 50% of the soluble leaf protein which undoubtedly makes it the most abundant protein in nature (84, 95). The sedimentation coefficient ($S_{20,w}^0$) for this protein ranges between 17-18 and diameter of 100-150 Å as estimated by electromicroscopy (88). In 1968, Anderson et al (3) demonstrated that RUBPCase could be divided into the following three categories: large (500,000), intermediate (360,000) and small molecular weight (120,000). Higher plants (88,170), green and blue-green algae (3) and the hydrogen bacteria (119,121,122) contain the large enzyme. RUBPCase from Thiobacillus denitrificans (120), Thiobacillus intermedius (144) and Chlorobium thiosulfatophilum (189) are of the intermediate size while the only small enzyme known is from

Rhodospirillum rubrum (3).. Gibson and Tabita (57,58) have reported that Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides contain both the large and intermediate forms.

Low temperatures ($0-4^{\circ}\text{C}$) appear to inactivate RUBPCase from tobacco and spinach but upon heating the enzyme solutions at 50°C for 20 to 30 minutes in the absence of reducing agent all activity is regained (33, 34, 84, 108, 178). Chollet and Anderson (34) were able to demonstrate that this reversible cold inactivation of tobacco RUBPCase is accompanied by a significant conformational change. By titrating the enzyme with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), they were able to show that during cold inactivation the number of sulphydryl groups exposed to the external environment increases, whereas the heat treatment results in the SH groups becoming less accessible to this reagent. They concluded that the heat activated enzyme is in a more compact state than the cold inactivated enzyme.

Comparison of the amino acid compositions (88, 122, 170) and immunological properties (158) indicated that the large subunit is quite different from the small subunit. However, the large subunit from different species appear to be the same with respect to amino acid composition (145, 193) whereas the small subunits from different

species vary in their amino acid composition (27,145,193).

The quaternary structure of RUBPCase from spinach was initially investigated by Rutner and Lane (158) and shown to be composed of large (55,000 molecular weight) and small (15,000 molecular weight) subunits. This was subsequently confirmed for the same enzyme isolated from other higher plants (88,106,170). The active site of the enzyme resides in the large subunit (128) whereas the function of the small subunit appears to be regulatory (129). Certain microbial and algal RUBPCases appear to lack the small subunit (144,188,189,190). Information from X-ray diffraction, electron microscopy, and optical diffraction suggests that the most likely structure of the crystalline tobacco RUBPCase is eight large subunits and eight small subunits, L_8S_8 (12). The molecule is thought to be arranged as a two layered structure, each having four large and four small subunits (Fig. 1). Immunological evidence suggests that the small subunits may be buried within the complex and the large subunit at the surface (60). Antibody to native RUBPCase does not react with the small subunit indicating that the determinant groups on the enzyme surface are primarily those located on the large subunit. Roy et al (157) have shown that the small subunits are in at least a binary molecular complex with one another by the use of cross

Fig. 1 Structure of RUBPCase depicting the relationship of the large and small subunits. Redrawn from Jensen and Bahr (84).

linking reagents. This is in support of a two layered structure for RUBPCase.

(1.3) Kinetic and Catalytic Properties

Photosynthetic organisms reduce CO_2 in the presence of ribulose -1,5-bisphosphate (RUBP) to give two molecules of phosphoglyceric acid (PGA) by the action of RUBPCase via the Calvin cycle (67). Recently, it became apparent that the enzyme from soybean could also convert RUBP to phosphoglycolate and PGA in the presence of oxygen (23, 133). This has now been confirmed for the enzyme from other sources and the presence of both a carboxylase and an oxygenase activity for RUBPCase has been firmly established (9, 94, 98, 109, 118). The carboxylase and oxygenase reactions are illustrated in Fig. 2. RUBPCase catalyzes the primary reaction of both photosynthesis and photorespiration thus having a direct influence in the net productivity of the whole plant. The chemical intermediate during carboxylation was shown by Siegel and Lane (169) to be 2-carboxy-3-ketoribitol-1,5-diphosphate. Subsequently, it was shown that the RUBP binding sites, one per large subunit, involve lysine residues by means of substrate affinity labels such as 3-bromo-1,4-dihydroxy-2-butanone-1,4-diphosphate (66, 132, 164, 165). Similar conclusions were reported by Whitman and Tabita (203) and Paech et al (134) with the

Fig. 2 Reactions catalyzed by RUBPCase.

use of pyridoxal-5-phosphate. It appears that sulfhydryl groups may participate in catalysis but their exact role has not been determined as yet (185,186,192).

From earlier work on the structure and function of RUBPCase, it was obvious that the apparent affinity of the isolated enzyme for CO_2 was too low to account for the observed rates of photosynthetic CO_2 fixation (7,10,83). In intact leaves and intact, isolated chloroplasts the apparent $K_m \text{CO}_2$ for carbon dioxide fixation was 10-20 μM (83) which is similar to the concentration of CO_2 in air at 25°C . Initial results for purified RUBPCase indicated that its $K_m \text{CO}_2$ values were between 70-600 μM (88,137). These fixation rates at atmospheric concentrations would be inadequate to explain the observed rates of CO_2 fixation in nature. Bahr and Jensen (10,83,84) observed that the carboxylase activity was equal to the rate of photosynthetic CO_2 fixation by intact chloroplasts when the kinetic and regulatory properties of the enzyme were measured immediately after its release from intact spinach chloroplasts. However, the kinetics of the RUBPCase released from the chloroplasts was not stable (83). Three minutes after release a new, lower, steady state rate with a $K_m \text{CO}_2$ of 500 μM was observed. This value was comparable to previous work with purified RUBPCase. Therefore, it appears that the

enzyme released from intact chloroplasts was a partially activated enzyme.

Enhancement of RUBPCase activity ($K_m \text{ CO}_2 = 20\text{--}40 \mu\text{M}$) was achieved by preincubating the enzyme with certain photosynthetic intermediates. Chu and Bassham (37,38,39) showed that effectors such as NADPH and PGA stimulated the enzyme only when present in the preincubation medium containing Mg^{2+} and 1mM HCO_3^- . Buchanan and Schurmann (28) reported activation of carboxylase activity, measured at 1mM HCO_3^- , pH 7.8, by fructose-6-P, ribulose-5-P, 6-P-gluconate, ribose-5-P, erythrose-4-P and xylose-5-P, all at 1mM concentration. The role of these intermediates in the physiological regulation of RUBPCase in intact chloroplasts has not been unequivocally demonstrated yet (38,84).

The active species involved in activation and catalysis is CO_2 rather than HCO_3^- (107). The following equation describes the relationship between CO_2 and HCO_3^- . Substrate is usually added in the form of HCO_3^- .

from which one calculates the concentration of CO_2 present using the Henderson-Hasselbach equation (115). RUBPCase shows time-dependent and order of addition-

$$\text{pH} = \text{pK}_a + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]} \quad (196).$$

dependent kinetics resulting from the activating effects

of Mg^{2+} and CO_2 and the inactivating effects of RUBP (5,10,38,99). In the absence of Mg^{2+} and CO_2 , the enzyme is essentially inactive. When RUBPCase is preincubated with RUBP and the reaction initiated with Mg^{2+} and CO_2 , a marked lag is observed in the early stages of the reaction (107,108). However, preincubation with Mg^{2+} and CO_2 results in a rapid initial rate of reaction (37,107,108). The activation of the enzyme by Mg^{2+} and CO_2 was recently characterized by Lorimer et al (107) as shown below. The final state of activation is

$$\begin{array}{l}
 \text{ENZ(inactive)} + \text{CO}_2 \xrightarrow{\text{slow}} \text{ENZ} \cdot \text{CO}_2 \text{ (inactive)} \\
 \text{ENZ} \cdot \text{CO}_2 + \text{Mg}^{2+} \xrightarrow{\text{fast}} \text{ENZ} \cdot \text{CO}_2 \cdot \text{Mg}^{2+} \text{ (active)}
 \end{array}$$

dependent on CO_2 , Mg^{2+} concentration and pH.

RUBP results in substrate inhibition at high concentrations and inactivates the enzyme in the absence of Mg^{2+} and CO_2 by allosterically affecting the binding of CO_2 and Mg^{2+} (39).

(1.4) Relationship Between Carboxylase and Oxygenase Activity

The rate of carboxylation of RUBP in the plant determines the rate of photosynthesis under conditions of saturating light (84). Similarly, the rate of RUBP oxygenation may determine the rate of glycolate production in photorespiration (23,84,109,133). The relative rates of the two reactions are regulated by the concentra-

tions of O_2 and CO_2 . The apparent K_m CO_2 and K_m O_2 vary differently with temperature (98) and pH (11). The ratio of phosphoglycolate produced to CO_2 fixed increased with increasing temperature (98). The pH optima of the two reactions are broad but may differ by as much as 0.2-0.3 pH units (84,118) the carboxylation being more acidic. Oxygen has been shown to be a competitive inhibitor of carboxylation and vice versa (11,98,99) which indicates that both reactions probably utilize the same active site. However, a recent report by Brändén (25) indicated that RUBPcarboxylase from parsley was separable from RUBP oxygenase by gel filtration on Sepharose 4B. The results indicated that the oxygenase was of slightly lower molecular weight than the carboxylase and contained bound copper. Further investigations are obviously required to resolve the authenticity of these results.

(1.5) Biosynthesis of RUBPCase

Through the specific and differential inhibition of 70S and 80S ribosomes by chloramphenicol and cycloheximide in barley plants, Criddle, Dau, Kleindopf and Huffaker (41,179) observed that the synthesis of the two major subunits of RUBPCase probably occurred at two separate cellular sites. These results have been confirmed by studies of ~~protein synthesis~~ in isolated, intact chloroplasts (22,48); therefore, the large subunit is

synthesized on 70S ribosomes and the small subunit on 80S ribosomes (48,79,84,93,96,105). Data are now available which support the conclusion that genetic information for directing the synthesis of the large subunit is present in the chloroplast genome whereas the nuclear genome directs the synthesis of the small subunit (22,41,156). Native RUBPCase is the result of the assembly of probably 8 large and 8 small subunits (12). Thus, formation of the native RUBPCase requires: (1) co-ordinated polypeptide synthesis (41,82,105), (2) transport of subunits to a common site which is undoubtedly the chloroplast and (3) the assembly of the multisubunit complex. Because several different, complex steps are involved in the synthesis and assembly of this enzyme, it is possible that protein synthesis is not the rate-limiting step and that changes in enzyme activity may reflect either assembly or transport-limited rates.

Experiments with barley (179) and Chlamydomonas reinhardtii (82) and intact chloroplasts (22) indicated that the de novo synthesis of RUBPCase requires light. In the dark, synthesis of the enzyme decreases but the enzymatic activity and protein concentration do not change significantly. These observations indicate an extremely slow turnover of the enzyme in the dark. This has been substantiated using a double label technique (82).

Since the large subunits are considered to be homologous (60,88), that is structurally similar, whereas the small subunits are not, it appears that during the evolution of this enzyme the genetic information for the large subunit has been conserved in the chloroplast while genetic variability has occurred in the nuclear genomes which code for the small subunit (84).

II. Winter Hardening

Winter hardiness is a term which includes all those properties of a plant that permit it to survive the stress of freezing (103). In the majority of cases, plants, being ectothermic organisms (80), are unable to avoid ice formation in their tissues at subfreezing ambient temperatures. In most plants cold hardiness appears to be related to tolerance of extracellular freezing (30,103,175). As the environmental temperature gradually decreases, ice formation is initiated in the intercellular spaces (72, 103) which lowers the extracellular water potential resulting in a gradient between the protoplasm and the intercellular spaces. This causes water to move out of the cell with concomitant extracellular ice crystal formation. Alternatively, a rapid drop in temperature usually leads to intracellular ice formation which causes irreversible mechanical damage and inevitably leads to cell death (6,101). In effect, freeze resistance is a form of drought

tolerance because removal of water from cells imposes a considerable dehydrative stress on the protoplasm (30,72, 103,176). Damage caused by freezing in general may be attributed to the interaction of the effects of the presence of ice in the tissue, dessication, and low temperature on cellular constituents (30).

(1.6) Physiological Characteristics Related to Plant

Hardiness

In the vast literature on frost hardiness many reports have demonstrated a parallel between the physico-chemical characteristics of a plant and its hardiness. For example, a consistent correlation has been found between soluble carbohydrate content and hardiness (68,103, 171,175,176). Any treatment which increases the sugar content appears to increase hardiness and any treatment that decreases sugar content lowers hardiness. Carbohydrates such as sucrose, glucose, fructose, raffinose and stachyose have been implicated in the cold-hardening of plants such as black locust tree, conifers and various cereals (68,136,171).

Another striking parallel was demonstrated between soluble protein content and frost hardiness in the cortical cells of black locust trees during both hardening and dehardening (26,103,171,172,173,174,176) and in chloroplasts of wheat during hardening (69). Furthermore, Heber and co-workers (71,73) demonstrated that chloroplast membranes from winter grown

spinach or rye released a protective principle which contained at least two, low molecular weight proteins. This work was extended by Volger and Heber (199) to include several proteins isolated from leaves of frost-resistant spinach and cabbage which were present in high enough concentrations to significantly contribute to the frost tolerance of these plants. Hardy plants have greater ability to incorporate labelled amino acids into water soluble proteins (103,154,155,176). This implicates protein synthesis in the cold-hardening process. Recently, Bixby and Brown (19) presented evidence that the ribosomal protein structure is altered during the induction of hardiness in black locust seedlings. Since protein synthetic mechanisms do appear to remain active during hardening (40,176), the ribosomes may be altered to function at lower temperatures. These results are in accord with studies indicating seasonal variations of RNA which parallel the variations in soluble proteins (176).

Burke and co-workers (29,30,63) demonstrated that hardiness can be correlated to the per cent of tissue water that can be frozen before death occurs. Hardy Frontier rye tolerated 62% of its unbound water frozen before injury occurred whereas non-hardy Manitou spring wheat was killed when only 18% of its unbound water was frozen (63).

(1.7) Injury Due to Extracellular Freezing

Evidence from many sources have indicated that membranes are the primary site of dessication injury resulting from intercellular ice formation (55,56,110). Williams and Williams (206) and Williams and Meryman (207) concluded that it is the removal of cell water and the associated reduction in volume that is responsible for membrane injury in dogwood cortical cells and spinach chloroplast grana. Photophosphorylation and to a lesser extent electron transport reactions of chloroplasts can be inactivated by freezing (70,71,74). Protection against this inactivation can be accomplished by various cryoprotectants such as sucrose, low molecular weight polypeptides or amino acids (70,71,73,74,75,76,199).

The phenomenon of increased unsaturation of fatty acids upon exposure to low temperatures has been investigated in plants resistant and sensitive to chilling injury (112,123,130,131,146,167,181) and various cultivars of hardy and non-hardy wheat (43,50,125,205). It was concluded that the ability to withstand freezing at low temperatures could not be accounted for by increased fluidity of membranes (125). In locust bark cells a general increase in the amount of cell membranes appears to be consistent with the development of extreme freezing tolerance (177).

Since proteins are the largest component of the dry matter of protoplasm, Levitt (104) proposed that they were a key to the understanding of freezing injury. He suggested that non-hardy plants are those whose membrane proteins irreversibly denature at cold-hardening temperatures and subsequently aggregate due to the formation of intermolecular disulfide bonds (103,104). However, hardy plants

possess proteins which remain in their native state at these hardening temperatures and resist sulfhydryl-disulfide interchange thus decreasing the possibility of inactivation due to intermolecular bond formation.

Levitt's sulfhydryl-disulfide hypothesis (102) has received support from the work of Gaff (54) in drought injury in cabbage and Cothren and Guin (39) on the effects of low, non-freezing temperatures on young cotton plants.

They showed a progressive decrease in reactive SH groups in the soluble protein fraction of unhardened plants during drought and low temperature stress.

Roberts (152) suggested that isozymic substitution plays an important role in cold-hardening. The deleterious effects of metabolic imbalance caused by thermally induced reduction in the rates of enzymatic reactions may be overcome by substituting isozymic forms of enzymes that have lower energies of activation than the form of the enzyme normally present at higher

temperatures. Roberts has observed that changes occur in the ratios of isozymes of several enzymes including invertase (153), peroxidase (150), and phosphatases (151) from wheat leaves during cold acclimation. However, only the changes in invertase appear to be correlated with cold-hardening.

To substantiate this hypothesis, it must be shown that the isozymic substitutions which do occur have adaptive advantages for cold resistance and that substitution of proteins with different primary structure are distinguished from those with the same primary structure but different conformation. In an attempt to gain some insight into the relationship between proteins and cold adaptation in plants this study became centered on Puma rye because it is a cereal that is able to survive temperatures as low as -30°C (113,126). In preliminary work electrophoretic effects of cold-hardening were detected in association with Fraction 1 protein. Since this RUBP carboxylase-oxygenase is the major plant protein, catalyzes the rate limiting reaction of CO_2 fixation in photosynthesis, and has no known isozymic forms, this enzyme was chosen for the study of the relationship between proteins and the low temperature adaptation of Puma rye. In the following pages are described results which demonstrate structural and functional changes that impart

important adaptative advantages to this enzyme and thus to the whole plant during cold-hardening.

MATERIALS AND METHODS

(2.1) Chemicals

Electrophoresis-grade acrylamide was obtained from Eastman and the following from Canal Industrial Corp.: N ,N ,N',N'-tetramethylethylenediamine and N',N'-methylene bisacrylamide. Sephadex was obtained from Pharmacia Fine Chemicals, Amido Black was purchased from J.T. Baker Chemical Co. and Coomassie Brilliant Blue R from Sigma. Similarly, ribulosebisphosphate (RUBP), ethylene diaminetetraacetic acid (EDTA), 5,5- dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT) and β -mercaptoethanol were obtained from Sigma. Ultra pure urea was purchased from Schwarz/Mann and sodium dodecyl sulfate (SDS) (Sequanal Grade) from Pierce Chemical Co. Ampholine (pH 5-8) was obtained from LKB-Produkter AB. [14 C] NaHCO₃ was purchased from New England Nuclear as was Aquasol. All other chemicals were of the highest grade available.

(2.2) Plant Material

Spring wheat (Triticum aestivum L. cv Marquis), winter wheat (cv Kharkov 22 M.C.) and rye (Secale cereale L. cv Puma) were germinated and grown at about 15 seedlings per pot in controlled environment rooms (78) in 14cm pots of Went's medium supplied with Hoagland's nutrient solution No. 1 (78) at 25°/20°C (day/night) as

described by Madowall (113). Illumination consisted of daylight fluorescent light supplemented with 16% of the fluorescent wattage as incandescent light at a total intensity of $75-90 \times 10^3$ ergs/cm² sec during 16/8 hr (light/dark) photoperiods. Plants grown continuously at 25°/20°C generally did not survive temperatures less than -4°C (4).

After 1 week usually 15 pots of each cultivar were transferred to a temperature of 4°/2°C (day/night) with other conditions closely matched, and grown for 90 days to provide maximum hardiness under these conditions according to previous tests (113). Control plants, usually 15 pots of each cultivar, were grown entirely at 25°/20°C (day/night) for a total of 14 days and are referred to as "unhardened". Those at 4°/2°C are called "hardened", although the extent of hardiness varied with cultivar.

(2.3) Isolation and Extraction of Intact Chloroplasts

For uniformity of physiological condition, only the uppermost fully expanded leaf blades were harvested. Age appeared to have no effect on the soluble proteins extracted from chloroplasts of leaves taken from unhardened plants of different ages. Typically 150 to 200gm of leaves (fresh weight) were washed, chilled, cut into small pieces about 1cm square and briefly ground with the use of a mortar and pestle in a continuous stream (5ml/min)

of isolation medium containing 0.4M sucrose, 20mM Tricine buffer (pH 8.0) with 5mM $MgCl_2$ and 20mM NaCl (126). The homogenate was filtered through eight layers of cheesecloth and one nylon sheet. The filtrate was centrifuged at 200 x g for 90 sec. The pellet was discarded, and the supernatant recentrifuged at 500 x g for 3min to obtain intact, class I chloroplasts as checked by phase-contrast microscopy (65,180). The preparations from unhardened leaves contained approximately 70-75% class I whole chloroplasts based on appearance under phase-contrast microscopy whereas preparations from hardened leaves contained about 35% class I chloroplasts. The chloroplasts were resuspended by vortexing in cold distilled water at a concentration of 0.4 to 0.6mg chlorophyll/ml and lysed for 3 hr at 4°C. After this time, the membranes, which included chloroplast thylakoids, lamellae and envelopes, were removed by centrifugation at 110,000 x g for 1 hr, washed, and extracted with 80% acetone. The soluble chloroplast proteins present in the 110,00 x g supernatant (about 50ml) were concentrated under N_2 pressure (30psi) in an Amicon ultrafiltration cell with the use of a UM-2 Diaflo membrane (147) in preparation for gel filtration and gel electrophoresis. All operations were performed at 4°C.

(2.4) Chlorophyll Determination

A volume of 0.2ml of a chloroplast suspension was brought to a final volume of 5.0ml with 80% acetone (v/v) to extract chlorophyll. The suspension containing a white protein precipitate was centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was decanted and its absorbance measured at 652nm. The chlorophyll concentration was calculated by employing the following equation (126):

$$C(\text{mg chlorophyll/ml}) = \frac{\text{Absorbance @652nm}}{36}$$

where the absorbance was measured in a cuvette with a 1cm path length.

(2.5) Preparation of Sucrose Gradients

Stepped sucrose gradients were prepared by carefully layering solutions of varying sucrose concentrations buffered with 0.1M Tricine (pH 8.0) in 30ml nitrocellulose centrifuge tubes. The compositions (expressed as % (w/v), volumes and order of addition were as follows: (1) 3ml of 60% sucrose, (2) 2ml each of 58, 56, 54, 51, 48 and 45% sucrose (3) and 3ml each of 42, 38, 34 and 30% sucrose for total volume of 27ml. Typically, sample volumes of 2.0 to 2.5ml were carefully layered at the top then centrifuged at 4,000rpm for 5 min and 10,000rpm for 10 min in an SW-25 swinging bucket rotor at 4°C.

(2.6) Fractionation of Soluble Chloroplast

Proteins on Sephadex G50

In anticipation of the presence of Heber's protein hardiness factors ($10-20 \times 10^3$ daltons) (72,199), the total soluble chloroplast proteins were fractionated on a Sephadex G-50 column (1.0 x 100cm) with a bed volume of 75ml. Protein samples were eluted with 5mM Tris buffer (pH 8.0 at room temperature) containing 20mM NaCl using an upward flow (5ml/hr) and collected in 2ml fractions at 4°C.

Extraction, electrophoresis, and fractionation of chloroplast proteins from each cultivar and growth condition were repeated one or more times.

(2.7) Polyacrylamide Gel Electrophoresis of Soluble Chloroplast Proteins

Concentrated samples of soluble chloroplast proteins from leaves of hardened and unhardened rye were electrophoresed on 7% polyacrylamide gels, pH 8.9, prepared in glass tubes (0.15 x 7.5cm) according to the method of Davis (42). Sample volumes of about 100 μ l (50 to 100 μ g of protein) of concentrated chloroplast protein extract containing bromophenol blue as a marker dye and 20% (w/v) sucrose were loaded on the tops of the gels. Electrode buffer which consisted of 0.025M Tris-0.2M glycine (pH) was carefully layered over the samples prior to electrophoresis. A current of 2mA/gel was applied until the marker dye was 0.5cm from the bottom of the gel. Proteins

in these gels were stained with 1% (w/v) Amido Black and destained in methanol: acetic acid: H₂O (45:10:45) at room temperature, then scanned in a Gilford Spectrophotometer Model 540 at 617nm.

(2.8) SDS Polyacrylamide Gel Electrophoresis of Chloroplast Membrane Polypeptides

After acetone extraction, membrane polypeptides were solubilized in 10mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% β -mercaptoethanol (90), heated for 2 min in a boiling water bath and subjected to SDS polyacrylamide gel electrophoresis according to the method of Weber and Osborn (200). No stacking gel was employed and the final concentration of polyacrylamide in the separating gel was 10% (w/v). Sample volumes of 100 μ l (100 to 250 μ g of protein) containing bromophenol blue and 10% glycerol were applied to the top of the gels. The electrode buffer which consisted of 10mM sodium phosphate buffer (pH 7.0) and 1% (w/v) SDS was carefully layered over the samples prior to electrophoresis. A constant current of 8mA/gel was maintained until the bromophenol blue marker dye was 0.5cm from the bottom of the gel. The proteins were fixed in 12.5% trichloroacetic acid, stained with 0.01% (w/v) Coomassie Brilliant Blue R and destained in methanol: acetic acid: H₂O (45:10:45) at room temperature, then scanned at 550nm

in a Gilford Spectrophotometer Model 540.

(2.9) Purification of RUBPCase from Leaves of Hardened
and Unhardened Puma Rye

The final procedure to obtain electrophoretically pure RUBPCase was as follows. In a typical preparation 200 to 250gm (fresh weight) of leaves were cut into small pieces, macerated in 100mM Tris-HCl (pH 8.1 at room temperature) buffer containing 1mM $MgCl_2$ and 10mM β -mercaptoethanol (5ml of buffer/gm fresh weight) for 30 sec at 0°C in a Servall Omni-Mixer set at maximum speed. The brei was filtered through 8 layers of cheesecloth after which the filtrate was centrifuged twice at 20,000 x g for 15 min at 4°C. All the RUBPCase activity of the supernatant was recovered by precipitation at 0°C for 30 min by $(NH_4)_2SO_4$ fractionation (44), the greatest amount of activity being present in the 25-50% saturated $(NH_4)_2SO_4$ fraction. This precipitate was stored overnight at -20°C to -25°C. This fraction was then dissolved in 75ml of 0.1M Tris- SO_4 buffer (pH 7.6 at room temperature) containing 0.2M $(NH_4)_2SO_4$, 1mM EDTA and 10mM β -mercaptoethanol followed by centrifugation at 88,000 x g for 60 min to remove residual material. The supernatant (1500 mg of protein) was filtered through Whatman No. 41 paper then applied to the top of a Sephadex G100 column (4.0 x 100cm) and eluted at 4°C with the above buffer in a downward

direction at a flow rate of 30ml/hr. The fractions containing RUBPCase activity were pooled and the protein was precipitated for 30 min at 0°C by the addition of solid ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation at 10,000 x g for 10 min, dissolved in the above buffer and then subjected to gel filtration on a Sephadex G200 column (5.0 x 100cm) in an upward direction at a flow rate of 15ml/hr. This consistently resulted in the separation of two protein peaks, the first being green in colour (minor peak) and the second light yellow (major peak). Both peaks had RUBPCase activity. The fractions of the major peak were pooled, precipitated and collected by centrifugation as described above. Samples (80mg of protein) were dissolved in 0.1M Tris-SO₄ buffer (pH 7.6 at room temperature) containing 20mM (NH₄)₂SO₄, 1mM EDTA and 10mM β-mercaptoethanol and applied to the top of a DEAE Sephadex A50 column (1.6 x 40cm) with SO₄⁼ as the counterion. The column was washed with 200ml of the above buffer to ensure that all the protein was bound, then eluted with 400ml of a linear gradient of 0.02M to 0.3M (NH₄)₂SO₄. The fractions from the single eluted peak were pooled, precipitated, collected and stored at -20°C until used.

In the course of a year of preliminary work, it was found that RUBPCase from Puma rye could not be crystallized

by the methods used for the tobacco enzyme (32). For this reason $(\text{NH}_4)_2\text{SO}_4$ fraction, gel filtration and ion exchange chromatography were employed as an alternative method for purification of this enzyme.

(2.10) Preparation of DEAE Sephadex A50

Fifty grams of DEAE Sephadex A50 (Cl^- form) was suspended and allowed to swell at room temperature for 48 hr in 1M $(\text{NH}_4)_2\text{SO}_4$, pH 7.6. The swollen gel matrix was repeatedly filtered and resuspended in the above solution until no Cl^- could be detected in the filtrate using the AgNO_3 test (198). The gel matrix was then equilibrated with buffer containing 0.1M Tris- SO_4 (pH 7.6 at room temperature) containing 20mM $(\text{NH}_4)_2\text{SO}_4$, 1mM EDTA and 10mM β -mercaptoethanol. The gel slurry was then poured in a column (1.6 x 40cm), packed and equilibrated at 4°C with the above buffer. Final equilibration of the column was deemed to have occurred when the conductivity, measured by a Yellow Spring Conductivity Bridge, of the eluant was the same as that of the starting buffer.

(2.11) Polyacrylamide Gel Electrophoresis of Purified RUBPCase

Purified RUBPCase from hardened and unhardened rye were electrophoresed on 6% polyacrylamide gels, pH 8.9, with a 2% polyacrylamide stacking gel prepared in glass tubes (0.5 x 7.5cm) according to the method of Davis (42).

Sample volumes of about 25 to 50 μ l (50 to 80 μ g of protein) containing bromophenol blue as a marker dye and 20% (w/v) sucrose were loaded to the tops of gels. Electrode buffer which consisted of 0.025M Tris-0.2M glycine (pH 8.3) was carefully layered over the samples prior to electrophoresis. A current of 2mA/gel was applied until the marker dye was 0.5cm from the bottom of the gel. Proteins in the gel were fixed at 60°C for 30 min in 12.5% trichloroacetic acid, stained in 1% (w/v) Amido Black @ 60°C for 30 min and then destained in methanol: acetic acid: H₂O (45:10:45) at 60°C until the background was clear.

(2.12) Polyacrylamide Gel Electrofocusing

Polyacrylamide gels (T, 6.24%; C, 3.85%), where $T = \frac{\text{gm acrylamide} + CL}{100 \text{ ml solution}}$ and $C = \frac{\text{gm cross linker (CL)}}{8 T}$ containing 4% Ampholine (pH 5-8) were prepared as described by Drysdale (47) and Righetti and Drysdale (149). Polymerization was accomplished using TEMED and ammonium persulfate and allowed to proceed to completion (about 1 hr) at room temperature. Aliquots of protein were applied to the top of the gels (0.5 x 6.5cm); then a solution of 2% sucrose was used to fill the remainder of the tube. With 750ml of anolyte (10mM H₃PO₄) and 750ml of catholyte (20mM NaOH) a current of 2mA/gel was maintained until a voltage of 350V was reached; this voltage was maintained at this value for 6 hr. The proteins in the gels

C

11

Fig. 3

The effect of Ampholine concentration on the gradient established during gel electrofocusing. Gels were prepared, electrofocused and the pH gradient determined as described in METHODS. pH gradients were established using 1% (○), 2% (□), 3% (▲) and 4% (●) (v/v) Ampholine (pH 5-8).

were fixed in 5% (w/v) trichloroacetic acid at 60°C, subsequently washed repeatedly with trichloroacetic acid and stained with 0.1% (w/v) Coomassie Brilliant Blue R for 30 min at 60°C. Destaining of the gels was accomplished as described above.

The pH gradient established in the gel was determined as described by Wrigley (208). Four per cent Ampholine was used because at this concentration the most stable, linear pH gradient was produced (Fig. 3). Unfixed gels were sliced into 2 or 4mm segments immediately after the completion of electrofocusing, washed with 2.0 ml of distilled water overnight and then the pH of the extracts measured. The pH gradient was reproducible for gels run simultaneously.

(2.13) SDS Polyacrylamide Gel Electrophoresis of Purified RUBPCase

Samples of purified RUBPCase from hardened and unhardened rye were dissolved in a solution containing 50mM Tris-HCl (pH 6.8 or 8.2), 1% SDS, 6M urea, 1% β -mercaptoethanol, 2mM EDTA and 10% glycerol. After heating in a boiling water bath for 2.0 min, all samples were cooled to room temperature and a drop of bromophenol blue

added prior to electrophoresis.

Polyacrylamide gels (0.5 x 6.5cm) were prepared by the method of Studier (184). The separating gel contained 0.15M Tris-HCl (pH 8.8), 0.1% SDS, 2mM EDTA and 10% acrylamide (30:0.8, acrylamide: bis) and was polymerized at room temperature with TEMED and ammonium persulfate. The stacking gel contained 63mM Tris-HCl (pH 6.8), 0.1% SDS, 2mM EDTA and 5% acrylamide. The electrode buffer consisted of 50mM Tris (pH 8.3), 0.38M glycine, 0.1% SDS, 2mM EDTA and a current of 2mA/gel was applied until the marker dye entered the separating gel and then increased to 4mA/gel for about 2 hr. The proteins were fixed in 12.5% (w/v) trichloroacetic acid at 60°C for 30 min; then the gels were washed with distilled water and stained with 0.1% Coomassie Brilliant Blue R at 60°C for 1 to 2 hr in a solution of methanol: acetic acid: H₂O (45:10:45). The gels were scanned at 617nm with a Gilford Spectrophotometer Model 540 and the relative proportions of the stained proteins were determined by integration of the peak areas on chart recordings using a Cybergraph Digitizer (Tallos) interfaced with a Wang 2200S programmable calculator.

(2.14) Molecular Weight Determination By SDS Polyacrylamide

Gel Electrophoresis

Molecular weights of the polypeptides of RUBPCase from hardened and unhardened rye were determined by comparing their mobilities to those of known molecular weights as described by Weber and Osborn (200). Samples of 20 μ l (10 to 30 μ g protein) were electrophoresed on 10% polyacrylamide gels, stained and destained as described above. Standard proteins used were: (1) bovine serum albumin (68,000), (2) catalase (60,000), (3) aldolase (39,500), (4) myoglobin (17,500), and (5) ribonuclease (13,700).

(2.15) Molecular Weight Determination by Gel Filtration on Sephadex G200

The molecular weights of RUBPCase from hardened and unhardened rye were compared by gel filtration on Sephadex G200 (1.5 x 100cm) using 20mM Tris-HCl (pH 8.1) containing 1mM MgCl₂ and 1mM β -mercaptoethanol as the eluting buffer. All protein samples (10mg protein in 1.0ml) were applied to the top of the column and eluted in a downward direction at a flow rate of 15ml/hr. The elution volumes of the two forms of RUBPCase were determined as described by Fischer (51) and compared to those of proteins of known molecular weights. The void volume (51) of the column was 37.0ml as determined with Blue Dextran 2000.

(2.16) Amino Acid Analysis

Samples of purified RUBPCase (8-10mg/ml) from hardened and unhardened rye were dialyzed against 3 litres of 25mM potassium phosphate buffer (pH 7.6) for 48 hr. at 4°C and then hydrolyzed (3-5mg) in sealed, evacuated ampoules with constant boiling HCl at 110°C for 24, 48 and 72 hr. The HCl was removed under vacuum, the residue taken up in 0.2M citrate buffer (pH 2.2) and the amino acid composition of samples of known dilution determined using a JEOL Type JLC-5AH automatic analyzer.

Methionine and cyst(e)ine were determined separately as their oxidation products by the performic acid procedure of Moore (127). Triplicate samples of RUBPCase (5mg) from hardened and unhardened rye were transferred into Pyrex glass tubes (18 x 150mm) and dried under vacuum. To each tube was added 3.0ml of a performic acid solution composed of a 9:1 mixture of formic acid: H_2O_2 , and the oxidation was allowed to proceed for 20 hr at 0°C and then stopped with the addition of 0.3ml of 48% HBr. The samples were dried under vacuum at 40°C in a Rotary Evapo-Mix and then hydrolyzed for amino acid analysis.

Tryptophan was determined spectrophotometrically by the method of Beaven and Holiday (14). The extinction coefficients of RUBPCase from hardened and unhardened Puma rye were determined at 280 and 294nm in 0.1N NaOH using a Gilford 2000 spectrophotometer.

(2.17) Titration of Sulfhydryl Groups with 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB)

Titration of native RUBPCase with DTNB was performed at 26°C in 0.1M Tris-SO₄ buffer (pH 7.6) in a final volume of 1.0ml. The reaction was initiated by the addition of 0.95ml of a solution of dethiolated enzyme (0.50-0.60mg/ml) to 0.050ml of a 10mM solution of DTNB in 0.1M potassium phosphate buffer (pH 7.0). The reaction was continuously monitored and recorded using a Beckman Recording Spectrophotometer Model DK equipped with a thermostatted cell compartment by following the change in absorbance at 412nm of liberated 2-nitro-5-thiobenzoate. To calculate the number of SH groups titrated, a molar extinction coefficient of 13,600M⁻¹cm⁻¹ was used (49,64).

The following procedure was used for the titration of the enzymes in the presence of denaturing agents. The enzyme solutions were heated in the presence of 1% SDS for 2.0 min in a boiling water bath, cooled to 26°C and titrated with DTNB. When 5M urea was present in the reaction mixtures without SDS it was added at 26°C, 15 min prior to titration with DTNB. However, in the

presence of both urea and SDS the samples were pre-heated prior to titration as described above. All absorbance values at 412nm were corrected using blanks containing 0.1M Tris-SO₄ (pH 7.6), with or without denaturing agent and DTNB. Purified RUBPCase from hardened and unhardened rye had negligible absorbance at 412nm.

The kinetics of titration of the enzymes with DTNB were analyzed by log vs time plots according to the method of Frost and Pearson (53).

Molar concentrations of the purified enzymes were based on a molecular weight of 552,000 as determined by SDS electrophoresis and a subunit composition of L₈S₈ (12,193).

(2.18) Determination of Disulfide Bonds with DTNB

The method of Cavallini et al (31,64) was used to liberate sulfhydryl groups by the reduction of RUBPCase with NaBH₄ in the presence of 8M urea and N₂.

(2.19) Bicarbonate Activation of RUBPCase from Hardened and Unhardened Puma Rye

A 0.5ml aliquot of purified enzyme (2mg protein/ml) was applied to the top of a column of Sephadex G25 (medium) (1.0 x 30cm) equilibrated with 0.1M Tris-SO₄ buffer (pH 8.6) containing 20mM MgSO₄, 10mM NaHCO₃ and 1mM DTT at 23°C. The eluate was collected at rate of

60ml/hr and kept on ice until used. The time required for the protein to come through the column was about 20 min. This established the conditions necessary for full activation of the enzyme by the criterion of Lorimer et al (107,108).

(2.20) Measurement of RUBPCase Activity

RUBPCase activity was measured by the method of Lorimer and co-workers (107) at 25°C in sealed, N₂-purged test tubes. To 0.43ml of carboxylase buffer containing 0.1M Tris-SO₄-NaOH (pH 8.2), 5mM DTT and 20mM MgSO₄ was added 50μl of 0.2M [¹⁴C] NaHCO₃ (0.25μCi/μmole) and 10μl of 20mM RUBP. The reaction was initiated by the addition of 10μl of bicarbonate-activated enzyme (50 to 75μg protein) and stopped by the addition of 0.1 ml of 2N HCl. The contents were quantitatively transferred to a scintillation vial, and dried. The residue was dissolved in 1.0ml of distilled water to which was added 10.0ml of Aquasol then counted in an Ansitron Spectrometer set at maximum efficiency for the ¹⁴C isotope.

(2.21) Preparation of Carboxylase Buffer

The method of Lorimer et al (108) was used. A solution of 0.1M Tris base was adjusted to pH 4 by the addition of concentrated H₂SO₄ then purged with N₂ for about 10 min. Subsequently the pH was readjusted to pH 8.2 with carbonate-free NaOH (92). Before use the

solution was thoroughly purged with N_2 in a sealed serum bottle for about 1 hr. This resulted in an anaerobic, CO_2 free 100mM Tris- SO_4 -NaOH buffer (pH 8.2) containing 5mM DTT and 20mM $MgSO_4$.

(2.22) Protein Determination

Protein content was routinely made by the method of Lowry (111) using bovine serum albumin as the standard and absorbance measured at 750nm. A typical standard curve is depicted in Fig. 4.

Fig. 4 Typical standard curve used for Lowry protein determinations. Experimental conditions are described in METHODS.

RESULTS

Preliminary Investigations

(3.1) The Effect of Low Growth Temperature on Chloroplast Proteins

Photophosphorylation and to a lesser extent electron transport reactions of chloroplasts can be inactivated by freezing (72,75,76). Heber and co-workers (70,71,74,199) demonstrated that a number of soluble proteins from leaves of winter-grown spinach and cabbage were capable of protecting isolated thylakoid membranes against inactivation during freezing. In contrast to the functional approach of Heber and co-workers a general polyacrylamide gel electrophoretic survey of the effects of cold-hardening on all detectable proteins in chloroplasts of certain cereals was performed. Fig. 5 illustrates the polyacrylamide gel electrophoresis patterns of polypeptides solubilized from the chloroplast membranes by SDS. They were generally similar for all cultivars and growth conditions, and resembled those published for chloroplast lamellae by Klein and Vernon (90), Anderson and Levine (2) and Park and Nolan (135). Genetically based differences could be seen between cultivars in the relative densities of some protein bands. However, there was no clear effect of growth at low temperature on membrane polypeptide composition. In contrast, the thylakoid

Fig. 5

Spectrophotometer scans of chloroplast membrane proteins solubilized by SDS, electrophoresed on 10% SDS polyacrylamide gels and stained with Coomassie Brilliant Blue as described in METHODS. The profiles are, left to right, from the wheat cultivars Marguis (M), Kharkov (K) and rye (R) cultivar Puma, in the unhardened state (NH) in the top row and in the cold hardened state (H) in the bottom row. The cultivar proteins were applied to the gels at concentrations of 100, 250, and 210 μ g, respectively, in 100 μ l.

ABSORBANCE (550 nm)

membranes of chloroplasts from hardened (RH) and unhardened rye (RNH) banded at different positions (Fig. 6) when centrifuged in a stepped sucrose gradient. The results indicate that the thylakoids from hardened rye were less dense than those from unhardened rye.

Fig. 7 illustrates the electrophoretic patterns of the water-soluble chloroplastic proteins. Six to eight protein bands were visible and resembled those in the literature (22,46). The last peak of the rye protein (Fig. 7, arrows) probably reflects a genetic difference between rye and wheat. Between the cathodic end and the large, densely staining bands, one smaller band was visible in all unhardened material but the resolution of the spectrophotometer was insufficient to distinguish this band from the large one in the unhardened rye samples. After cold-hardening, however, an additional, more slowly migrating band appeared in this region in all cases.

To increase resolution, the soluble chloroplastic proteins were fractionated on Sephadex G-50 (fine) and the results obtained for RH and RNH chloroplasts are shown in Fig. 8. Identical data were obtained from all materials. The three peaks were eluted and their constituents fractions pooled, concentrated, and analyzed by polyacrylamide gel electrophoresis. Peak A had RUBPCase

Fig. 6 Relative densities of thylakoid membranes of chloroplasts from hardened (H) and unhardened (NH) Puma rye. Five ml samples of isolated chloroplast suspensions (0.4 to 0.6mg chl/ml) was layered on the top of a stepped, 30-60 % (w/v) sucrose gradient as described in METHODS. Centrifugation was at 4°C at 4000rpm for 5 min and 10,000rpm for 10 min in a SW-25 swinging bucket rotor.

Fig. 7 Spectrophotometer scans of gels of chloroplast soluble polypeptides electrophoresed on 7% polyacrylamide gels and stained with Amido Black as described in METHODS. Labelling is identical to that of Fig. 5. The cultivar proteins were applied to the gels at concentrations of 1mg/ml with a sample load of 100 μ l.

The significance of the arrows in the rye scans is explained in the text.

ABSORBANCE (617 nm)

Fig. 8

Profiles of fractions of soluble chloroplast proteins from cold-hardened (RH) and unhardened (RNH) rye eluted from a column of Sephadex G-50 (fine) (1.0 x 100cm) using a 5mM Tris buffer (pH 8.0) containing 20mM NaCl. Protein concentration was determined spectrophotometrically at 660nm by the method of Lowry (111). Peaks A, B and C are described in the text.

activity. The electrophoretic resolution of peak A from Puma rye is shown as a typical example in Fig. 9. The initial or slowest migrating band from all samples was the largest and is known to be Fraction 1 protein (22,46). In preparations from unhardened plants, as in the case of the RNHA (Fig. 9, solid line), the large band was more diffuse and it preceded a smaller band (arrow on solid line), which migrated as Fraction 1 protein. This phenomenon was observed in all three cultivars.

When the combined fractions of peak B were electrophoresed, several protein bands appeared one of which occurred only in the most hardy samples, namely Kharkov winter wheat and hardened Puma rye. Results for unhardened Marquis spring wheat, hardened Marquis spring wheat and hardened Kharkov winter wheat are shown in Fig. 10 (I) in which the new band (large arrow) is apparent only in hardened Kharkov. The second band in the unhardened Marquis gel (small arrow) appeared to be an expression of the non-hardy state as it was absent from cold-hardened Marquis and did not occur in the unhardened winter cereals. Fig. 10 (II) shows the hardiness indicator protein band (arrow) from hardened Kharkov winter wheat at the same position as one (arrow) in a gel containing proteins from cold-hardened Puma rye, and it was absent in gels from the unhardened plants. The gel preparation

Fig. 9

Spectrophotometric scans of soluble proteins eluted from Sephadex G-50 as peak A (Fig. 7), electrophoresed on 7% polyacrylamide gels and stained with Amido Black as described in METHODS. Comparison of preparations from hardened (dashed line) and unhardened (solid line) rye chloroplasts. Small protein loads (50 μ g) and a large absorbance span were used to distinguish the chief band (Fraction I protein).

Fig. 10

Photographs of gels of peak B proteins eluted from Sephadex G-50. Proteins were run on 7% polyacrylamide gels and stained with Amido Black as described in METHODS. Parts I and II were obtained with gels prepared from two different lots of Canalcro acrylamide. Gels are identified by labels as in Fig. 5. The large arrows mark the protein bands which were present only in extracts from chloroplasts of cold-hardened Kharkov winter wheat and hardened Puma rye. The small arrow marks the protein band characteristic of the chloroplasts from unhardened Marquis spring wheat.

I	KH	RNH	RH
II	KH	RNH	RH

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used for the examples shown in Fig. 10 (I) allowed better separation than that in Fig 10 (II).

Electrophoresis did not resolve any protein constituents of Peak C. This low molecular weight fraction was yellow in colour.

To elucidate the apparent change in Fraction I protein from chloroplasts during hardening RUBPCase was assayed in the soluble extracts of isolated chloroplast from hardened and unhardened Puma rye as a function of the extraction medium. Highest activity was obtained with 20mM Tris-HCl (pH 8.3) as the extracting buffer (Table 1). The enzyme from hardened rye extracts lost 18% of its activity when extracted into distilled water (final pH of 7.75) and 10% when extracted into 20mM Tris-HCl at pH 7.75. However, RUBPCase from unhardened rye extracts lost 80-90% of its activity when extracted into either distilled water or 20mM Tris-HCl buffer at pH 7.75.

A single protein band, the presence of which was independent of the extraction medium and similar to that illustrated in Fig. 11 (gel 3), was evident for RUBPCase upon electrophoresis of soluble chloroplast extracts from hardened rye on 6% polyacrylamide gels. Unhardened rye chloroplasts extracted into 20mM Tris-HCl (pH 8.3) showed one protein band for RUBPCase (Fig. 11, gel 3). However, a large diffuse band (Fig. 11, arrow), similar to the one

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Table 1. Effect of pH of the Extraction Medium on Ribulose-1,5-Diphosphate Carboxylase Activity

Extraction Medium	Final pH	RUBP Carboxylase Activity	
		(μ moles HCO_3^- fixed/mg protein/hr.)	RNH
*20mM Tris-HCl	8.30	2.48	2.40
*20mM Tris-HCl	7.75	2.10	0.48
Distilled H_2O	7.75	2.04	0.30

*Tris-HCl extraction medium also contained 1mM MgCl_2 and 1mM β -mercaptoethanol. Extraction time was 3 hr at 4°C .

Fig. 11 Photograph illustrating the results obtained when the soluble extracts of chloroplasts of unhardened (RNH) Puma rye were electrophoresed on 6% polyacrylamide gels as described in METHODS. Protein was stained with Amido Black. Large arrow marks the position of the large diffuse band. The gels represent the electrophoretic patterns of the soluble extracts obtained when chloroplasts were extracted in 20mM Tris-HCl containing 1mM $MgCl_2$ and 1mM β -mercaptoethanol at pH 7.75 (gel 1) or pH 8.3 (gel 3) or distilled water (gel 2) (final pH 7.75).

described previously, preceded the RUBPCase band only in unhardened samples extracted from chloroplasts either in distilled water (gel 2) or 20mM Tris-HCl buffer at pH 7.75. (gel 1). Thus this large diffuse band was associated with a loss in activity of the enzyme. These preliminary data illustrated discernable differences in the soluble chloroplast protein fraction during cold-hardening. The most quantitatively striking change was centered about Fraction 1 protein or RUBPCase. Since this enzyme is present in such large quantities in plants and proper assay procedures have been well established, RUBPCase was the most appealing protein for further study of low temperature adaptation in plants.

Studies on the Structural Properties of
Purified RUBPCase from Hardened and Unhardened Puma rye

(3.2) Enzyme Purification

Because of the difficulty of isolating whole, intact chloroplasts in high yields from cold-hardened rye leaves, the purification of RUBPCase after extraction from chloroplasts was not ideal for obtaining reasonable quantities of this enzyme. Therefore purification from total soluble leaf proteins was attempted. The crude preparations were prepared by macerating leaves in buffer, then removing the residual insoluble material by centrifugation as described in METHODS. The residual material from cold-

hardened leaves was more difficult to remove by centrifugation than that from unhardened leaves which agrees with the data for membrane density presented earlier.

RUBPCase purification was initiated by $(\text{NH}_4)_2\text{SO}_4$ fractionation of this crude preparation. As indicated in Table 2, 95% of the enzyme activity was recovered in the 25-50% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction. The 0-25% saturated fraction contained mainly green, insoluble material which was discarded. Although the 50-95% saturated fraction contained very little RUBPCase activity, further investigation by polyacrylamide gel electrophoresis revealed some striking differences between the proteins of this fraction from hardened and unhardened plants, as illustrated in Fig. 12 (arrows). Further work is required to elucidate the significance of these changes.

After the 25-50% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was redissolved in buffer and the green suspension subjected to high speed centrifugation, the resulting brown supernatant was subjected to gel filtration on Sephadex G100. This resulted in the profile illustrated in Fig. 13 A for hardened rye (RH) and Fig. 14 A for unhardened rye (RNH). In both cases all the RUBPCase activity was present in the one major peak with an elution volume of about 400ml. When the fractions of this peak were applied to a Sephadex G200 column (Fig. 13 B; Fig. 14 B), two

Table 2. $(\text{NH}_4)_2\text{SO}_4$ Fractionation of Total Water-Soluble Leaf Proteins from Hardened and Unhardened Rye

$(\text{NH}_4)_2\text{SO}_4$ Fraction	RUBPCase Activity* (cpm/mg protein)	
	RH	RNH
0-25%	101	85
25-50%	2512	2341
50-95%	41	30

* Activity was measured as described in METHODS after the respective $(\text{NH}_4)_2\text{SO}_4$ precipitates were redissolved in 0.1M Tris- SO_4 buffer (pH 7.6) containing 0.2M $(\text{NH}_4)_2\text{SO}_4$, 10mM β -mercaptoethanol and 1mM EDTA. Activity represents that of the unactivated enzyme.

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Fig. 12 Polyacrylamide gel electrophoresis of the 50-95% $(\text{NH}_4)_2\text{SO}_4$ fraction from hardened (RH) and unhardened (RNH) Puma rye. Protein samples (100 μg) were electrophoresed on 6% polyacrylamide gels and stained with Amido Black as described in METHODS. Arrows represent apparent differences in the proteins present in the two samples.

RH

RNH

Fig. 13

Purification of RH RUBPCase. (A) Elution profile of the 88,000 x g supernatant after gel filtration through Sephadex G-100. (B) Elution profile of the G-100 RUBPCase peak after gel filtration through Sephadex G-200. (C) Elution profile of the major G-200 RUBPCase peak after ion exchange chromatography through DEAE Sephadex A-50 as described in METHODS. (o-o-o) Absorbance at 280nm; (●-●-●) RUBPCase activity.

Fig. 14 Purification of RNH RUBPCase. Labelling in
the same as for Fig. 13.

protein peaks were discernable with the major peak having an elution volume of about 500ml and the minor peak an elution volume of about 450ml. The major peak was a clear, pale yellow solution whereas the minor peak was a greenish solution; both peaks had RUBPCase activity. All of the green colour associated with the minor peak appeared to be bound to the protein since the green colour was still associated with the protein after precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 50% saturation. This indicated that during this isolation procedure, a certain proportion of the protein, mainly RUBPCase, probably had chlorophyll bound to it. This was substantiated when a solution of this protein was scanned at wavelengths between 400nm and 900nm. The results shown in Fig. 15 indicate an absorption peak at about 670nm for a solution of the minor peak but no such peak in the scan of a solution of the major peak. This absorption peak at 670nm is indicative of chlorophyll. Similar results were obtained for hardened and unhardened material except that there was always more protein-bound chlorophyll in hardened preparations. Since gel filtration separates proteins strictly on a molecular weight basis, the RUBPCase in the minor peak appeared to have a higher molecular weight than the RUBPCase in the major peak. This difference in apparent molecular weight was undoubtedly due to the binding of

Fig. 15. Spectrophotometric scans of protein solutions of hardened rye from the minor peak (a) and major peak (b) obtained after gel filtration through Sephadex G-200. The protein solutions were scanned in 0.1M Tris-SO₄ buffer (pH 8.1) containing 0.2M (NH₄)₂SO₄, 10mM β-mercaptoethanol and 1mM EDTA using a Beckman DK-1A Recording Spectrophotometer.

chlorophyll. For this reason, only the RUBPCase in the major peak was further purified by ion exchange chromatography using the anion exchanger DEAE Sephadex A50 with SO_4^- as the counterion. As shown in Fig. 13 C and Fig. 14 C a single protein peak having RUBPCase activity was eluted. However, if Cl was used as the counterion, a single protein peak was obtained having no enzyme activity. Both the hardened and the unhardened preparations resulted in clear colourless protein solutions after this purification step. Table 3 summarizes the purification of RUBPCase from hardened and unhardened rye. The specific activities of pure, HCO_3^- activated RH and RNH RUBPCase were 180 and 100 nmoles HCO_3^- fixed/mg/min respectively which are similar to that reported for the tobacco enzyme (178). From a fresh weight of about 200mg, approximately 100mg of purified RUBPCase was obtained which represented final yields of between 15 and 20% for the enzyme from rye. Fig. 16 illustrates the purity of RH and RNH RUBPCase after successive steps of separation. After ion exchange chromatography of RH (gel 6) and RNH RUBPCase (gel 7) a major protein band was visible. The minor protein band present in purified samples was not another type of protein but a polymeric form of RUBPCase (46,182,194) produced during electrophoresis because upon gel filtration through Sepharose 4B, which separates molecules

Table 3. Purification of RUBPCase from Hardened (H) and Unhardened (NH) Rye

Purification Step	Total Vol. (ml)		Total protein(mg)		Specific Activity (nmoles HCO ₃ fixed/mg/min)		Total Activity (specific activity x total protein)		% Yield	
	H	NH	H	NH	H	NH	H	NH	H	NH
CRUDE Homogenate	1000	1100	2850	22200	10.2	12.8	28,070	28,160	100	100
25-50% (NH ₄) ₂ SO ₄ Fraction	120	100	1500	1210	17.7	20.3	26,550	24,563	91	87
Sephadex G100 Eluate	150	125	487.7	450	44.7	30.1	21,803	13,545	75	48
Sephadex G200 Eluate	125	100	147.6	110	68.9	49.8	10,175	5,478	35	19
DEAE Sephadex A50 Eluate	98	90	87.8	80	69.5	51.2	6,102	4,096	21	15
HCO ₃ ⁻ -Mg ²⁺ Activation					180	100				

Activity was measured as described in METHODS.

Fig. 16 Polyacrylamide gels illustrating the purification steps for hardened Puma rye. Protein samples (50-80 μ g) were electrophoresed on 6% polyacrylamide gels and stained with Amido Black as described in METHODS. Large arrow (gel 7) represents the position of RUBPCase. Gel 1, crude sample; gel 2, 25-50% $(\text{NH}_4)_2\text{SO}_4$ fraction; gel 3, 88,000 x g supernatant; gel 4, RH RUBPCase peak from Sephadex G-100 column; gel 5, RH RUBPCase peak from Sephadex G-200 column; gel 6, RH RUBPCase peak from DEAE Sephadex A50 column; gel 7, RNH RUBPCase peak from DEAE Sephadex A50 column.

of molecular weights between 10^5 and 10^6 , only a single protein peak occurred consistently. When this protein was electrophoresed on 6% acrylamide gels, the prominent RUBPCase band was again accompanied by the polymeric form. Thus it was concluded that RH and RNH RUBPCase was purified until it alone was evident upon one dimensional polyacrylamide gel electrophoresis.

(3.3) Amino Acid Composition

The results of amino acid analysis of RUBPCase from hardened and unhardened rye are presented in Table 4. The data are presented as nearest integral number of residues per 552,000 molecular weight and as relative molar ratios of phenylalanine. A comparison of the amino acid compositions of RH and RNH RUBPCase revealed no significant differences. Furthermore they were similar to those of RUBPCase isolated from sugar beet (148), spinach (2, 192) and tobacco (86,158). RH and RNH RUBPCase contained 780 ± 25 and 799 ± 28 basic amino acids respectively, and 1037 ± 34 and 1053 ± 41 acidic amino acids respectively. This indicated that these two enzymes were acidic in nature. From these amino acid data and the published data for amino acid side chain hydrophobicities (18), it was possible to calculate the average hydrophobicity ($H\phi_{AV}$) of RUBPCase from hardened and unhardened rye. The results of such a calculation are presented in Table 5. The

Table 4. Amino Acid Composition of RUBP-
Carboxylase from Puma Rye

Amino Acid	Nearest Integral Number of Residues Per 552,000 M. Wt.		Relative Molar Ratios of Phenylalanine	
	RH $\pm$ SEM ^a	RNH $\pm$ SEM ^b	RH	RNH
LYS	310 $\pm$ 10	315 $\pm$ 12	1.08	1.10
HIS	140 $\pm$ 4	146 $\pm$ 5	0.52	0.51
ARG	330 $\pm$ 11	338 $\pm$ 11	1.15	1.18
ASP ^d	477 $\pm$ 16	481 $\pm$ 20	1.68	1.69
THR ^d	354 $\pm$ 22	360 $\pm$ 19	1.24	1.26
SER ^d	210 $\pm$ 11	228 $\pm$ 14	0.73	0.80
GLU	561 $\pm$ 18	572 $\pm$ 21	1.96	2.00
PRO	310 $\pm$ 14	311 $\pm$ 13	1.08	1.09
GLY	532 $\pm$ 18	550 $\pm$ 19	1.86	1.92
ALA	488 $\pm$ 16	501 $\pm$ 16	1.71	1.75
VAL	377 $\pm$ 15	384 $\pm$ 12	1.32	1.34
ILE	244 $\pm$ 9	244 $\pm$ 7	0.85	0.85
LEU ^d	433 $\pm$ 14	541 $\pm$ 16	1.51	1.58
TYR ^d	245 $\pm$ 11	256 $\pm$ 11	0.86	0.90
PHE ^c	286 $\pm$ 9	287 $\pm$ 9	1.00	1.00
TRP ^c	191 $\pm$ 5	192 $\pm$ 2	0.67	0.67
$\frac{1}{2}$ CYS ^e	91 $\pm$ 2	90 $\pm$ 1	0.32	0.31
MET ^e	77 $\pm$ 4	75 $\pm$ 1	0.27	0.26

a Average of 6 samples.

b Average of 7 samples.

c Determined spectrophotometrically. Average of 6 determinations.

d Corrected for loss during hydrolysis by the method of Moore and Stein (127)

e Determined as their oxidation products. Average of 3 samples.

Table 5. Effects of Cold-Hardening on the
Average Hydrophobicity of RUBPCase
from Puma Rye

Amino Acid	Amino Acid Side Chain Hydrophobicities (Kcal/residue) *	HØ**±SEM(Kcal)	
		RH	RNH
Trp	3.00	581±16	583± 5
Ileu	2.95	728±26	724±25
Tyr	2.85	630±18	636±24
Phe	2.65	766±23	759±26
Pro	2.60	816±26	810±39
Leu	2.40	1050±33	1073±35
Val	1.70	649±25	657±22
Lys	1.50	471±15	473±20
Met	1.30	100± 5	97± 1
½ Cys	1.00	91± 2	90± 1
Ala	0.74	366±14	372±14
Arg	0.75	243± 8	254± 9
Thr	0.45	149± 4	150± 6
Total Hydrophobicity(Kcal)		6641±216	6696±229
Total Number of amino acid residues per molecule		5676±209	5689±221
HØ _{AV} (cal/residue)		1170±14	1177±10

* Taken from Bigelow (18)

** Hydrophobicity

Fig. 17

Molecular weight determination of native RUBP Case from hardened (●) and unhardened rye (●). Gel filtration through Sephadex G-200 was used as described in METHODS. Standard proteins used were myoglobin (1), ovalbumin (2), hexokinase (3), catalase (4) and apoferritin (5) with known molecular weights of 17,800, 45,000, 99,000, 240,000 and 480,000 respectively. The void volume was determined by Blue Dextran 2000.

average hydrophobicities for RUBPCase from hardened and unhardened rye were 1170 ± 14 and 1177 ± 10 cal/residue respectively. These values agree with the calculated value of 1000-1200 cal/residue for globular proteins (18).

(3.4) Molecular Weight and Charge of Native RUBPCase from Hardened and Unhardened Rye

The results of the molecular weight determinations of the enzymes from hardened and unhardened rye by gel filtration on Sephadex G200 are presented in Fig. 17. A plot of V_e/V_o for proteins of known molecular weights versus the logarithm of their molecular weights resulted in a straight line. From this standard curve and the determination of V_e/V_o for the enzyme from hardened and unhardened rye a molecular weight of 560,000 was calculated for both enzymes. This agrees with the published molecular weight of this enzyme from other higher plants (89). This similarity in molecular weights was confirmed by polyacrylamide gel electrophoresis using the method of Hedrick and Smith (77). According to the criteria established by these authors, proteins having identical molecular weights but different charges are characterized by parallel lines in a plot of $\log R_m$ versus gel concentration. In addition, the slope of such plots is directly related to the molecular weights of the proteins.

Fig. 18 illustrates the results of such a plot for RH and RNH RUBPCase. Since this resulted in two parallel lines it was concluded that the molecular weights of the two proteins were the same but their charges differed. Thus the two forms of RUBPCase, one found in cold-hardened rye and the other in unhardened rye, can be termed charge isomers (77). To confirm this, pure RH and RNH RUBPCase were electrofocused on 6% polyacrylamide gels using 4% Ampholine (pH 5-8). If the two forms of RUBPCase have different net charges, their isoelectric points must also differ. As shown in Fig. 19 (gel 2) the two proteins are clearly separable on the basis of charge. Furthermore, since samples of RH and RNH RUBPCase showed only one stained protein band upon electrofocusing, it was concluded that the samples were homogeneously pure. The isoelectric points of RH and RNH RUBPCase, determined from Fig. 20, were 6.4 and 6.3 respectively. These values agree with the acidic nature of this protein as indicated by amino acid analysis and with the isoelectric point for RUBPCase from Chlamydomonas reinhardtii reported by Iwanij and co-workers (81).

(3.5) Tertiary Structure of RUBPCase from Hardened and Unhardened Rye

In contrast to the common amino acid composition of RUBPCase from hardened and unhardened rye, the

Fig. 18 Determination of relative size and charge of RUBPCase from hardened (o) and unhardened (●) Puma rye. Enzyme preparations (120 μ g) were dissolved in 20mM Tris-HCl buffer (pH 8.1) containing 1mM MgCl₂ and 1mM β -mercaptoethanol and electrophoresed on 3, 4, 5, 6 and 7% polyacrylamide gels as described in METHODS. Proteins were stained with Amido Black. Relative mobility was measured as described by Hedrick and Smith (77).

Fig. 19 Results of polyacrylamide gel electrofocusing of RUBPCase from hardened and unhardened rye. Proteins were focused on polyacrylamide gels (T, 6.24%; C, 3.85%) containing 4% Ampholine (pH 5-8) for 6 hr at room temperature, fixed in 5% trichloroacetic acid, stained with methanol:acetic:H₂O (45:10:45) as described in METHODS. (gel 1) Purified RNH RUBPCase (15µg); (gel 2) 1:1 (v/v) mixture of RH and RNH RUBPCase; (gel 3) purified RH RUBPCase (10µg).

RNH

1

RNH+RH

2

RH

3

Fig. 20

Determination of the pH gradient established during polyacrylamide gel electrofocusing.

Unfixed gels were sliced into 2 or 4mm segments immediately after completion of electrofocusing and each extracted in 2.0ml of distilled water overnight. The pH of the extracts were then determined. The pH gradient was reproducible for gels run simultaneously.

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reactivities of their sulfhydryl groups were very different (Fig. 21). The SH groups of the enzyme from unhardened plants were much more reactive towards 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). After 30 min. at 26°C, approximately half as many SH groups were titrated in the enzyme from hardened plants (24 ± 1.0) as compared to the same enzyme from unhardened plants (46 ± 0.8). During the reaction of these SH groups with DTNB, the carboxylase activity of neither enzyme was impaired (Fig. 21) so that the sulfhydryl groups titrated during this period had no catalytic function.

The reaction of the free SH groups with DTNB (Fig. 22) was described as the linear plot of a first-order reaction which was at least biphasic indicating the presence of fast and slow titrating SH groups as observed by others (34,100). The data in Fig. 22 cover the first 15 min. of titration but linearity continued up to 30 min. The slope of a plot of $\log(a-x)$ versus time (1,100) gives k' , the pseudo first-order rate constant for the titration of SH groups with 0.5mM DTNB. In this case, the slope of the linear portion of the curve represents the rate constant for the slow titrating SH groups, k'_{slow} . This rate constant was calculated to be $0.120 \pm 0.03 \text{ min}^{-1}$ and $0.088 \pm 0.03 \text{ min}^{-1}$ for RH and RNH RUBPCase respectively.

In an attempt to react all free SH groups with

Fig. 21 Titration of sulfhydryl groups of native RUBP Case from hardened (o) and unhardened (●) rye. The dethiolated enzymes (0.80-1.00nmol/ml) were titrated at 26 °C in 0.1M Tris-SO₄ buffer (pH 7.6) with DTNB at a final concentration of 0.5mM as described in METHODS. Each point represents the average of 5 titrations with standard error of the means less than ±1 SH group. Change of enzyme activity was tested and is represented by normalized plots for the more active hardened (Δ) and less active unhardened (▲) enzymes. After various incubation times of enzyme samples at 26°C in the presence and absence of DTNB, RUBPCase activity was measured at 25°C as the rate of HCO₃ fixed over the initial 2 min after the addition of 10-20μl aliquots (0.070mg protein) to assay mixtures containing 80mM Tris-SO₄-NaOH (pH 8.2), 18mM MgSO₄, 20mM [¹⁴C] NaHCO₃ (0.25μCi/μmole), 0.4mM RUBP and 4mM DTT as described in METHODS.

RUDP CARBOXYLASE ACTIVITY
(% OF CONTROL)

Fig. 22 Pseudo first-order plots for the titration of native RUBPCase from hardened (o) and unhardened rye (●) with conditions for the reaction of DTNB with the proteins identical to those for Fig.21. Labelling of the ordinate is similar to that of Lane (100) where "a" is the number of SH groups titrated after 30 min and "x" the number of SH groups titrated at given times. The slope of the linear part of the curve equals the pseudo rate constant for the slow phase (k'_{slow}).

DTNB, RH and RNH RUBPCase were titrated in the presence of denaturing agents to unfold the proteins (Fig. 23; A,B). Titration in the presence of either 1% SDS or 5M urea resulted in an increase in both the initial rate and extent of reaction. A total of 60 SH groups were titrated in RH RUBPCase in the presence of 1% SDS or 5M urea, with a much faster initial rate of titration in the presence of the latter. In RNH RUBPCase, there were consistently more sulfhydryl groups titrated in the presence of SDS (73 SH groups) than urea (63 SH groups). The initial rate of titration was again much faster in the presence of urea. Interestingly, SDS and urea were antagonistic since for both forms of RUBPCase the initial rates and extents of titration were lower in the presence of both denaturing agents (Fig. 23, C). Table 6 summarizes the calculated pseudo first-order rate constants for the slow titrating SH groups (k'_{slow}) in the presence of these denaturants. In the presence of 1% SDS the slow titrating SH groups of RH RUBPCase reacted with DTNB at a rate which was 2.3 times slower than those of RNH RUBPCase. However, in the presence of 5M urea the slow titrating SH groups of RH RUBPCase reacted with DTNB 1.5 times faster than those of RNH RUBPCase. Apparently the extent of unfolding of the native enzymes from two different sources in the presence of these denaturants were substantially different. To

Fig. 23

The effect of denaturing agents on SH group titration of RUBPCase from hardened (o) and unhardened rye (●). The dethiolated enzymes (0.90-1.0nmol/ml) were titrated at 26°C in 0.10M Tris-SO₄ buffer (pH 7.6) with DTNB at a final concentration of 0.5mM in the presence of (A) 1% SDS, (B) 5M urea and (C) 1% SDS and 5M urea, with all samples prepared as described in METHODS.

Table 6. The Effects of Denaturants on the Pseudo-First-Order Rate Constant for the slow titrating SH groups (K'_{slow})

Denaturing Agent	K'_{slow} (min. ⁻¹)	
	RH	RNH
none	0.093	0.089
1% SDS	0.374	0.867
5M Urea	0.322	0.222
1% SDS + 5M Urea	0.402	0.429

K'_{slow} was determined as described for Fig. 22

verify this, the effect of denaturant concentration on enzyme activity and k'_{slow} was investigated. In the presence of 0.1mM SDS, when SDS/protein was 0.23, RH RUBPCase retained full activity but the activity of RNH RUBPCase was lessened by 55% (Fig. 24). When the SDS/protein was greater than 0.23, RH RUBPCase lost considerable activity and at 1mM SDS (SDS/protein = 2.3) both preparations were completely inhibited. Therefore RH RUBPCase was more stable towards low concentrations of SDS. The effect of the ratio of SDS to protein on k'_{slow} was similarly dependent on the form of the enzyme (Fig. 25). The rate constant for the titration of RH RUBPCase doubled, from 0.17 min^{-1} to 0.32 min^{-1} , when the SDS/protein was increased from 0.23 to 23.0. In contrast, k'_{slow} for RNH RUBPCase quintupled when the SDS/protein was increased from 0.23 to 2.3 then remained constant at 0.88 min^{-1} when SDS/protein was further increased to 23.0. Clearly the tertiary structure of RH RUBPCase was more stable to low concentrations of SDS.

Published reports of the amino acid analyses of RUBPCases from several different sources of higher plants (89,186,192) estimate 90 to 96 cysteine residues per molecule and no intramolecular disulfide bonds (89). Assuming a total of 90 free SH groups on the basis of the amino acid analyses of rye RUBPCase (Table IV), the 60 to

Fig. 24 The effect of SDS on RUBPCase activity. Enzyme samples (40 μ g) of hardened (o) and unhardened (●) Puma rye were dissolved in 20mM Tris-HCl buffer (pH 8.1) containing 1mM $MgCl_2$ and 1mM β -mercaptoethanol and assayed, as described in METHODS, for carboxylase activity in the presence of various SDS concentrations.

Fig. 25

The effect of SDS concentration on the rate of reaction of the slow titrating sulfhydryl groups of RUBPCase from hardened (o) and unhardened (●) rye. The conditions for the experiment were the same as those for Fig. 23A except that the SDS concentration was varied between 0 and 1% (w/v) resulting in SDS/protein as indicated on the abscissa. In the presence of the denaturant the enzymes were titrated for 14 minutes and the rate constant (k'_{slow}) at each concentration of SDS calculated as described in the legend of Fig. 22. In the absence of SDS (control), the enzymes were titrated for 30 minutes.

73 sulfhydryl groups titrated in denatured RH and RNH RUBPCase (Fig. 23 A,B) indicated that even when the enzyme were fully dissociated all the free SH groups were not available for reaction with DTNB. To ensure that the concentration of DTNB was not limiting, several different concentrations of the reagent were used at constant protein concentrations. Fig. 26 illustrates the results of such an experiment using RH RUBPCase in the presence of 1% SDS. DTNB reached a saturating concentration when the ratio of DTNB/protein was between 125 and 325. In all experiments with this reagent, ratios of about 500 were used and therefore DTNB concentration was not rate-limiting.

To determine whether the presence of disulfide bonds could account for the discrepancy in the number of SH groups determined by amino acid analysis (88) or titration with DTNB, the method of Cavallini *et al* (31,64) was used to detect disulfide bonds using DTNB after reduction of the proteins with NaBH_4 in the presence of 8M urea. Table 7 shows that reduction of the enzyme from hardened and unhardened plants resulted in no change in the number of SH groups titrated. Therefore disulfide bonds are probably not involved in stabilizing the tertiary structure of RUBPCase from rye.

(3.6) Quaternary Structure of RUBPCase from Hardened and Unhardened Rye

Fig. 26

The effect of DTNB concentration on the titration of SH groups. RH RUBPCase (0.90n moles/ml) was titrated with varying concentrations of DTNB in the presence of 1% SDS as described in METHODS. The number of SH groups titrated per mole of enzyme after 15 minute reaction times was calculated and plotted as a function of the ratio of DTNB to protein.

Table 7. Determination of Disulfide Bonds
With DTNB

Reaction Time With NaBH ₄ (min)		titrated/552,000 Mol wt
RH	0 (control)	60.5
	30	61.4
	60	59.9
	90	59.9
RNH	0 (control)	62.0
	30	57.5
	60	57.0
	90	57.0

Presence of disulfide bonds was determined as
described in METHODS.

RUBPCase from spinach leaves was initially shown by Rutner and Lane(158) to be composed of large(55,000 molecular weight) and small(15,000 molecular weight) subunits. This was subsequently confirmed for the same enzyme isolated from other sources(89,170). The results in Fig. 27 (gels 3 and 4) indicate that the subunit structure of RUBPCase from hardened rye is similar to that reported for spinach and other higher plants. The molecular weights of the large and small subunits were estimated by SDS polyacrylamide gel electrophoresis(Fig. 28) to be $54,000 \pm 800$ and $13,900 \pm 548$ respectively (Table 8). This was not affected by pH. Since the subunit composition of native RUBPCase is thought to be L_8S_8 (12,192), the molecular weight of the the enzyme from Puma rye was calculated to be 552,000 which agrees with the value of 560,000 determined by Sephadex G200 chromatography. When the same enzyme from unhardened rye was subjected to SDS polyacrylamide gel electrophoresis, a polypeptide of $109,000 \pm 1000$ molecular weight (D in Fig. 27, gel 1 and 2) appeared in addition to the large (LS) and small(SS) subunits of molecular weight $55,000 \pm 500$ and $14,600 \pm 678$ respectively (Table 8). Furthermore, in contrast to the enzyme from hardened rye, the large subunit of RNH RUBPCase appeared to be sensitive to pH (Fig. 27, gel 1 and

Fig 27

SDS polyacrylamide gel electrophoresis of the active form of RH and RNH RUBPCase. The proteins were dissolved in a solution containing 50mM Tris-HCl, 1% SDS, 6M urea, 1% β -mercaptoethanol, 2mM EDTA and 10% glycerol at pH 6.8 (gel 1, RNH RUBPCase; gel 3, RH RUBPCase) or pH 8.2 (gel 2, RNH RUBPCase; gel 4, RH RUBPCase) then heated for 2.0 min. Electrophoresis was performed using separating gels of 10% polyacrylamide with stacking gels of 5% polyacrylamide. The proteins were fixed with 12.5% trichloroacetic acid, stained with 0.1% Coomassie Brilliant Blue R and destained with methanol:acetic acid:H₂O (45:10:45) as described under METHODS. D, dimer; LS, large subunit; X, 47,000 mol. wt. polypeptide; SS, small subunit.

Fig 28

Molecular weight determination of the subunits of RH (x) and RNH RUBPCase (o) by SDS polyacrylamide gel electrophoresis. Mobilities of the polypeptides of RH and RNH RUBPCase were compared to proteins of known molecular weight by the method of Weber and Osborn (139). Conditions for electrophoresis are described under METHODS. Protein standards were: (1) bovine serum albumin (dimer, 136,000); (2) bovine serum albumin (monomer, 68,000); (3) catalase (60,000); (4) aldolase (39,500); (5) myoglobin (17,800); (6) ribonuclease (13,700). D, dimer; LS, large subunit; X, 47,000 mol. wt. polypeptide; SS, small subunit.

Table 8. Summary of Molecular Weights of Polypeptides of RUBPCase from Hardened (RH) and Unhardened (RNH) Rye

Polypeptide	Active Form		NaCl-Inactivated Form	
	RH	RNH	RH	RNH
Large Subunit (LS)	54,800±800 ^a	55,000±500	55,200±580	54,250±750
Small Subunit (SS)	13,900±548	14,600±678	13,500±678	14,450±519
Dimer (D)	np ^b	109,000±1000	np	108,000±1780
Anomalous (X)	np	np ^b pH 8.2 46,500±500 ^b pH 6.8	47,600±510	46,500±289

^a Average of at least 3 determinations ±SEM

^b not present

2). When the latter was dissolved at pH 6.8 a new protein band (X) of $46,000 \pm 500$ molecular weight appeared (Fig. 27, gel 1). After the gels were scanned (Fig. 29) and the peak areas of the chart recordings were integrated (Table 9) it was evident that the presence of this new polypeptide (X) resulted in a decrease of the proportion of the large subunit present relative to the small subunit. The large subunit was the probable precursor of this anomalous polypeptide. No change was observed when the pH of the sample solution was shifted back from 6.8 to 8.2 indicating that this pH effect was irreversible (Fig. 30).

Previously, Trown (195) and Steer and co-workers (182) reported that RUBPCase was irreversibly inactivated by NaCl and stabilized by $(\text{NH}_4)_2\text{SO}_4$. Present work with the enzyme from hardened and unhardened rye agreed with these results. When RH and RNH RUBPCase was eluted from DEAE Sephadex A50 with a linear NaCl gradient (0.02M to 0.3M) complete and irreversible inactivation of both enzymes occurred. Furthermore, when these NaCl-inactivated enzyme preparations were electrophoresed on SDS polypeptide gels (Fig. 31, and 32), a 47,000 molecular weight protein band appeared in both cases. The presence of this polypeptide in samples from both hardened and unhardened rye was not affected by pH.

Fig. 29 Spectrophotometric scans of SDS polyacrylamide gels of RUBPCase from hardened (RH) and unhardened (RNH) Puma rye. Labelling and conditions for electrophoresis are the same as those described in the legend of Fig. 27. Gels were scanned at 550nm in a Gilford Spectrophotometer Model 540. The bars represent 0.1 absorbance units.

RH pH6.8

RNH pH6.8

RH pH8.2

RNH pH8.2

Table 9. Relative Proportions of Polypeptides of the Active Form of RUBPCase After Dissociation with SDS

pH	Polypeptide	Average Integrated Peak Areas		Relative Proportions of Polypeptides (D:LS:X:SS)	
		RH	RNH	RH	RNH
6.8	D	np ^a	1478±143 ^b		
	LS	9945±100	6414±303	0:10	:0:1
	X	np	2929±199		1:5:3
	S	923±167	1232±143		:1
8.2	D	np	341±117		
	LS	9532±107	10806±265	0:10	:0:1
	X	np	np		0.2:10
	S	917±113	1058±163		:0:1

^a not present

^b Average of 5 integrations ±SEM

Fig. 30

Irreversibility of the pH effect on the large subunit of RNH RUBPCase. Conditions for electrophoresis are the same as for Fig. 27. (Gel 1), sample was dissolved in 50mM Tris buffer (pH 6.8) and heated (control); (Gel 2), sample was dissolved in 50mM Tris buffer (pH 6.8), adjusted to pH 8.2 and heated; (Gel 3), sample was dissolved in 50mM Tris buffer (pH 6.8), heated, cooled to room temperature then adjusted to pH 8.2. D, dimer; LS, large subunit; X, 47,000 mol. wt. polypeptide; SS, small subunit.

Fig. 31

SDS electrophoresis of the NaCl-inactivated forms of RH and RNH RUBPCase. Conditions for electrophoresis and sample preparation are the same as for Fig. 27. Gels 1 and 3 contain RNH RUBPCase; gels 2 and 4 contain RH RUBPCase. D, Dimer; ES, large subunit; X, 47,000 mol. wt. polypeptide; SS, small subunit.

Fig. 32 Spectrophotometric scans of polyacrylamide gels of NaCl-inactivated RUBPCase from hardened (RH) and unhardened (RNH) rye. Conditions for electrophoresis are the same as described for Fig. 27. Labelling is the same as for Fig. 31. Gels were scanned at 550nm in a Gilford spectrophotometer Model 540. Bars represent 0.3 absorbance units.

RH pH6.8

RNH pH6.8

RH pH8.2

RNH pH8.2

(3.7) The Effects of Freezing and Thawing on the
Structure of Purified RUBPCase from Hardened and
Unhardened Rye

An important property of protein molecules which are subjected to extremely low temperatures is resistance to denaturation due to freezing and thawing. Since the large subunit of RH RUBPCase was less susceptible to dimer formation than RNH RUBPCase (Fig. 27 and Fig. 29) the effects of freezing and thawing of solutions of the native enzyme on the large subunit was investigated (Figs. 33 and 34). In contrast to the results of SDS gel electrophoresis with reducing agent present (Fig. 33; a and Fig. 34; a), in the absence of β -mercaptoethanol a polymeric form (molecular weight $> 110,000$) of the large subunit was formed in both RH and RNH RUBPCase. In addition, the dimeric form of the large subunit was not produced under these conditions in RH RUBPCase samples but did appear in the RNH RUBPCase samples. In both cases, the anomalous 47,000 molecular weight polypeptide X appeared as well as the 55,000 molecular weight large subunit. However, there was more of the large subunit present relative to the anomalous polypeptide X in the samples of RH RUBPCase than in RNH RUBPCase samples. After five freeze-thaw cycles (Fig. 33; 5 and Fig. 34; 5) at -20°C there was still large subunit and polypeptide X present in RH RUBP.

Fig. 33 The effects of freezing and thawing on the structure of purified RUBPCase from hardened rye (RH). (a) Purified RH RUBPCase prepared for SDS electrophoresis at pH 8.2 in the presence of reducing agent as described in METHODS. All other scans represent samples of RH RUBPCase which were prepared for SDS electrophoresis at pH 8.2 in the absence of reducing agent. Prior to electrophoresis, these samples were subjected to the indicated number of freeze-thaw cycles in 0.1M Tris-SO₄ buffer (pH 8.2) containing 0.2M(NH₄)₂SO₄ and 1mM EDTA. Samples were frozen at -20°C for 30 min and thawed at room temperature. All gels were scanned at 550nm using a Gilford Spectrophometer Model 2200.

RH

LS

SS

a

P

LS

X

SS

FREEZE-THAW CYCLES

0

1

2

3

5

Fig. 34

The effects of freezing and thawing on the structure of purified RUBPCase from unhardened rye (RNH). Labelling and experimental conditions are the same as for Fig. 33.

RNH

LS

P

FREEZE-THAW CYCLES

0

1

2

3

Case samples but not in RNH RUBPCase samples. The decrease in amounts of large subunit and polypeptide X resulted in concomitant increases in the large polymeric forms. However, the small subunit appeared to be unaffected in all RH and RNH RUBPCase samples.

Functional Properties of RUBPCase from Hardened and
Unhardened Rye

(3.8) Temperature Stability

In an attempt to assess the significance of the structural changes observed in RUBPCase during low temperature adaptation of rye, certain important functional or catalytic properties of the two forms of RUBPCase were compared. One important property studied in relation to cold-hardening was thermal stability. In a preliminary experiment, the activity of crude, unactivated RUBPCase from hardened and unhardened rye was measured during storage at -25°C as a 50% $(\text{NH}_4)_2\text{SO}_4$ precipitate. The activity of the preparation from the hardened source remained constant over a period of 28 days (47nmol HCO_3^- fixed/mg protein/min.) while that from the unhardened source decreased by 50% (41 to 20nmol HCO_3^- fixed/mg protein/min.) in this time (Fig. 35). When the crude enzymes from both sources were dissolved in 20mM Tris-HCl, pH 8.1 containing MgCl_2 and β -mercaptoethanol at 1mM, they lost 85% of their carboxylase activity after 14 days. To

Fig. 35

Stability of crude RUBPCase activity at -25°C . The 25-50% $(\text{NH}_4)_2\text{SO}_4$ fraction was prepared from hardened and unhardened Puma rye as described in METHODS. This fraction from hardened rye was stored as a precipitate (Δ) and in solution (ϕ) at -25°C ; similarly for unhardened rye, as a precipitate ($\blacktriangle$) and in solution ($\bullet$). The solutions contained the 25-50% $(\text{NH}_4)_2\text{SO}_4$ fractions dissolved in 20mM Tris-HCl buffer (pH 8.1) containing 1mM MgCl_2 and 1mM β -mercaptoethanol. Enzyme activity was determined as described in METHODS.

confirm a differential stability to low temperature, a similar experiment was performed with purified RH and RNH RUBPCase. As illustrated in Fig. 36 similar results were obtained when the pure enzymes were stored at -20°C as $(\text{NH}_4)_2\text{SO}_4$ precipitates. The specific activity, measured after HCO_3^- activation, of the RH RUBPCase remained constant over a period of about a month whereas the enzyme from unhardened rye lost about 87% of its activity after 30 days at -20°C .

The decay of RNH RUBPCase activity was a first-order process with a calculated rate constant (k) of 0.111 day^{-1} (Fig. 37). Using this value for k , the half-life ($t_{1/2}$) of the carboxylase activity of RNH RUBPCase at -20°C was calculated to be 6.2 days.

All the activity of RNH RUBPCase was reacquired by heating at 50°C for 20 to 40 min (Fig. 36) indicating that this loss in carboxylase activity due to low temperature was reversible.

Fig. 38 illustrates the kinetics of heat activation of both RH and RNH RUBPCase after storage as an $(\text{NH}_4)_2\text{SO}_4$ precipitate at -20°C for one month. As indicated, RH RUBPCase activity was stable from 0 to 40 min at 50°C and did not substantially increase at this temperature. After incubation of RNH RUBPCase at 50°C for 20 to 40 min all the original carboxylase activity (0.09 moles

Fig. 36

Stability of the carboxylase activity of purified RUBPCase from hardened (○) and unhardened (●) rye at -20°C as 50% $(\text{NH}_4)_2\text{SO}_4$ precipitates. Samples were HCO_3^- activated at 23°C in the presence of 1mM DTT as described in METHODS. Enzyme activity was measured at 25°C as described in METHODS. (■) Specific activity of RNH RUBPCase after heating for 20 min at 50°C .

Fig. 37 Log specific activity vs. time plot for the decay of the carboxylase activity of RNH RUBPCase when stored at -20°C as a 50% $(\text{NH}_4)_2\text{SO}_4$ precipitate. Conditions are the same as that described for Fig. 36.

3 3

OF/DE

Fig. 38

Kinetics of heat activation of RUBPCase from hardened and unhardened rye after storage for 24 days at -20°C as a 50% $(\text{NH}_4)_2\text{SO}_4$ precipitate. (o) HCO_3^- activated RUBPCase from hardened rye incubated at 50°C in the presence of 1mM DTT. Aliquots were removed at various intervals for the determination of specific activity at 25°C . (●), (▲) HCO_3^- activated RUBPCase from unhardened rye incubated at 25° and 50°C , respectively. Aliquots were removed at various times for the determination of specific activity at 25°C .

HCO_3^- fixed/mg/min) was regained. The RNH RUBPCase activity increased by 450% after 40 min at 50°C . Heat reactivation of RNH RUBPCase was substantially slower at 25°C such that in 2hr the activity of RNH RUBPCase increased 100%.

The stability of RH and RNH RUBPCase stored in a buffered solution [0.1M Tris- SO_4 (pH 8.2), 0.2M $(\text{NH}_4)_2\text{SO}_4$, 10mM β -mercaptoethanol, and 1mM EDTA] was also studied (Fig. 39). The activity of RH RUBPCase proved to be more stable at 0°C than the same enzyme from unhardened plants. In 12 days at this temperature RH RUBPCase activity decreased by 30% while that of RNH RUBPCase decreased by 70%. The calculated rate constants for the decay in carboxylase activity at 0°C (Fig. 40) were 0.026 day^{-1} and 0.113 day^{-1} for RH and RNH RUBPCase respectively, and calculated values of $t_{1/2}$ were 26.7 and 6.1 days respectively, indicating that the enzyme from cold-hardened plants is much more stable at 0°C than the enzyme from unhardened plants. In both cases, all activity was regained after heating the enzyme samples at 50° for 20 min in the presence of DTT.

When both enzyme preparations were dissolved in the above buffer and incubated at 25°C , there was a 30% increase in activity of both enzyme preparations after 2hr (Fig. 39). This thermal activation was stable for only about 24 hr in RH RUBPCase in contrast to about 48 hr in

Fig. 39 Stability of the carboxylase activity of purified RUBPCase at 0°C and 25°C. (○), (Δ) Samples of HCO₃⁻ activated enzyme from hardened rye stored in 0.1M Tris-SO₄ buffer (pH 8.6) containing 20mM MgSO₄, 10mM NaHCO₃ and 1mM DTT at 0° and 25°C, respectively. Aliquots were removed at specific intervals and the specific activities determined at 25°C. (●), (▲) Samples of HCO₃⁻ activated enzyme from unhardened rye stored in solution at 0° and 25°C respectively. Aliquots were removed at specific times for the determination of specific activity. (□), (■) Heat activation at 50°C RUBPCase from hardened and unhardened rye, respectively. All results are normalized with respect to original specific activity. (100% activity for RH and RNH RUBPCase was 190 and 100nmoles HCO₃⁻ fixed/mg protein/min respectively.

Fig. 40 Log activity vs. time plots for the decay of the carboxylase activity of RH($\square$) and RNH($\blacksquare$) RUBPCase at 0°C. Experimental conditions were the same as in Fig. 39.

RNH RUBPCase. After this time, the enzyme activity of both RH RUBPCase and RNH RUBPCase was completely and irreversibly abolished at 25°C.

(3.9) Bicarbonate Activation of RH and RNH RUBPCase

It is known that RUBPCase from spinach is activated by incubation with HCO_3^- and Mg^{2+} (5,28,36,37,38,99). The time courses for carboxylase activity by the two forms of the enzyme from rye leaves are shown in Fig. 41 for RUBPCase preparations with or without pre-activation by HCO_3^- and Mg^{2+} . Only the pre-activated reaction began without any discernable lag and proceeded linearly for up to 2min as observed by others (108). This occurred in reactions catalyzed by either RH or RNH RUBPCase. When the HCO_3^- and Mg^{2+} activated and unactivated enzymes were titrated with DTNB a marked increase in reactivity of SH groups was observed in the activated enzymes from both hardened and unhardened rye (Fig. 42).

Bicarbonate activation of RH and RNH RUBPCase was independent of temperature (Fig. 43). Both enzymes were activated equally at 4°C and 23°C.

Fig. 44 illustrates the effect of pH on the HCO_3^- and Mg^{2+} activation. RH and RNH RUBPCase were activated at 23°C, as described in METHODS, at various values of pH and the enzymatic activity was measured at 25°C and pH

Fig. 41

Time course for HCO_3^- activation of RUBPCase from hardened (RH) and unhardened (RNH) rye. Samples were HCO_3^- activated in the absence of DTT as described in METHODS. Unactivated enzymes were prepared by passing 0.5ml aliquots of purified RUBPCase dissolved in 0.1M Tris- SO_4 buffer (pH 8.6) through a Sephadex G25 column equilibrated with the same buffer at 23°C. Enzyme activities were measured as described in METHODS. (o) and (●) represent HCO_3^- activated enzymes, (Δ) and (▲) represent unactivated enzyme preparations.

Fig. 42 Effect of HCO_3^- activation on SH titration with DTNB. Preparation of HCO_3^- activated and unactivated enzyme samples from hardened (RH) and unhardened (RNH) rye were the same as for Fig. 41. Sulfhydryl groups were titrated as described in METHODS. (o) and (●) represent unactivated enzyme preparations; (Δ) and ($\blacktriangle$) represent HCO_3^- activated enzyme preparations.

Fig. 43 Effect of temperature on HCO_3^- activation of RUBPCase. Activation was achieved in the presence of 1mM DTT at either 4°C or 23°C as described in METHODS. (o) and ($\blacktriangle$) represent HCO_3^- activation of RH RUBPCase at 0° and 23°C respectively. ($\bullet$) and ($\blacktriangledown$) represent HCO_3^- activation of RNH RUBPCase at 0° and 23°C respectively. Enzyme activity is expressed as $\mu\text{moles HCO}_3^-$ fixed/mg protein.

Fig. 44

Effect of pH on HCO_3^- activation of RH RUBPCase. Activation was accomplished at pH 7.0 (■), 7.5 (□), 8.0 (●), 8.5 (△) and 9.0 (▲) at 23°C in the presence of 1mM DTT as described in METHODS. Enzyme activity was measured at 25°C and pH 8.2 as described in METHODS. Inset represents the same results with specific activity (nmoles HCO_3^- fixed/mg protein/min) plotted as a function of the pH of HCO_3^- activation.

NMOLES HCO_3^- FIXED / MG PROTEIN

300

200

100

30

60

90

120

TIME (SEC)

SPECIFIC ACTIVITY

200

100

7

8

9

pH

8.2. For both forms of the enzyme, maximum activity was observed between pH 8.5 and 9.0 in agreement with data for the spinach enzyme (107).

(3.10) Effect of pH on RUBPCase Activity

The initial rate (v) of HCO_3^- and Mg^{2+} activated RH and RNH RUBPCase was measured as a function of pH (Fig. 45). The results indicated a pH optimum between 8.2 and 8.5 for both enzymes and this is consistent with results from other laboratories (84,88). In contrast, pH had significantly different effects on the apparent $K_m \text{ CO}_2$ of RH and RNH RUBPCase (Fig. 46). In general, as pH was increased the apparent $K_m \text{ CO}_2$ decreased; that is, the apparent affinity of RH and RNH for CO_2 was directly related to changes in pH between 7.0 and 9.0. This is consistent with the results of Lorimer et al (107) using spinach RUBPCase, and Ogren et al using the soybean enzyme (133). The $K_m \text{ CO}_2$ of the enzyme from hardened plants was, however, less sensitive to alterations of pH between 7.0 and 8.2 and apparently more sensitive between pH 8.2 and 9.0 than the $K_m \text{ CO}_2$ of the enzyme from unhardened plants. This difference in sensitivity to pH was even more dramatic when the $pK_m \text{ CO}_2$ was plotted against pH (Fig. 46, inset) which indicated a change in the ionization of an essential group(s) in the active sites of the two forms of RUBPCase. However, the intersection of the asymptotes

as

Fig. 45

Effect of pH on the initial rate (v_0) of the carboxylase reaction of RUBPCase from hardened (o) and unhardened (●) rye. Enzyme samples were HCO_3^- activated at pH 8.6 and 23°C in the presence of 1mM DTT as described in METHODS. Enzyme activity was measured at 25°C and various pH's as described in METHODS. Reaction time was 60 sec.

Fig. 46

Effect of pH on the K_m CO_2 for RH (o) and RNH (●) RUBPCase. Fresh enzyme preparations were HCO_3^- activated at $23^\circ C$ in the presence of 1mM DTT as described in METHODS. All initial rates were determined from 60 sec incubations at $25^\circ C$. Apparent $K_m HCO_3^-$ was determined by varying the HCO_3^- concentration between 1 and 20mM while holding the RUBP concentration constant at 0.4mM. Each point represents the average of three K_m determinations at each pH with the error bars representing $\pm SEM$. $K_m CO_2$ was calculated from $K_m HCO_3^-$ data.

In set represents the $pK_m CO_2$ ($-\log K_m CO_2$) as a function of pH.

which is an approximation of the pK_a of the essential group(s) (45), indicated that the ionizable group(s) in the active sites of both enzyme forms must be the same with a pK_a of about 8.2.

(3.11) Effect of Temperature on RUBPCase Activity

Since the temperature to which hardened and unhardened rye plants were adapted had profound effects on the stability of RUBPCase to low temperature, it was of interest to determine if temperature could differentially affect the apparent K_m CO_2 of RUBPCase from rye. Fig. 47 illustrates that in general as the temperature was increased, the apparent affinity of RH and RNH RUBPCase for CO_2 decreased, that is, the apparent K_m CO_2 increased with temperature. However, there was a significant difference in the degree to which K_m CO_2 of RH and RNH RUBPCase was affected by temperature. Below $10^\circ C$, the apparent affinity of RH RUBPCase for CO_2 was consistently higher than that of RNH RUBPCase. In contrast, above $10^\circ C$, the apparent affinity of RH RUBPCase was consistently less than that of RNH RUBPCase.

After V_{max} for different temperatures was determined from the y-intercept of the reciprocal plots and the log V_{max} was plotted against the inverse of temperature, the activation energies (E_a) of RH and RNH RUBPCase were calculated for the carboxylation reaction from the slopes

Fig. 47

Effect of temperature on the K_mCO_2 for RH (o) and RNH (●) RUBPCase.

Fresh enzyme preparations were HCO_3^- activated at $23^\circ C$ in the presence of 1mM DTT as described in METHODS. All initial rates were determined from 60 sec incubations at pH 8.2 at various temperatures. The apparent $K_mHCO_3^-$ was determined by varying the HCO_3^- concentration between 1 and 20mM while holding the RUBP concentration constant at 0.4mM. The apparent $K_mHCO_3^-$ was calculated from the slope and V_{max} . To obtain highly reproducible results below $10^\circ C$, greater amounts of enzyme (200 to 250 μg protein) were used per assay. Each point represents the average of three K_m determinations at each temperature with error bars representing $\pm SEM$. K_mCO_2 was calculated from the $K_mHCO_3^-$ data.

according to Arrhenius (1) (Fig. 48). The points could be joined by a smooth curve or conceivably by two straight lines with a discontinuity at about 15°C . On the assumption of a break at 15°C , the activation energies of both enzymes were greater below this temperature and lower above this temperature. A break at 15°C is consistent with the work of Badger and co-workers on RUBPCase from Atriplex glabruscula (8).

Fig. 48

Arrhenius plot of the effect of temperature on V_{max} for the carboxylase reaction of RH (o) and RNH (●) RUBPCase. Experimental conditions are the same as those described for Fig. 48. V_{max} was determined from the y-intercept of the Lineweaver-Burk plots. Each point represents the average of three V_{max} determinations at each temperature with the error bars representing $\pm SEM$. Activation energies were calculated from the slopes of the broken lines.

DISCUSSION

Heber, Santarius and co-workers (72,73,74,199) demonstrated that certain proteins washed from chloroplast membranes of winter grown spinach or rye and a number of other soluble proteins from leaves of wintergrown spinach and cabbage protected isolated thylakoid membranes against inactivation during freezing. In contrast to the functional approach of Heber and co-workers, a general polyacrylamide gel electrophoretic survey was commenced in an attempt to study the effects of cold-hardening on all detectable chloroplast proteins.

(4.1) Effects of Cold-Hardening on the Soluble Proteins and Membranes of Chloroplasts

Soluble proteins and membrane polypeptides were separated from chloroplasts isolated intact from Marquis spring wheat, Kharkov winter wheat and rye plants, listed in increasing ability to resist freeze damage (113). At the level of resolution used in this study, no effects of growth at low temperature were observed among chloroplast membrane polypeptides. However, differences in the relative densities of the thylakoid membranes after cold-hardening were apparent (Fig. 6). This may be indicative of a change in the composition of the thylakoid membrane. Further work is required to elucidate this effect of cold-hardening.

A surprising difference between chloroplasts from hardened and unhardened rye was the greater susceptibility of the former to mechanical disruption. The gentle, manual grinding method employed for the isolation of whole chloroplasts routinely resulted in 35% Class I chloroplasts from cold-hardened plants whereas unhardened plants routinely gave 70-80% Class I chloroplasts as judged by phase contrast microscopy. According to Hall (65), Class I chloroplasts have retained their outer envelope intact whereas Class II have damaged envelope membranes. This suggests that under identical isolation conditions the envelope membranes of chloroplasts from cold-hardened plants are more sensitive to mechanical stress than those of chloroplasts unhardened plants. Methods for the isolation and purification of envelope membranes are well advanced (85,142) making it possible for a comprehensive study of the structure, composition, and function of chloroplast envelope membranes during cold-hardening. These membrane effects were observed in all cultivars studied.

In contrast to the membrane polypeptides, several changes in the soluble, chloroplast proteins were observed. One of the rapidly migrating rye proteins (Fig. 7, RNH and RH, arrow) had no counterpart in the two wheat cultivars. Further work is required to establish this as a

real, qualitative, genetic difference between these cultivars. A varietal effect of growth at low temperature took the form of diminution or disappearance of a Marquis protein eluted from Sephadex G-50 in the B peak (Fig. 10 I, MNH arrow).

Chloroplast proteins that are involved in a mechanism of freeze resistance would be expected to be present only, or in greater proportion, in hardy rye and winter wheat. Such a protein band was found among those in the Sephadex G-50 B (Fig. 8) which would place it in the molecular weight range of 1.5 to 30×10^3 Daltons. Since it was detected neither in any unhardened samples nor in the spring wheat that acquired only a low level of cold-hardiness, this band may contain a protein involved in freeze resistance. The existence of such a protein is not unexpected since small ($10-20 \times 10^3$ Daltons), heat-stable proteins have been extracted from spinach and rye chloroplasts of cold-grown plants (72,73,74).

Some general changes among the soluble proteins appeared following growth at low temperature in all cultivars. These changes centered on the Fraction 1 protein band (Figs. 7 and 9), the large slowly migrating band of soluble protein. A new small band of protein was observed close to the origin after growth of all cultivars at low temperature (Fig. 7). It was distinct from the next small

band, on the same side of Fraction 1, that was obtained from cold-hardened and unhardened materials alike. These smaller bands probably represent polymeric forms of Fraction 1 protein and their increase in number has been noted by Draper and Watson (46) in total soluble leaf protein of cold-hardened perennial rye grass. After passage through Sephadex G-50, the small initial bands were no longer evident but all hardened material displayed only one peak in the region of the Fraction 1 protein band (Fig. 9) but two were present in all unhardened material. In an attempt to determine the significance of this apparent change in Fraction 1 protein or RUBPCase, enzyme activity was assayed in soluble extracts of isolated rye chloroplasts from hardened (RH) and unhardened (RNH) plants. The results indicated that the larger protein band in the RUBPCase fraction from RNH chloroplasts was an inactive artifact of extraction at pH less than 8.0. More importantly, this result indicated a differential sensitivity of RH and RNH RUBPCase to the pH of the extraction medium.

(4.2) The Effects of Cold-Hardening on the Structure of RUBPCase from Puma Rye

Preliminary evidence indicated that the major changes which occurred in the soluble fraction of chloroplastic protein were centered on RUBPCase, thus this protein was a

logical choice for further investigation in relation to cold-hardening. In order to perform in depth physical-chemical analyses of the structure of this enzyme, relatively large quantities were required in highly purified form. Whole, intact chloroplasts were difficult to isolate from hardened rye leaves; therefore, the purification of RUBPCase after extraction from chloroplasts was not feasible. With the published information for the purification of this enzyme from spinach (137,169), it appeared more promising to attempt the purification from total soluble leaf extracts from Puma rye. The method of successive gel filtration through Sephadex G100 and G200 followed by ion exchange on DEAE Sephadex A50 was found more than adequate for the purification of RUBPCase from rye leaves (Table 3, Figs. 16 and 19). The specific activity increased after each purification step, and final yields were comparable to those for RUBPCase from spinach (137) using DEAE cellulose and hydroxylapatite chromatography. Since only one protein band was observed for purified RH and RNH RUBPCase upon electrofocusing, this proved that the preparations were homogeneously pure.

Conformational changes known to occur in phosphorylase b (91), oxyhemoglobin and carbonmonoxyhemoglobin (62) and β -lactoglobulins (140) indicate that a change in reactivity of SH groups towards DTNB or any other

sulfhydryl reagent is a reliable method for detecting changes in their steric environments (209). Ellman (49) proposed that the rate of reaction of DTNB with SH groups is influenced by their number in anionic form (S^-), and also by their differing pK values. However, Kleppe and Damjanovich (91) indicated that this is not the full explanation for the difference in reactivity observed in phosphorylase b and concluded that steric hindrance plays the most important role with respect to reactivity of SH groups toward DTNB in proteins. Therefore the reactivity of the SH groups of native RH and RNH RUBPCase towards this specific, sulfhydryl reagent was employed for the purpose of measuring possible conformational changes occurring in this enzyme during cold-hardening of rye plants. The sulfhydryl groups of RH RUBPCase were less accessible to DTNB than those of RNH RUBPCase (Fig. 21) which may be a result of differential steric hindrance. This indicated that RH RUBPCase had a more compact structure than RNH RUBPCase. Chollet and Anderson (34) showed that the in vitro, cold-inactivation of crystalline, tobacco RUBPCase was associated with an enhanced accessibility of SH groups to DTNB. In contrast, in vivo adaptation of Puma rye to low temperature was associated not with a more opened, native structure for RUBPCase but rather a more compact structure.

The data for the titration of sulfhydryl groups of RH and RNH RUBPCase are consistent with a change in conformation during cold-hardening. This was corroborated by the effects of chaotropic agents, such as SDS, on the denaturation of the two forms of RUBPCase. First, the structure (Fig. 25) and carboxylase activity (Fig. 24) of RH RUBPCase were less sensitive to SDS than that of RNH RUBPCase. Secondly, data on the quaternary structure of the two forms of the enzyme indicated alterations in the stability, but not molecular weight, of the large subunit to pH (Fig. 27) and freezing and thawing (Figs. 33 and 34) with no apparent changes in the small subunit. An alternative to a conformational explanation is that the observed heterogeneity of the large subunit was due to proteolysis during purification. Degradation of the large subunit with no apparent effects on the small subunit was reported by Gray and Kekwick (60) to occur during the purification of RUBPCase from French bean in the absence of protease inhibitors. Unlike the results of Gray and Kekwick, the specific activity of RH and RNH RUBPCase increased with purification (Table 3) and resulted in single protein bands upon electrofocusing after DEAE Sephadex chromatography (Fig. 19). The native, NaCl-inactivated enzyme preparations were indistinguishable from the active forms of RUBPCase upon electrophoresis on 6% polyacrylamide gels

(Fig. 16) unlike a consequence of proteolysis. Furthermore, the high affinity of both enzyme forms for substrate (CO_2) and their regulatory properties were consistent with those observed for the same enzyme from other sources (84) which indicate the retention of native structures during the purification of RH and RNH RUBPCase. Moreover, heterogeneity of the large subunit of RUBPCase from Hydrogenomonas eutropha has been observed by Purohit and McFadden (143) under conditions identical to those employed for the enzyme from unhardened rye, and was shown to be independent of proteolysis. It is the author's opinion that this heterogeneity is an artifact, but of sample preparation for SDS polyacrylamide gel electrophoresis rather than proteolysis. (See Appendix I for further proof)

Although the conformations of RH and RNH RUBPCase appeared to be different, their amino acid compositions were not (Table 4). The analytic data indicated the presence of 90-93 half-cystinyl residues per mole of enzyme. This is in agreement with results for the tobacco and spinach RUBPCase (88) and it has been postulated that all half-cystinyl residues are present as free sulfhydryl groups in the native enzyme. Chollet and Anderson (33) showed that all of the sulfhydryl groups of crystalline, tobacco RUBPCase were accessible to DTNB in the presence of 1% SDS. In contrast, not all SH groups of RH or RNH

RUBPCase were accessible to DTNB under any of the denaturing conditions used (Fig. 23 A,B,C). Similar results were reported by McFadden and Kuehn (121) who showed that of the 93-97 half-cystines present in RUBPCase from Hydrogenomonas eutropha and Hydrogenomonas facilis only 35-37 were titratable with DTNB in the presence of 8M urea. Since there was no change in the number of SH groups titrated after reduction with NaBH_4 in the presence of 8M urea, disulfide bonds were not involved in stabilization of the tertiary structure of either RH or RNH RUBPCase (Table 7) and thus can not account for the discrepancy between the number of SH groups determined by amino acid analysis and that determined by titration with DTNB. Alternative explanations are: (1) the denaturants used may mask SH groups in some way (2) small metabolites be bound to RH and RNH RUBPCase might mask SH groups (3) urea and SDS cause dissociation of RH and RNH RUBPCase but not complete unfolding of polypeptides under the conditions employed. Further investigations is required to resolve these questions.

Although amino acid analysis indicated similar primary compositions, the net charge of RH and RNH were different (Fig. 19). This may be due to a change in conformation which would alter the pK_a 's of certain ionizable groups in the protein and therefore result in a

change in charge. Alternatively, the amide groups of asparagine and glutamine are labile to acid hydrolysis and therefore these residues did not appear in the amino acid analyses. Differences in aspartate/asparagine and glutamate/glutamine could account for the difference in net charge between RH and RNH RUBPCase. Fingerprinting of the two forms of this enzyme could resolve this question.

(4.3) The Effects of Cold-Hardening on the Functional Properties of RUBPCase

Differences in the photosynthetic temperature dependence of higher plants native to habitats of contrasting thermal regimes were reported by Bjorkman, Mooney and Ehleringer (20) and Berry (16). Further work by Pearcy, Berry and Fork (138) and Berry, Fork and Garrison (17) indicated that differences in photosynthetic performance at low temperatures appear to be related principally to changes in RUBPCase stability while that at higher temperatures is principally due to the thermal stability of photosystem II.

Earlier work by Takabe and Akazawa (191) showed that the active site of RUBPCase resides in the large subunit. Since this subunit was shown to be a locus of change in rye RUBPCase during cold-hardening, various catalytic properties were investigated in relation to temperature

and pH. The K_m CO_2 data of Fig. 47 are very similar to the results reported for photosynthetic temperature dependence of Atriplex sabulosa and Tidestromia oblongifolia (20). The former, adapted to a cool, coastal environment, is capable of high photosynthetic rates at low and moderate temperatures (10° - $30^{\circ}C$) only, whereas the latter, adapted to a hot, desert environment, is capable of high photosynthetic rates at moderate to high temperatures (25° - $45^{\circ}C$) only. Plants were photosynthetically most efficient in the temperature range to which they were adapted. A similar trend was observed for RUBPCase from rye adapted to growth at different temperatures. RUBPCase purified from rye plants adapted to growth at low temperature (2° - $4^{\circ}C$) had higher affinity for CO_2 between 0° and $5^{\circ}C$ than the enzyme purified from plants adapted to growth at moderate temperatures (20° - $25^{\circ}C$). In contrast, RNH RUBP Case had higher affinity for CO_2 between 20° - $30^{\circ}C$ than RH RUBPCase. Furthermore, the carboxylase activity of the enzyme from cold-hardened rye was much more stable to low temperature than that from unhardened plants (Figs. 36 and 39). Thus growth at cold-hardening temperatures resulted in a form of RUBPCase which was more efficient at CO_2 fixation in the temperature range of 2° - $4^{\circ}C$ than the form isolated from plants grown at moderate temperatures and vice versa.

Salcheva and co-workers (159) and Sawada et al (160, 161, 162, 163) have reported that isolated, intact chloroplasts from cold-hardened wheat have higher rates of CO₂ fixation at 20°C than those from unhardened wheat. This has been attributed to higher RUBPCase activity (159). The results presented here are consistent with this since the specific activity of RH RUBPCase was always about double that of RNH RUBPCase at 25°C. Therefore the temperature regime to which rye plants have been adapted has a profound effect on RUBP carboxylase activity and efficiency.

Although the affinity of RH and RNH RUBPCase for CO₂ had been altered during growth at low temperature, chemical group(s) ionizable between pH 7.0 and 9.0, and involved in the active site had not (Fig. 46). The pKa of these group(s) was approximately 8.2 as indicated by the pH at which the asymptotes of the pKa vs pH curves (Fig. 46) intersect (45). The most likely candidate is free SH groups with a pKa of 8.3-8.6 at 25°C (45). Early work by Trown and Rabin (195) implied the direct involvement of SH groups in the mechanism of action of RUBPCase. However, the most recent evidence (134) indicates that the epsilon amino group of lysine (pKa = 9.4-10.6) is essential for both carboxylase and oxygenase activity. Data presented in Fig. 21 also indicates that exposed SH

groups were not directly involved in carboxylation since RUBPCase activity remained unaffected after titration with DTNB for up to 30 min. However, the K_m CO_2 for both RH and RNH RUBPCase was extremely sensitive to the ionization state of sulfhydryl groups ($SH \rightleftharpoons S^-$) (Fig. 46) which indicates that SH groups must be integrally involved in the active site, possibly through the maintenance of a proper tertiary structure.

HCO_3^- - Mg^{2+} activation of RUBPCase from hardened and unhardened rye appeared to have been affected little by in vivo adaptation to low temperature. However, activation due to HCO_3^- and Mg^{2+} clearly resulted in the loosening of the structure of both RH and RNH RUBPCase (Fig. 42). Although Kwok and Wildman (97) have shown a conformational change upon binding of RUBP by tobacco RUBPCase, the increased reactivity of SH groups after HCO_3^- - Mg^{2+} activation (Fig. 42) is the first direct evidence for a conformational change accompanying RUBPCase activation.

(4.4) Possible Relevance of this Work to the Oxygenase-Carboxylase Relationship

Besides fixing CO_2 , RUBPCase also catalyzes an oxygenase reaction producing phosphoglycolate with the subsequent formation of glycolate, the substrate for photorespiration (23,84,109,133). All evidence indicates that O_2 is a competitive inhibitor of the carboxylase and

vice versa implying that both reactions utilize the same active site (84,109). However, Brändén (25) recently reported the chromatographic separation of carboxylase and oxygenase activity in RUBPCase from parsley. When no reducing agent was used during extraction and purification, the protein having oxygenase activity eluted immediately after the protein having carboxylase activity on a Sepharose 6B column, which means that the former had a slightly smaller molecular weight than the latter. This is reminiscent of the presence of an anomalous 47,000 molecular weight polypeptide from both RH and RNH RUBP Case (Figs. 33 and 34) in the absence of reducing agent for which it was shown that the large subunit was the probable precursor (Table 9). Without reducing agent, there was a heterogenous population of 55,000 and 47,000 molecular weight large subunits in rye which could possibly result in the presence of 550,000 and 466,000 molecular weight RUBPCases respectively, assuming unchanged subunit composition of L_8S_8 for both proteins. The larger protein may be "Brändén's carboxylase" and the smaller "Brändén's oxygenase" after oxidative impairment. Further work is required to resolve this question.

(4.5) Relevance of this Work to Cold-Hardening

According to the sulfhydryl-disulfide hypothesis of Levitt (102) freezing injury may result from the

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denaturation of protoplasmic proteins after formation of intermolecular disulfide bonds induced by the concentrating effect of dehydration. This suggests that freeze resistance opposes intermolecular sulfhydryl-disulfide interchange. Sulfhydryl group titration indicated that the SH groups of RH RUBPCase are less accessible to DTNB than those of RNH RUBPCase. Consequently, the protein from hardened rye would be expected to have a lower probability of forming intermolecular disulfide bonds. This was corroborated by the fact that the large subunit of RH RUBPCase never forms a dimer (Figs. 27 and 31); the large subunit of RH RUBPCase was also more resistant to polymerization due to the formation of intermolecular disulfide bonds induced by freeze-thawing (Figs. 33 and 34). Furthermore, the greater structural stability of RH RUBPCase resulted in a concomitant functional stability to low temperature (Figs. 35, 36 and 39). This is the first direct, structural and functional evidence in support of Levitt's sulfhydryl-disulfide hypothesis.

It has been postulated that the ability of tissues to bind water is correlated with their cold-hardiness (71). The work of Macdowall and Buchanan (114) with wheat grown to tolerate different degrees of freezing indicated an increase in bound water in the more hardened tissue. There have been suggestions that proteins of differing

structure have different amounts of bound water associated with them, and that small alterations in the structure of a protein might cause changes in its ability to bind water (15). The change in conformation of RUBPCase during cold-hardening of Puma may be highly significant in this respect.

Work by Shomer-ilan and Waisel (168) indicated that the amino acid composition and thus the calculated average hydrophobicity of RUBPCase from cabbage decreased during cold-hardening in support of a suggestion by Chou and Levitt (35) that hydrophobicity may be an important factor in the cold-hardening process. The results presented by Shomer-ilan et al are questionable on several grounds. First, on the basis of the total number of amino-acid residues reported to be present in cabbage RUBPCase (~ 3800) and using an average molecular weight of 100/ amino acid, the calculated molecular weight of cabbage RUBPCase is 380,000 which is far below the expected molecular weight of 500,000-600,000 reported for higher plants. Secondly, the average hydrophobicities of 800 cal/residue is far below the expected value of 1000-1200 cal/residue for such a large molecular weight, multi-subunit, globular protein (18). The data for pure rye RUBPCase disagree with the results of Shomer-ilan et al and it is concluded that the hydrophobicity did not play

a significant role in RUBPCase during the cold-hardening of Puma rye.

Conformational changes observed in rye RUBPCase during cold-hardening do not appear to be related to the isozymic substitution hypothesis as postulated by Roberts (152) since no changes in activation energies between RH and RNH RUBPCase were observed. This is consistent with the reports of Bjorkman and Percy (21) for RUBPCase from various C₃ and C₄ species adapted to different thermal environments, and Weidner and Salisbury (201) for the same enzyme from several spring wheat varieties grown at low and moderate temperatures, but it is contrary to the report of Phillips and McWilliam (141) which indicated that the activation energies of RUBPCase were lower in plant species adapted to low temperatures.

(4.6) Summary

Important structural and concomitant functional changes in RUBPCase have been identified in relation to the cold-hardening of Puma rye. The large subunit was the locus for these alterations with no apparent changes in the small subunit. The conformational changes observed by SH titration are in direct support of Levitt's sulfhydryl-disulfide hypothesis, and indicate that proteins are an integral component of the mechanism of freeze resistance. ~~The results indicate that Puma rye RUBPCase can~~ exist as two charge isomers the presence of which depends on the temperature at which the protein was synthesized in vivo.

In conclusion , the structural and functional changes shown to occur in rye RUBPCase impart significant, adaptive advantages to this important, regulatory, photosynthetic enzyme and thus to the plant during the cold-hardening process.

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APPENDIX I

Proof that Polypeptide X is not due to Proteolysis

As shown in Fig.33 and 34 (scan 0), polypeptide X was evident when purified samples of RH and RNH RUBPCase were prepared for SDS polyacrylamide gel electrophoresis in the absence of reducing agent. However, complete conversion to that shown in Fig.33(scan a) and Fig.34(scan a) respectively occurred after addition of 2% mercaptoethanol to the same RH and RNH RUBPCase samples. The large subunit and small subunit only were evident in the RH samples and the large subunit dimer, large subunit, and small subunit only were evident in the RNH samples. This proves that the effect of reducing agent was completely reversible, and that polypeptide X can not be due to proteolysis.

The above proof is valid if and only if the polypeptide X formed in the absence of reducing agent is the same polypeptide formed, irreversibly, by NaCl or a decrease in pH. Further experiments are required to elucidate the structure, function and composition of these polypeptides.

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