Cold-Hardiness-Specific Glutathione Reductase Isozymes in Red Spruce

Thermal Dependence of Kinetic Parameters and Possible Regulatory Mechanisms

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The thermal dependence of kinetic parameters has been determined in purified or partially purified preparations of cold-hardiness-specific glutathione reductase isozymes from red spruce (Picea rubens Sarg.) needles to investigate a possible functional adaptation of these isozymes to environmental temperature. We have previously purified glutathione reductase isozymes specific for nonhardened (GR-1NH) or hardened (GR-1H) needles. Isozymes that were distinct from GR-1NH and GR-1H, but appeared to be very similar to each other, were also purified from nonhardened (GR-2NH) or hardened (GR-2H) needles (A. Hausladen, R.G. Alscher [1994] Plant Physiol 105: 205–213). GR-1NH had 2-fold higher \( K_m \) values for NADPH and 2- to 4-fold lower \( K_m \) values for oxidized glutathione (GSSG) than GR-2NH, and a similar difference was found between GR-1H and GR-2H. However, no differences in \( K_m \) values were found between the hardiness-specific isozymes GR-1NH and GR-1H. There was only a small effect of temperature on the \( K_m \) (GSSG) of GR-1H and GR-2H, and no significant temperature effect on \( K_m \) (NADPH) or \( K_m \) (GSSG) could be found for the other isozymes. These results are discussed with respect to "thermal kinetic windows," and it is proposed that the relative independence of \( K_m \) values to temperature ensures adequate enzyme function in a species that is exposed to extreme temperature differences in its natural habitat. A variety of substrates has been tested to characterize any further differences among the isozymes, but all isozymes are highly specific for their substrates, NADPH and GSSG. The reversible reductive inactivation by NADPH (redox interconversion) is more pronounced in GR-1H than in GR-2H. Reduced, partially inactive GR-1H is further deactivated by \( {H_2}{O_2} \), whereas GR-2H is fully reactivated by the same treatment. Both isozymes are reactivated by GSSG or reduced glutathione. It is proposed that this property of GR-2H ensures enzyme function under oxidative conditions, and that in vivo the enzyme may exist in its partially inactive form and be activated in the presence of increased levels of GSSG or oxidants.

The temperature dependence of kinetic parameters of enzymes has been implicated as an important part of the biological adaptation of an organism to a particular climate (Somero, 1978). Teeri and Peet (1978) showed that apparent \( K_m \) values of malate dehydrogenase from plant populations of different climates are at a minimum over the temperature range normally encountered during a growing season. GRs from plants adapted to different environmental temperatures had minimal \( K_m \) values for NADPH within the optimal growth temperature range of that species or population (Kidaribi et al., 1990; Mahan et al., 1990). These observations and similar findings for other enzymes have led to the introduction of the term TKW to describe the relationship between optimal growth temperature and temperature-dependent enzyme function (Burke et al., 1988; Mahan et al., 1990).

GR catalyzes the NADPH-dependent reduction of GSSG, thereby maintaining the antioxidant GSH in its reduced, active form. It is well established that photooxidation is a mechanism of low-temperature-induced injury to plants (Gilles and Vidaver, 1990). Therefore, it is necessary for antioxidant enzymes to function adequately at low temperatures. Several studies have shown an increase in GR activities during cold hardening of conifers, and high activities throughout winter in dormant tissue (Esterbauer and Grill, 1978; Anderson et al., 1992; Doulis et al., 1993). Recently, the presence of multiple GR isozymes has been demonstrated in several plant species, and in the accompanying paper we demonstrate the occurrence of GR isozymes specific for the state of cold hardness of red spruce (Picea rubens Sarg.) (Hausladen and Alscher, 1994). Guy and Carter (1984) found that the kinetic characteristics of GR isolated from hardened spinach leaves were different from GR present in nonhardened tissue. \( K_m \) values for NADPH or GSSG were lower at 5°C than at 30°C in GR from hardened leaves, whereas no difference in \( K_m \) values at different temperatures was found.

Abbreviations: CAP, cold-acclimation protein; CoASSG, mixed disulfide between glutathione and coenzyme A; \( AS^o \), Gibbs free energy of activation; \( AS^f \), entropy of activation; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GR, glutathione reductase; GR-1H, GR isozyme 1 from hardened needles; GR-2H, GR isozyme 2 from hardened needles; GR-1NH, GR isozyme 1 from nonhardened needles; GR-2NH, GR isozyme 2 from nonhardened needles; hGSSG, homo-glutathione, oxidized; hGSH, homo-glutathione, reduced; TKW, thermal kinetic window.
in GR from nonhardened leaves. They proposed that acclimation influences the enzyme to function optimally at ambient growth temperatures. Electrophoretic studies of GR from spinach leaves have indicated the presence of a different complement of isozymes in hardened and nonhardened tissue (Guy and Carter, 1984).

In light of these results, the measurement of kinetic characteristics on what might be a heterogeneous enzyme population seems insufficient to describe the thermal dependence of kinetic constants and to interpret the significance for optimum function at ambient temperatures. Studies in plant (Teeri, 1980; Kidambi et al., 1990; Mahan et al., 1990) and animal (Low et al., 1973; Johnston and Goldspink, 1975) systems have shown a correlation between kinetic properties or thermodynamic activation parameters of enzymes and environmental temperature. However, in all of these studies different species or populations from different habitats were compared. Any differences found between enzymes may therefore also reflect a wide evolutionary diversity resulting from factors other than temperature adaptation.

The occurrence of hardness-specific GR isozymes in red spruce offers a unique opportunity to overcome the problems encountered when interpreting temperature-dependent enzyme function from organisms of a wide phylogenetic diversity. In the study reported here, we have focused on the temperature dependence of kinetic parameters in isozymes found in cold-hardened or nonhardened tissue within one species. The aim is to characterize the functional difference between these isozymes with respect to temperature adaptation of a plant species the foliage of which experiences extreme temperature differences between -20 and +30°C during the course of a year in its natural habitat (Sheppard et al., 1989). In an effort to further characterize differences between the isozymes, we have also studied substrate specificities and possible regulatory mechanisms in purified or partially purified GR isozymes.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, all chemicals were from Sigma. GR isozymes from hardened and nonhardened red spruce (Picea rubens Sarg.) needles have been purified or partially purified as described in Hausladen and Alscher (1994). Homo-glutathione was kindly provided by Dr. I.K. Smith (Ohio State University). hGSSG was produced from hGSH by oxidation with 1 mM H₂O₂ at pH 8.0 for 40 h at room temperature. The oxidation was followed by titration with DTNB (extinction coefficient = 13.6 mm⁻¹ cm⁻¹; Jocelyn, 1987), and the concentration of hGSSG was measured using yeast GR and GSH as standards (Griffith, 1980). After 40 h of incubation at room temperature, spectrophotometric determination with DTNB detected 1% of the sulfhydryl groups initially present, while total glutathione was found at its initial concentration.

Assays and Data Analysis

GR was assayed as described previously (Hausladen and Alscher, 1994). The pH of the assay buffers was adjusted at the desired assay temperatures. Unless otherwise indicated, the assay temperature was 25 ± 0.1°C. Kinetic parameters were measured in duplicate or triplicate assays using five concentrations each of NADPH (1-50 μM) and GSSG (10-500 μM). Reaction rates were measured by recording 10 data points per second and were calculated from the linear portion of the slope. Measurements were initiated within 5 s after mixing the reactants. Kₘ values were calculated by iterative fitting of the reaction rates to the rate equation for a ping-pong kinetic mechanism (Montero et al., 1990) using the computer program PENNZYME (University of Pennsylvania). Kₘ values at different temperatures and between isozymes were compared by one-way or two-way analysis of variance. Kₘ and Vₘₐₓ values for hGSSG were measured at a fixed NADPH concentration of 50 μM and five concentrations of hGSSG between 0.05 and 1.0 mM. Data analysis was as described above using the rate equation for a unikinetic mechanism. Assays for Arrhenius plots were performed in 2.5°C intervals at saturating substrate concentrations (50 μM NADPH, 1 mM GSSG). Comparisons of slopes for each isozyme after grouping data as ±22.5°C and ±22.5°C and between isozymes were done by multiple linear regression. Thermodynamic activation parameters for GR-2H were calculated according to Lehrer and Baker (1970). Data given by Kidambi et al. (1990) were used to calculate thermodynamic activation parameters of GRs of the two legumes alfalfa and Onobrychis vicifolia.

Thioredoxin from Spirulina sp. was reduced by incubation in sealed tubes for 1 h in N₂-saturated 0.1 M Tris, pH 7.5, containing 1 mM EDTA and 5 mM DTT. GR was incubated with reduced thioredoxin in individual tubes for each assay to be performed, after flushing the tubes with N₂ for 5 min. When additions were made during the incubation, the tubes were flushed with N₂ again.

RESULTS

Kinetic Properties and Thermodynamic Activation Parameters

To study the functional adaptation of GR isozymes to environmental temperature, Kₘ values were determined over a range of temperatures. Analysis of variance results for differences in Kₘ values between isozymes and with temperature are summarized in Table I. Kₘ values for NADPH were significantly higher for GRs 1 than for GRs 2, and the difference was up to 5-fold in the isozymes from hardened needles at low temperatures (Table I, Fig. 1, A and B). GR-1H had higher Kₘ(NADPH) values than GR-1NH, but no difference was found between GR-2NH and GR-2H. No significant effect of temperature on Kₘ(NADPH) was found for either isozyme (Table II). Kₘ(GSSG) was 2- to 3-fold higher for GRs 2 than for GRs 1. There was no difference in Kₘ(GSSG) between GR-1NH and GR-1H (Table I, Fig. 1, C and D). A significant effect of temperature on Kₘ(GSSG) was found for GRs 1, but not for GRs 2 (Table II). No attempts were made to model the temperature dependence of Kₘ(GSSG) for GRs 1, but visual examination of Figure 1, C and D, shows an increase over the range of temperatures used, with no indication of a minimum.
Cold-Hardiness-Specific Glutathione Reductase Isozymes: Kinetics

Table 1. Statistical analysis of differences in $K_m$ values for NADPH and GSSG of red spruce GR isozymes

Isozymes were compared by two-way analysis of variance, controlling for the effect of temperature. Values are probabilities of no difference between isozymes.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>NADPH</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GR-2NH</td>
<td>GR-1H</td>
</tr>
<tr>
<td>GR-1NH</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>GR-2NH</td>
<td>n.d.</td>
<td>0.36</td>
</tr>
<tr>
<td>GR-1H</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

* n.d., Not determined.

Arrhenius plots showed breaks at 22.5°C for all isoforms (Fig. 2). When data from the Arrhenius plots were grouped by temperature, assuming a break point at 22.5°C, multiple linear regression showed significantly different slopes above and below 22.5°C for each isofrom. No significant differences among slopes were found among the isoforms. Table III shows that the differences in activation energy of GR-2H at low and high temperatures are based on large differences in $\Delta S^\ddagger$, whereas the $\Delta G^\ddagger$ is nearly the same at 10 and 35°C. $\Delta G^\ddagger$ values obtained for legume GRs (Kidambi et al., 1990) are nearly identical to that of GR-2H, and $\Delta S^\ddagger$ values are intermediate to those of GR-2H at low and high temperatures.

Substrate Specificity

Several known substrates and effectors of GR were tested to establish possible differences between the GR isozymes. All isoforms were highly specific for GSSG, CoASSG was reduced at a very low rate by all isoforms, and no activity was found using oxidized thioredoxin from *Spirulina* sp. instead of GSSG. hGSSG was reduced by all isoforms, however, with different kinetic parameters. $V_{max}/K_m$ ratios for hGSSG were 1.9 to 6.7% of those for GSSG, and $V_{max}$ for hGSSG was between 30 and 60% of that for GSSG (Table IV). No activity was observed for either isofrom when NADH was used instead of NADPH. Ca$^{2+}$ and Mg$^{2+}$ (both 5 mM) had no effect on activity. NADP$^+$ inhibited activity of all isoforms to the same extent, when either saturating or non-saturating concentrations of NADPH were present.

Inactivation of GR by NADPH

Preincubation with NADPH led to a time- and concentration-dependent decrease in activity, which was more pronounced in GR-1H than in GR-2H. After 3 h of incubation with 0.2 mM NADPH, activities of GR-1H and GR-2H decreased by 50 and 30%, respectively (Fig. 3). In GR-1H, activity could be restored to almost 100% by either 5 mM GSH or 1 mM GSSG and to about 60% by 5 mM DTT. GSH, GSSG, or DTT fully restored activity of GR-2H. DTT-reduced thioredoxin had only a minor effect on reversal of NADPH inactivation.
Table II. Statistical analysis of a temperature effect on $K_m$ values for GSSG and NADPH of red spruce GR isozymes

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>NADPH</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR-1NH</td>
<td>0.22</td>
<td>0.038</td>
</tr>
<tr>
<td>GR-2NH</td>
<td>0.52</td>
<td>0.58</td>
</tr>
<tr>
<td>GR-1H</td>
<td>0.64</td>
<td>0.012</td>
</tr>
<tr>
<td>GR-2H</td>
<td>0.17</td>
<td>0.068</td>
</tr>
</tbody>
</table>

The effect of temperature was analyzed separately for each isozyme by one-way analysis of variance. Values are probabilities for no temperature effect.

inactivation when compared with DTT treatment alone. NADPH-inactivated GR-1H was further deactivated by 1 mM $H_2O_2$, whereas the same treatment led to complete reactivation of GR-2H (Fig. 4).

Preincubation with 5 mM GSH or 1 mM GSSG for 3 h completely protected both GR-1H and GR-2H from NADPH inactivation (0.2 mM). However, preincubation of GR-1H with 5 mM DTT or 4 $\mu$M of DTT-reduced thioredoxin had no effect 4 h after NADPH was added and restored activity to about 60% of an untreated control after 18 h. The same treatment protected GR-2H from NADPH inactivation (Fig. 5).

DISCUSSION

Temperature Dependence of Kinetic Parameters

GR activities in spruce needles have been shown to increase during cold hardening and the onset of dormancy (Esterbauer and Grill 1978; Anderson et al., 1992; Doulis et al., 1993). Since our study shows the appearance of a new isoform during this period, while the nonhardened-specific isoform disappears, we investigated kinetic parameters of the GRs over a range of temperatures. Most enzymes operate under nonsaturating substrate concentrations; therefore, substrate binding is usually of greater importance for the characterization of enzyme function under physiological conditions than its $V_{\text{max}}$. It is reasonable to assume that this relationship holds true for GR as well, because chloroplastic glutathione concentrations have been shown to be about 5 mM in pea (Gillham and Dodge, 1986). With about 5 to 10% of the total glutathione pool being in the oxidized form (Smith, 1985), GSSG concentrations in chloroplasts under normal conditions are expected to be in the range of 0.2 to 0.5 mM. Assuming that these values hold true for conifers as well, the range of $K_m$(GSSG) values of 0.025 to 0.15 mM found in our study for all isoforms and temperatures indicates that, under unstressed conditions, the GRs operate slightly above half-maximal saturation with GSSG.

The subject of temperature adaptation of enzymes has received considerable attention, but in all studies different species or populations from contrasting climates have been compared, where factors other than temperature adaptation may contribute to differences in kinetic parameters (Teeri, 1980; Simon et al., 1983; Dubuc et al., 1988; Kidambi et al., 1990; Mahan et al., 1990). This study investigates the temperature dependence of isozymes that are specific for the state of cold hardness within one species. Both GR-1NH and GR-1H have $K_m$ values for GSSG that are 2- to 4-fold lower than for GR-2, but the $K_m$ values of GR-1NH are not significantly different than those from GR-1H. Similarly, the $K_m$(NADPH) of GR-1NH and GR-1H are different from those of GRs 2. There was a significant difference in $K_m$(NADPH) between GR-1NH and GR-1H. However, since this difference was rather small, a physiological importance of this finding is doubtful. The $K_m$(NADPH) of red spruce GRs is independent of temperature between 10 and 35°C. This is in contrast to data reported for a number of broadleaf plants (Kidambi et al., 1990; Mahan et al., 1990), where the change was up to 9-fold over the temperature range between 10 and 35°C.

Based on earlier work by Teeri (1980), Burke et al. (1988) have introduced the concept of TKWs, which relates the temperature at which $K_m$ values of an enzyme are at a minimum with optimal adaptation to the environmental temperature. GRs from species of contrasting climates have been shown to have TKWs for the $K_m$(NADPH) within their normal growth temperatures (Kidambi et al., 1990; Mahan et al., 1990), and Anderson et al. (1992) have suggested a TKW for pine GR. Annual plants, such as those used by Kidambi et al. (1990) or Mahan et al. (1990), are likely to experience only a narrow range of temperatures during their life cycle, whereas perennial conifers growing at high altitudes in the southern Appalachians are exposed to temperatures ranging between −20 and +30°C during the course of 1 year (Sheppard et al., 1989). It may be that the temperature independence of $K_m$(NADPH) for GRs in red spruce ensures adequate enzyme function at all temperatures. A similar conclusion has been reached by Teeri and Peet (1978), who found $K_m$ values of malate dehydrogenase to be less dependent on temperature in a plant population growing in an environment with large temperature changes than in a population growing in a mild climate with relatively constant temperatures.

The concept of TKWs implies that low $K_m$ values are an advantage for enzyme function, which is true only at low substrate concentrations. As mentioned above, this is likely to be the case for GR as well. However, in situations where both enzyme and substrate levels are highly variable depending on physiological conditions, it is difficult to make predic-
Table III. Thermodynamic activation parameters for CRs from three plant species

Data for alfalfa (Medicago sativa L.) and sanfoin (O. viciifolia Scop.) are calculated from data given by Kidambi et al. (1990). Parameters have been calculated according to Lehrer and Baker (1970).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature</th>
<th>kcat</th>
<th>Activation Energy</th>
<th>Enthalpy</th>
<th>∆S‡</th>
<th>∆G‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>s⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR-2H</td>
<td>10</td>
<td>55</td>
<td>56</td>
<td>54</td>
<td>-0.020</td>
<td>60</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>35</td>
<td>440</td>
<td>46</td>
<td>43</td>
<td>-0.055</td>
<td>60</td>
</tr>
<tr>
<td>Sanfoin</td>
<td>10</td>
<td>90</td>
<td>51</td>
<td>48</td>
<td>-0.037</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>520</td>
<td>51</td>
<td>48</td>
<td>-0.037</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>90</td>
<td>49</td>
<td>47</td>
<td>-0.041</td>
<td>59</td>
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<td>35</td>
<td>490</td>
<td>49</td>
<td>47</td>
<td>-0.042</td>
<td>59</td>
</tr>
</tbody>
</table>

* E.U., Entropy units = kal mol⁻¹ degree⁻¹.

Tables about the physiological significance of kinetic parameters based on K_m values alone. Several studies have shown such variability in both CR activities and GSSG contents of conifer needles. GSSG levels in spruce are severalfold higher under oxidative conditions (Mehlhorn et al., 1986). Similarly, CR activities are substantially increased during winter in conifers (Esterbauer and Grill, 1978; Anderson et al., 1992; Doulis et al., 1993). Furthermore, a correlation of the temperature used, defining a TKW is of limited value for the red spruce CR isozymes.

In summary, although there is a difference in K_m values for both substrates between CR-1NH or CR-1H and CR-2, at this point no conclusion can be made about a functional difference of the hardiness-specific CR isoforms with respect to the temperature dependence of their kinetic parameters. Considering the variability of GSSG concentrations in conifers, it may be an advantage if CR isozymes with different K_m values are present to allow for efficient substrate turnover at both high and low substrate concentrations.

No differences in the activation energies were found between the CR isoforms, and the range between 37 and 57 kJ mol⁻¹ degree⁻¹ is within the values reported for CRs from alfalfa and O. viciifolia (Kidambi et al., 1990). The break points found at 22.5°C indicate that some conformational change occurs within the protein at this temperature.

Most studies on enzyme adaptation to environmental temperature have considered K_m, V_max, or V_max/K_m ratios, but not other thermodynamic parameters. When k_cat values are known, activation energies can be used to calculate thermodynamic activation parameters of an enzyme. ∆G‡ measures the energy difference between the enzyme-substrate complex and the transition state of an enzymatic reaction, and is therefore used to describe the "catalytic efficiency" of an enzyme. ∆S‡ measures the entropy change during formation of the enzyme substrate complex from enzyme and free substrate. Calculation of ∆G‡ and ∆S‡ for CR-2H shows that the differences in activation energies at low and high temperatures are due to differences in ∆AS‡, and despite the break points in Arrhenius plots, ∆G‡ is the same at all temperatures. The ∆G‡ value for CR-2H is almost identical to the values for alfalfa and sanfoin, whereas considerable differences exist in ∆S‡. The observation that differences in activation energies are based largely on ∆AS‡ are in agreement with data obtained using lactate dehydrogenases from a wide variety of animal species or fish myofibrillar ATPases (Low et al., 1973; Johnston and Goldspink, 1975). The range of ∆G‡ values for legume and spruce CRs is in the range found for lactate dehydrogenases from poikilothermic animals, but considerably lower than the values for mammals or birds (Low et al., 1973).

Substrate Specificities of Red Spruce CRs

In an effort to characterize differences among the red spruce CR isoforms, several known substrates and effectors of CR have been tested. A slight difference was found in

Table IV. Substrate specificity of red spruce CR isozymes using GSSG and hGSSG as substrates

For V_max/K_m values, only comparisons within rows are appropriate, since not all k_cat values are known.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>K_m</th>
<th>V_max</th>
<th>V_max/K_m</th>
<th>hGSSG</th>
<th>V_max</th>
<th>V_max/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-H</td>
<td>845 ± 23</td>
<td>7.66 ± 1.16</td>
<td>0.0091</td>
<td>56.2 ± 3.06</td>
<td>26.9 ± 0.69</td>
<td>0.48</td>
</tr>
<tr>
<td>2-H</td>
<td>1222 ± 266</td>
<td>167 ± 23.3</td>
<td>0.14</td>
<td>138.9 ± 5.93</td>
<td>285 ± 11.3</td>
<td>2.0</td>
</tr>
<tr>
<td>1-NH</td>
<td>667 ± 4.4</td>
<td>12.2 ± 0.42</td>
<td>0.018</td>
<td>72.5 ± 4.59</td>
<td>28.7 ± 0.78</td>
<td>0.40</td>
</tr>
<tr>
<td>2-NH</td>
<td>1069 ± 334</td>
<td>62.9 ± 11.7</td>
<td>0.059</td>
<td>131.1 ± 8.48</td>
<td>130 ± 4.27</td>
<td>0.99</td>
</tr>
</tbody>
</table>
substrate binding and turnover of hGSSG between GR-1NH and the other GR isozymes, but there was no other indication of differences in substrate specificity. hGSH (γ-Glu-Cys-Ala) is a GSH analog found in certain legumes (Klapheck, 1988). It has been shown that the enzymes catalyzing its synthesis from γ-glutamyl-Cys are highly specific for their respective substrates, Gly or Ala, but very little information is available on the specificity of GR for hGSSG or GSSG. Klapheck (1988) presents data suggesting a higher affinity of yeast GR for hGSSG than for GSSG, whereas Carnegie (1963) showed that extracts from Phaseolus aureus and purified yeast GR reduce hGSSG and GSSG at comparable rates. A detailed study of GSSG binding of human GR with synthetic GSSG derivatives showed that relatively few interactions between the glycyl moiety and the enzyme exist when GSSG is bound to GR (Janes and Schulz, 1990).

Considering these data, it is surprising to find that, in contrast, red spruce GRs are highly specific for GSSG, binding hGSSG with only 2 to 6% of the affinity found for GSSG. Although Carnegie (1963) assayed yeast or legume GR only under Vₘₐₓ conditions, it is evident that the substrate specificities of those two GRs are markedly different from those of the red spruce GRs. Klapheck (1988) has evaluated the standard cycling GSH assay with yeast GR (Griffith, 1980) for its usefulness in assaying hGSH. This assay essentially consists in assaying the enzyme under nonsaturating substrate concentrations, since it is based on a continuous oxidation of GSH by DTNB, and rereduction of GSSG by GR. The rate of DTNB reduction is proportional to the GSH concentration in the assay, and GSSG concentrations are low at all times. Klapheck's data show a 2.6-fold steeper slope of the calibration curve for hGSH than for GSH, indicating that Vₘₐₓ/Kₘₐₓ ratios of yeast GR for hGSSG are higher than for GSSG. Red spruce GRs show the opposite behavior, with Vₘₐₓ/Kₘₐₓ ratios for hGSSG that are only 2 to 6% of those for GSSG. It will be interesting to see whether other plants outside the legume family, which presumably do not contain hGSH, have GRs as specific as those of red spruce and whether legumes possess GRs that are specific for hGSSG.

One other known substrate of GR is CoASSG. The high Kₘ values for this substrate in Spirulina maxima (Rendón et al., 1986; Schirmer and Krauth-Siegel, 1989) make it unlikely that this activity is of physiological significance, but it has been used to characterize the GSSG binding site of GR (Schirmer and Krauth-Siegel, 1989). CoASSG was reduced by all red spruce GRs at about 1% of the rate observed with GSSG as a substrate; however, there was no indication of a difference between the isozymes. Since none of the substrates tested provided evidence of differences in substrate specificity, it can be concluded that none of the GR isozymes has an activity other than the NADPH-dependent reduction of GSSG.

Figure 3. Inactivation of GR-1H (○) and GR-2H (●) by NADPH. GRs were incubated for 3 h at different NADPH concentrations (A) and for different time periods in 0.2 mM NADPH (B).

Figure 4. Reactivation of NADPH-inactivated red spruce GRs. A, GR-1H; B, GR-2H. GRs were incubated for 3 h in the presence of 0.2 mM NADPH, then the following additions were made: ○, no addition; ●, 5 mM DTT; ▲, 4 μg/100 μl thioredoxin in 5 mM DTT; △, 5 mM GSH; □, 1 mM GSSG; ■, 1 mM H₂O₂. Data are averages of two experiments.
Kalt-Torres et al. (1984) reported that Mg²⁺ and Ca²⁺ have a stimulatory effect on GR activity, but no evidence for this was found for red spruce GRs. Red spruce GRs are competitively inhibited by NADP⁺, as has been found for the spinach enzyme (Halliwell and Foyer, 1978). At nonsaturating NADPH concentrations all isozymes were inhibited by NADP⁺ to the same extent. Therefore, no differences in competitive inhibition by NADP⁺ were indicated, and inhibition constant values were not determined.

Redox Interconversion of GR

Several authors have reported an inhibitory effect of NADPH on GR activity (Halliwell and Foyer, 1978; Guy and Carter, 1984; Mahan and Burke, 1987; Wingsle, 1989). The effect is time dependent; therefore, it does not seem to be substrate inhibition. It is more likely that the loss of activity results from the reduction of GR by NADPH. This process has been termed “redox interconversion” in *E. coli* GR (Mata et al., 1985), and the reduction of an intramolecular disulfide bridge has been proposed as a mechanism (Schirmer and Krauth-Siegel, 1989; Peinado et al., 1991). The NADPH concentrations used to inactivate the GRs in this study are several orders of magnitude higher than the *Kₗ₅* values for this substrate, but represent realistic in vivo concentrations found in chloroplasts (Bielawski and Joy, 1986; Gillham and Dodge, 1986). The greater susceptibility of GR-1H than GR-2H to this inactivation suggests a structural difference between these isozymes, restricting electron transfer from NADPH to the site of inactivation.

As was found for *E. coli* and corn GR (Mata et al., 1985; Mahan and Burke, 1987), red spruce GR-1H and GR-2H can be reactivated by GSSG. Since reactivation can be achieved not only by reductants, such as GSH or DTT, but also by GSSG, it is not clear whether this is a direct effect or one that occurs after reduction/oxidation of these compounds. In *E. coli*, reactivation of GR by GSSG is achieved at lower concentrations and at a faster rate than by GSH; therefore, the mechanism of reactivation is more likely to be a reoxidation of GR by GSSG (Mata et al., 1985). This was further investigated by using H₂O₂ as a nonspecific oxidant for GR. As expected, NADPH-inactivated GR-2H was fully reactivated by H₂O₂, but, surprisingly, GR-1H was further deactivated by the same treatment. Although a mechanism for this effect can only be speculative, a possible physiological importance should be considered. From the available data on chloroplastic GSSG, GSH, and NADPH concentrations (Gillham and Dodge, 1986), it is difficult to predict whether GR in vivo predominates in the oxidized or less active reduced form, since the concentrations of these metabolites are high enough to achieve both deactivation and reactivation. The in vivo concentrations of these substrates are highly variable, depending on light regime (Bielawski and Joy, 1986) or the presence of oxidative stress (Smith et al., 1985); therefore, the in vivo redox state of GR will likely vary with environmental conditions. It is possible that increases in GR activities observed after oxidative stress (Mehlhorn et al., 1986, 1987; Schmidt and Kunert, 1986) are at least partially the result of an activation of GR by increased levels of GSSG and H₂O₂.

The effect of H₂O₂ on reduced GR-2H may constitute an advantage in oxidative stress resistance, since H₂O₂ has been shown to inactivate chloroplast or mitochondrial enzymes in concentrations in the range of those used for the incubation of GR-2H (Hossain and Asada, 1984; Verniquet et al., 1991). This property of GR-2H ensures adequate functioning of the enzyme in the face of oxidative stress, and the inactivation of GR-1H under the same conditions shows that resistance to H₂O₂ is not an intrinsic property of GRs.

Effect of Thioredoxin on GR

Recently, it has been shown that GR activity in barley leaves increases upon illumination in the same way as NADP-malate dehydrogenase, suggesting that the enzyme is light regulated (Foyer et al., 1991). Since light activation of NADP-malate dehydrogenase is known to be mediated through thioredoxin (Scheibe and Anderson, 1981), the effect of this compound on red spruce GRs was tested. Thioredoxin was reduced by DTT prior to assay. An effect of thioredoxin in the presence of DTT can be detected by a faster rate of reductive activation of enzymes than with DTT alone (Cséke and Buchanan, 1986). Only moderate increases in GR activity were achieved within several minutes after addition of thioredoxin to untreated (oxidized) enzyme (data not shown), and similarly, only a minor additive effect of thioredoxin was observed on NADPH-reduced GR when compared with DTT treatment alone. Oxidized thioredoxin is known to deactivate...
malate dehydrogenase (Scheibe and Anderson, 1981), but no effect of oxidized thioredoxin on GR was found (data not shown). These data make a thioredoxin-mediated regulation of spruce GRs unlikely, although it should be kept in mind that thioredoxins are fairly specific for their target enzymes (Cséke and Buchanan, 1986).

**SUMMARY AND CONCLUSIONS**

This study and the preceding paper demonstrate the occurrence and characteristics of hardness-specific GR isozymes in red spruce. The identification of GR-1H as a CAP of known physiological function suggests a role for this GR in cold hardiness. It will be interesting to see whether other plants induce a GR isozyme during cold hardening. We have demonstrated that GR-1H and GR-2H are different gene products with considerable structural differences. GR-1NH and GR-1H are different from each other, based on their physical characteristics. However, it remains to be seen whether GR-2NH and GR-2H are different or identical, since none of the methods employed here has revealed significant differences between these two isozymes. The temperature dependence of kinetic parameters of red spruce GRs is very small when compared with that of enzymes from plants native to moderate climates, and we conclude that this provides an advantage in an environment of extreme temperature variability. Unlike many other enzymes, GR-2H is remarkably stable to oxidizing conditions, a property that ensures adequate functioning as an antioxidant enzyme. The N-terminal sequences provide a tool for the isolation of GR genes and, consequently, for the study of the induction of GR genes during hardening and oxidative stress.

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**LITERATURE CITED**


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