Occurrence of Nicotinamide Adenine Dinucleotide Phosphate-linked Glyoxylate Reductase in Nonphotosynthetic Xylem Tissue of Perennials

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ABSTRACT

Xylem extracts of poplar tree contained glyoxylate reductase specific for NADPH. By isoelectric focusing in the pH ranges 3.5 to 10 or 4 to 6, the enzyme exhibited a single peak of activity at pH 5.4. The enzyme showed essentially no activity toward hydroxy pyruvate, pyruvate, or NADH. The reaction was optimal at pH 6.0 in phosphate buffer and the activity profile exhibited a sharp and narrow pH profile with half-maximal activities at about pH 7.0. The K_m of the enzyme for glyoxylate was 0.11 millimolar. The xylem tissue of poplar tree exhibited high levels of enzyme activity (30 micromoles per gram dry weight per hour) even in the wintering stage and a slight change in activity occurred in spring and fall at the time when metabolism transition occurs.

Previous studies have shown that glucose-6-P and 6-P-gluconate dehydrogenase activities were higher in the wintering poplar xylem than in the budding ones and that the changes in the activities occurred in spring and fall during the transition of growth from the wintering to growing stage and vice versa (13).

The increase and decrease of the two enzymes activities indicate that the cells change their synthetic activity as they require a variety of substrates during growth and differentiation. The increased activities of the above two dehydrogenases at the time of metabolism transition from growing to wintering stage in the fall imply that the cells require a large amount of NADPH to maintain the cells in a reduced state, together with additional building blocks for the stem.

Since NADPH serves as a carrier of electrons from catabolic reactions to reductive reactions, a variety of coupled reactions would be present and functioning in the wintering perennials. At present little is known about the reactions which utilize NADPH in wintering plants. The present report provides evidence that the xylem tissue, the nonphotosynthetic tissue of the stem, contains an enzyme specific both for NADPH and glyoxylate. Studies were also carried out to study the activity of the enzyme during differentiation through a year life cycle of perennials.

MATERIALS AND METHODS

Chemicals and Enzymes. NADP, NADPH, NAD, NADH, pyruvate, and triose-P isomerase were from Boehringer-Mannheim GmbH; glycerate dehydrogenase (spinach leaves), hydroxy pyruvate (lithium salt) and glycolaldehyde were purchased from Sigma Chemical Co. Glycolic acid, oxaloacetic acid, glyoxal, dihydroxyacetone, glyceraldehyde, and other chemicals were purchased from Nakarai Chemicals Ltd.

Plant Materials and Crude Enzyme. Populus gelrica was grown in the field. Portions of 1- or 2-year-old twigs located about the middle to two-thirds toward the apex were sampled and used on the same day. Trees (about 12 years old) were cut in May and they were cut into logs about 1.5 m long starting from the basal part of the trunk. The material located about 4 m tall (about 20 cm in diameter) of the tree was transferred to a cold room and stored at -20 C. Homogenization mixtures contained 1.0 g of small excised pieces of xylem tissue, 0.2 g polyal AT, 1.0 g sea sand, and 3.5 ml 0.05 M Tris-HCl (pH 7.6) containing 2 Mm DTT. Gridding of the mixture was carried out automatically in a cooled mortar for about 3 min (14). The slurry was pressed through a sheet of gauze and the resultant extract was centrifuged at 14,000g for 5 min. In another set of experiments, homogenization mixtures contained 1.0 g of excised pieces of xylem tissue, 0.2 g polyal AT, 1.0 g sea sand, and 3.5 ml 0.25 M sucrose containing 25 Mm Tris-Mes (pH 7.0), 2 Mm DTT, and 3 Mm EDTA. Grinding of the mixture and extraction of crude enzyme were carried out as above. After centrifugation at 1,000g for 10 min to remove cell debris, the extract was centrifuged at 14,000g for 10 min to spin down chloroplasts and mitochondria and then at 105,000g for 30 min to spin down microsomes. Finally the supernatant was centrifuged in buckets of a swing-out rotor at 369,000g (60,000 rpm) for 30 min at 4 C in a Beckman 12 65B centrifuge.

Enzyme Activity Measurement. Glyoxylate reductase activity was assayed according to Zelitch and Gotto (24), in the following mixture, which contained 75 mM phosphate buffer (pH 6.4), 0.12 mM NADPH or NADH, 3 mM glyoxylate, and enzyme solution in a total volume of 1.5 ml. The reaction was initiated by the addition of glyoxylate and measurements were carried out with a Beckman DB-GT recording spectrophotometer, thermostated at 25 C by circulation of a water-ethanol solution. Hydroxypyruvate reductase activity was assayed by the procedure of Tolbert et al. (20); the reaction mixture contained 75 mM Tris-HCl (pH 7.2), 0.12 mM NADH, 3 mM hydroxpyruvate, and enzyme solution in a total volume of 1.5 ml. The reaction was initiated by the addition of hydroxypyruvate. Malate dehydrogenase activity was assayed by the procedure of Ochoa (10); the reaction mixture contained 75 mM Tris-HCl (pH 7.2), 2 mM EDTA, 0.12 mM NADH, 0.4 mM oxaloacetate, and enzyme solution in a total volume of 1.5 ml. The reaction was initiated by the addition of oxaloacetate. Glucose-6-P and 6-P-gluconate dehydrogenase activities were measured as described previously (14). Diaphorase activity was assayed by the procedure described by Forti (6); the reaction mixture contained 75 mM Tris-HCl (pH 7.6), 1 mM ferricyanide, 0.12 mM NADPH, and enzyme solution in a total volume of 1.5 ml. Assay conditions of reduced NADP dehydrogenase (pyridine nucleotide quinone reductase) were similar to those described by Wosilait and Nason (22). In 1.5 ml, the reaction
mixture contained 75 μmol K-phosphate (pH 7.2), 0.4 μmol p-benzoquinone, 0.12 μmol NADPH, and enzyme solution. A correction was made for the nonenzymic rate.

**Isoelectric Focusing.** An LKB electrofocusing column (8100-1, 110-ml effective volume) was used. The column was cooled by circulation of a thermostated water-ethanol mixture (0°C) through the jacket. Separations were carried out in a pH range 3.5–10 or 4–6, using 1% LKB Ampholine (carrier ampholites), 2 mM DTT, and sucrose gradient from 0 to 50% (top to bottom). Xylem extracts were prepared by grinding 1.0 g of twig xylem in the presence of 3.5 ml of 20 mM Tris-HCl (pH 7.6) containing 2 mM DTT and 0.05% Triton X-100, 0.2 g Polyclar AT, and 1.0 g sea sand. After centrifugation at 14,000g for 10 min, the extract was centrifuged at 105,000g for 30 min. The supernatant, equivalent to 5 g dry weight xylem, was layered into the column. No purification procedures were included to avoid selective removal of any protein fraction with the reductase activity. A current (570 v, 14 mamp at the start and 1,600 v, 4 mamp at the end) was applied and the duration was for about 16–18 h. When isoelectric focusing was completed the entire column was fractionated in 2-ml portions, and the enzyme activities in each fraction assayed. The pH of the fraction was determined at 20°C using a Beckman expandamatic pH meter. Protein was determined by adding aliquots of the solution into a final volume of 2 ml of 2% sulfosalicylic acid, followed by measurement at 600 nm after standing for 10 min at room temperatures and BSA was used as the standard.

**RESULTS**

Xylem extracts contained a NADPH-linked glyoxylate reductase and also exhibited a low level of the reductase activity with NADH. A portion of the latter activity may have been attributed to a NADH-linked hydroxypyruvate reductase present in the extract, since this NADH-linked enzyme is known to reduce glyoxylate as well (7, 20).

Activity levels of the NADPH-linked glyoxylate reductase were followed throughout the year (Fig. 1). At the stage when active growth occurred in June, the xylem tissue began to exhibit an increase in activity and until late August the activity seemed to remain at the same level. After differentiation of cambium to xylem, a slight decrease in the activity occurred and after November and throughout winter the activity remained at the same level.

At the time of the onset of growth in May a decrease in the activity occurred in 2-year-old xylem, concomitant with the slight rise in the activity in the differentiating xylem cells.

In an effort to see whether the NADPH-linked enzyme is composed of isoenzymes, the xylem extract was separated by the method of isoelectric focusing. The isoelectric focusing profile of the extract in a pH range 3.5–10 is shown in Figure 2. Pooled fractions (pH about 5–6) from a set of experiments in the pH range 3.5–10 were dialyzed for 2 h at 0°C against cold distilled H2O and electrofocused in a pH range 4–6 (Fig. 3). In both cases...
The enzyme activity with NADPH was present only as one peak at pH 5.4. The major activity was located within 0.1 pH unit, indicating that the NADPH-linked enzyme is not present in multiple molecular forms. A typical result of the purification procedure is shown in Table 1. By isoelectric focusing, an enzyme fraction with a 40-fold increase in specific activity was obtained in one fraction (2 ml) with recovery of about 20% of the starting material.

Using isoelectric focused enzyme, the pH optimum in phosphate buffer was found to be 6.0 for poplar xylem enzyme (Fig. 4). The highest rate of the activity was always observed between pH 5.8 and 6.2. The pH optimum was rather sharp and narrow and at pH values 7.0 and 8.0 the activity was decreased to about 55 and 23% of the maximum. The xylem glyoxylate reductase from poplar exhibited a strict substrate specificity (Table II). Of the nine substrates examined, glyoxylate was the only substrate for the enzyme. It is also true that NADH does not seem to serve as the substrate for the reaction.

The $K_m$ value for the xylem glyoxylate reductase was determined with the isoelectric focused enzyme. A low $K_m$ (0.11 mM) glyoxylate for the xylem glyoxylate reductase was obtained (data not shown).

It is known that the equilibrium of NADH-linked enzyme

Table I. Purification of NADPH-linked Glyoxylate Reductase from Xylem of Poplar Twigs

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total Activity (µmol/min/mg)</th>
<th>Specific Activity (µmol/min)</th>
<th>Purification</th>
<th>Enzyme Recovery (%)</th>
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<tr>
<td>14S</td>
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<td>2.23</td>
<td>0.05</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>105S</td>
<td>14.5</td>
<td>2.32</td>
<td>0.16</td>
<td>3.2</td>
<td>104</td>
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Isoelectric focusing

<table>
<thead>
<tr>
<th>No. of fraction</th>
<th>Total Activity (µmol/min)</th>
<th>Specific Activity (µmol/min)</th>
<th>Purification</th>
<th>Enzyme Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0.35</td>
<td>0.10</td>
<td>0.28</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>0.35</td>
<td>0.21</td>
<td>0.61</td>
<td>11.7</td>
</tr>
<tr>
<td>19</td>
<td>0.21</td>
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<td>39.0</td>
</tr>
<tr>
<td>20</td>
<td>0.27</td>
<td>0.06</td>
<td>0.22</td>
<td>4.1</td>
</tr>
<tr>
<td>21</td>
<td>0.27</td>
<td>0.06</td>
<td>0.22</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of pH on NADPH-glyoxylate reductase activity. Standard assay system in 50 mM K-phosphate buffer was utilized; assay temperature was 25 C.

Fig. 5. Distribution of enzyme activities in different age of xylem tissue of poplar stem. Extracts were prepared from frozen-stored logs sampled in May. Standard assay conditions were used. Other experimental conditions were the same as described in Figure 4. PNQR: reduced NADP dehydrogenase (pyridine nucleotide quinone reductase).

The reaction is far removed from the formation of glyoxylate and the reaction essentially functions in the formation of glycolate at the expense of NADH (23). The equilibrium for the reaction catalyzed by xylem glyoxylate reductase was determined from the experiments in which the amount of NADPH formed from added NADP (0.24 µmol) was measured in the presence of a large excess of glycolate (200 µmol). With 50 mM K-phosphate (pH 7.6) and using 0.08 unit of isoelectric focused enzyme, the amount of NADPH produced in the experiments was too small (about 0.5 nmol) for accurate measurement. Yet, the determination of the reversibility of the reduction of glyoxylate supported the reversibility of the reaction.

The distribution of the glyoxylate reductase activity in annual rings was determined. The results indicated that the activity of the xylem glyoxylate reductase was higher in the newly synthesized xylem tissue and decreased toward the inner portion of the xylem (Fig. 5). The annual ring of the 6 year old still exhibited an

Table II. Substrate Specificity of Xylem NADPH-linked Glyoxylate Reductase from Poplar

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyoxylate (NADPH)</td>
<td>0.66</td>
<td>100</td>
</tr>
<tr>
<td>Glyoxylate (NADH)</td>
<td>0.66</td>
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</tr>
<tr>
<td>Hydroxyproprionate (NADPH)</td>
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<td>2</td>
</tr>
<tr>
<td>Hydroxyproprionate (NADH)</td>
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<td>0</td>
</tr>
<tr>
<td>Glycoxyl (NADPH)</td>
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<td>2</td>
</tr>
<tr>
<td>Acetaldehyde (NADPH)</td>
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</tr>
<tr>
<td>Glycolaldehyde (NADPH)</td>
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<td>0</td>
</tr>
<tr>
<td>Dihydroxyacetone (NADPH)</td>
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<td>0</td>
</tr>
<tr>
<td>Glyceraldehyde (NADPH)</td>
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<td>0</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P (NADPH)</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P + triose-P isomer (30 units) (NADPH)</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Pyruvate (NADPH)</td>
<td>2.0</td>
<td>0</td>
</tr>
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</table>
appreciable amount of the reductase activity and of reduced NADP dehydrogenase (pyridine nucleotide quinone reductase) activity (22). The annual ring of 8 year old also exhibited an appreciable amount of diaphorase activity (6).

DISCUSSION

A NADH-linked glycolylyl reductase in spinach leaves was first described and crystallized by Zelitch (23). At about the same time a NADH-linked hydroxypyruvate reductase (d-glyceraldehyde dehydrogenase) in parsley leaves was reported by Stafford et al. (18). d-Glyceraldehyde dehydrogenase was isolated from spinach leaves by Holzer and Holldorf (7), and Laudahn (9). From these results, it was generally accepted that the same NADH-linked reductase was involved in the reduction of hydroxypropanoate to d-glyceraldehyde and of glycolylyl to glycolate. In later work, it was shown that the enzyme is located in leaf peroxisomes and utilizes NADPH at a rate slower than NADH (20).

A NADPH-linked glycolylyl reductase in higher plants was first reported to be present in tobacco leaves and partially purified by Zelitch and Gotto (24) and the intracellular localization of the enzyme was shown to be in chloroplasts (4, 16, 20).

The present report provided evidence that the xylem of poplar twigs and stem contained glycolylyl reductase and that isoelectric focusing studies revealed the presence of a glycolylyl reductase with a strict substrate specificity toward NADPH and glycolylyl in the nonphotosynthetic tissue of the trees. Of 34 perennials (including trees and shrubs) studied, xylem tissues of the 33 plants contained the enzyme activity, whereas in the living bark only 22 plants exhibited the enzyme activity (S. Sagisaka, unpublished data).

The xylem enzyme is unique in its strict substrate specificity, and the activity profile (Fig. 4) is different from those NADH-linked enzymes isolated from spinach and tobacco leaves (20, 24). The xylem enzyme exhibited a low $K_m$ (0.11 mm) for glycolylyl. The affinity for glycolylyl of the NADPH-linked xylem enzyme is about 100 times as great as that previously reported for the NADH-linked enzyme (7, 23), and is about the same for NADPH-linked glycolylyl reductase from spinach leaves (24).

It is likely that only a single species of NADPH-glycolylyl reductase is present in the fraction, since a single charge change in proteins generally resulted in a change of about 0.2 unit in their isoelectric point (19) and proteins with a difference of pI of 0.2 pH could have been separated by the isoelectric focusing procedure (Figs. 2 and 3). After electrofocusing for 18 h at 0 C, 20% of the activity was present in one fraction and total recovery was about 50% of the initial. To inhibit protease action, addition of phenyl-methylsulfonyl fluoride (1.7 mm final concentration) to the homogenization mixture did not improve the recovery at the stage of isoelectric focusing.

After differential centrifugation of xylem homogenate, the distribution of the NADPH-glycolylyl reductase was measured. The activity was present in the upper fraction after centrifugation at 369,000 g for 30 min, indicating that the reductase is readily soluble plastid enzyme in poplar xylem. The plastids may well be fragile, resulting in loss of plastid enzyme (4, 16). Enzymes readily solubilized such as hydroxypropanoate reductase and serine-glycolylyl (pyruvate) aminotransferase may well be the same plastid enzymes of poplar xylem and released from the plastids during homogenization (S. Sagisaka, unpublished data).

Previously I showed that glucose-6-P and 6-P-glucanate dehydrogenase activities in wintering poplar tissues were higher than in budding ones (13, 14). The elevated level of reducing power in terms of NADPH-generating activity of the pentose-P cycle in the winter stage is one of the characteristics of perennials such as poplar. In xylem tissue, most of NADPH could be synthesized from the pentose-P cycle activity and perhaps, to a lesser extent from isocitric dehydrogenase. Since the xylem of poplar twigs exhibits a very low but distinct glycolate oxidase activity (S. Sagisaka, unpublished data), it seems probable that a portion of the NADPH formed in xylem tissue is metabolized by way of glycolate oxidation and glycolate oxidation to generate peroxides (15). The coupled reactions act as a shuttle of reducing equivalents from NADPH to O$_2$, resulting in regeneration of NADP. The glycolate oxidase reaction may have occurred in organelles and involved in the reactions requiring peroxide, such as lignin and flavonoid synthesis and IAA metabolism. The xylem of poplar contains also high levels of enzyme activities, such as reduced NADP dehydrogenase (pyridine nucleotide quinone reductase) and diaphorase (Fig. 5). NADPH could be reoxidized to NADP by the action of these enzymes. In addition to the NADPH-linked glycolate reductase, the occurrence of the plastid enzymes suggests that sucrose is synthesized from glycolate in xylem tissues (8, 12).

The formation of glycolate by photosynthetic CO$_2$ assimilation was described first by Benson and Calvin (2). It was further shown that glycolate production by leaves was enhanced by high light intensity, low CO$_2$ concentration, and high O$_2$ partial pressure (1, 3, 5, 21). In xylem, a nonphotosynthetic tissue, the reaction by which glycolate or glycolate is synthesized is not known. Xylem cells do not contain appreciable amounts of chloroplasts. The xylem tissue may not have, if any, such a reaction system as in leaves and it appears probable that a reaction different from the leaf system is taking place. Glycolate formation from the transketolase-C$_2$ complex has been studied by Plaut and Gibbs (11) and Shain and Gibbs (17). This reaction is one of the possible candidates of the glycolate formation with nonphotosynthetic properties of xylem tissue.

LITERATURE CITED