myo-Inositol 1-Phosphate Synthase Inhibition and Control of Uridine Diphosphate-α-glucuronic Acid Biosynthesis in Plants

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ABSTRACT

Of the eight intermediates associated with the two pathways of UDP-glucuronic acid biosynthesis found in plants, only UDP-glucuronic acid inhibited myo-inositol 1-phosphate synthase (EC 5.5.1.4), formerly referred to as α-glucose 6-phosphate cyloaldolase. Inhibition was competitive. An attempt to demonstrate over-all reversibility of the synthase indicated that it was less than 5% reversible, if at all.

Biosynthesis of UDP-glucuronic acid in plants proceeds via UDP-glucose oxidation or via MI oxidation followed by MI oxidation (17). There is mounting evidence that the MI oxidation pathway is the major path of carbon from hexose to uronic acid and pentose components of plant polysaccharides during certain stages of development (17, 30), while at other stages oxidation of UDP-glucose appears to predominate (28, 29). Information regarding the activities of phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, and glucuronokinase in Lilium longiflorum pollen has been gathered by Dickinson and his colleagues (8-10, 16). The activities of MI oxygenase and UDP-glucuronic acid pyrophosphorylase have not been studied in pollen but have been reported in other plant tissues (15, 27, 28). Regulation of these two pathways may play a major role in the metabolic control of carbohydrate interconversions associated with plant growth (22).

Feedback inhibition contributes to the control of enzymes involved in the UDP-glucose oxidation pathway. In L. longiflorum pollen and in other tissues, both UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase are inhibited by UDP-glucose, UDP-galactose, UDP-xyllose, UDP-glucuronic acid, and UDP-galacturonic acid. UDP-arabinose also inhibits the second enzyme, and UDP-xyllose is a particularly effective allosteric inhibitor of MI oxygenase (5, 8, 16, 21). Less information is available regarding inhibition of enzymes associated with the MI oxidation pathway. MI oxygenase from rat kidney is inhibited by D- and L-myoinositol-1, myo-inositol-2, and D- and L-epi-inositol-2 (6). Glucuronokinase from L. longiflorum pollen is inhibited by glucuronic acid 1-phosphate and UDP-glucuronic acid (10). No inhibition is observed in the activity of UDP-glucuronic acid pyrophosphorylase from barley seedlings when this enzyme is tested in the presence of 2 mM glucose, glucose 6-phosphate, glucose 1-phosphate, MI or glucuronic acid or in the presence of 1 mM UDP-xyllose, UDP-glucose, UDP-galacturonic acid, or UDP-glucuronic acid (28).

Phosphoglucomutase is competitively inhibited by excess concentrations of its substrates, glucose 1-phosphate and glucose 1,6-diphosphate (26). Glucuronic acid 1-phosphate binds to this enzyme and may prove to be a competitive inhibitor (25). The present report examines the inhibitory properties of intermediates and products of the two biosynthetic pathways to UDP-glucuronic acid toward MI 1-phosphate synthase, the first enzyme of the MI oxidation pathway, and compares the specific activity of this enzyme with those of other enzymes of the two pathways in so far as data are available.

MATERIALS AND METHODS

MI 1-phosphate synthase was prepared from Acer pseudoplatanus cells as previously described (18) with slight modification. To prepare large quantities of the enzyme, a 35 to 50% ammonium sulfate fraction from 600 g fresh weight of cells was further purified on a 2 × 30 cm column of DEAE-cellulose. With a linear gradient from 0 to 0.2 M sodium chloride in 600 ml of 0.02 M tris-HCl buffer, pH 8, containing 0.5 mM GSH, the active enzyme appeared between 0.12 to 0.17 M NaCl. This material was further purified on Sephadex G-200 as previously described. The specific activities of these preparations ranged from 10⁻² to 10⁻² U/mg of protein where U is amoles of product formed per min. Frozen solutions remained stable up to 3 months.

Experience has shown that active preparations of enzyme may be obtained from cells harvested and stored at −20 C for 8 months. The ammonium sulfate step of purification is also stable when stored as a frozen solution at −20 C for several months.

This enzyme was previously referred to as glucose 6-phosphate: 1-L-MI 1-phosphate cyclase (or cyloaldolase). Recently, Hoffmann-Ostenhof introduced a more appropriate name, MI 1-phosphate synthase, 1-L-MI 1-phosphate lyase (isomerizing) (EC 5.5.1.4) (24). This name has been adopted here.

1 This work was supported by Grant GM-12422 from the National Institutes of Health.
2 This work is dedicated to Professor Otto Hoffmann-Ostenhof on the occasion of his 60th birthday.
3 Where unstated, configurations of sugars refer to the naturally occurring form in plants.
4 Abbreviations: MI: myo-inositol.
In the enzyme assay for MI 1-phosphate synthase, 3.61 μmoles of glucose-1-14C 6-phosphate (10′ cpm), 1.5 μmoles of NAD+, 7.2 μmoles of NH4Cl, 50 μmoles of tris-HCl buffer at pH 8.0, and enzyme in a total of 1.5 ml were incubated for 2.5 hr at 30 C. Analysis of the product was as previously described (18, 19). In kinetic experiments, 1-hr incubations were used rather than 2.5-hr incubations to determine initial velocities. Enzymic activity remained linear up to 2.5 hr. To assay samples containing added MI, it was necessary to substitute paper chromatography in one direction for the regular two dimensional TLC. In these assays, separation was made on Whatman No. 1 paper in ethyl acetate-pyridine-water (10:6:5, v/v) for 20 hr. The MI area was cut out and assayed for radioactivity.

All samples were assayed for radioactivity by liquid scintillation counting with a toluene-Triton X-100 mixture (1:0.5, v/v) containing 4 g of PPO and 0.1 g of dimethyl POPPOP per liter of toluene.

Fructose 1-phosphate, MI, MI 2-phosphate (cyclohexyiammonium salt), UDP-glucuronic acid, and UDP-glucose were purchased from Sigma Chemical Co.; fructose 6-phosphate, sodium glucuronate, and sodium galacturonate from Nutritional Biochemical Co.; glucose 1-phosphate from Schwartz-Mann, intestinal alkaline phosphatase from Worthington Corp.; and glucose-1-14C 6-phosphate from New England Nuclear Corp.

d-MI 1-phosphate was prepared by acid-catalyzed migration of MI 2-phosphate (1). Organic phosphates were separated from Pi by gradient elution of Dowex 1 (formate) (31) and resolved by paper chromatography on prewashed Whatman No. 3 paper in 2-propanol-concentrated ammonium hydroxide-water (7:1.3, v/v) for 135 hr at 32 C. d-MI 1-phosphate concentrations were determined using the method of Bartlett (4).

1l-MI-U-14C 6-phosphate was prepared biochemically by incubating 49.6 mg of glucose-U-14C 6-phosphate (7 × 106 cpm) and 43 mg of NAD+ in 60 ml of 0.05 M tris-HCl, pH 8, for 3 hr with freshly prepared MI 1-phosphate synthase from 222 g of rat testes (2). The reaction was terminated with 250 ml of warm ethanol and centrifuged at 12,000g for 20 min. MI 1-phosphate was recovered from the supernatant as described by Eisenberg and Bolden (13). At the time it was used to measure reversibility, TLC showed 18% hydrolysis to free MI. About 1% of the radioactivity in this preparation was glucose 6-phosphate.

To test for reversibility of MI 1-phosphate synthase from A. pseudoplatanus, 1l-MI-U-14C 1-phosphate (195,530 cpm, 0.34 μmole) was incubated with enzyme as described in the assay, omitting glucose 6-phosphate. In the control, the enzyme was heat denatured before addition of label. After treatment with 0.2 mg of alkaline phosphatase and 4 μmoles of magnesium chloride, the reaction mixture was heated in boiling water for 5 min followed by centrifugation to remove denatured protein. The supernatant was passed through Dowex 50 (H+) and Dowex 1 (formate) columns to remove ionic components and separated by paper chromatography on Whatman No. 1 paper using a descending solvent system of ethyl acetate-pyridine-water (8:2:1, v/v) for 20 hr to separate glucose from MI which compounds were located by radioscanning, cut out, eluted, and assayed for radioactivity by liquid scintillation counting.

RESULTS AND DISCUSSION

The enzymic activities associated with the two pathways of UDP-glucuronic acid, as found in crude plant extracts, are listed in Table I. Missing from this list is the MI 1-phosphate specific phosphatase which has been obtained from rat testis (12) and yeast (7) but not from plant tissues. Raw extracts of A. pseudoplatanus, tested at pH 8, hydrolyzed 34% of the MI 1-phosphate produced by the synthase (18), an observation recently confirmed by additional studies (M. W. Loewus, unpublished observations). Until more information regarding specific or nonspecific phosphatase action on MI 1-phosphate in plant tissues has been obtained, the activity of this step in the MI oxidation pathway, relative to other enzymes involved, cannot be clearly established. Of the enzymes listed, MI 1-phosphate synthase and MI oxygenase had activities orders of magnitude less than the others. It would appear that the rate-limiting step in the conversion of glucose to UDP-glucuronic acid via MI occurs prior to glucuronic acid.

The low activity of MI 1-phosphate synthase posed the question of the existence of an unfavorable equilibrium due to reversibility of this enzyme. To test this, A. pseudoplatanus synthase was incubated with 1l-MI-U-14C 1-phosphate and NAD+. In duplicate experiments, 99% of the counts were recovered in MI 1-phosphate from both active enzyme and boiled control. The other 1%, recovered as glucose 6-phosphate, was no larger than the original contamination. The experimental error was such that a reverse reaction of less than 5% of the forward reaction would be detected. Therefore the low activity of the synthase was not a result of significant reversibility. The partial reaction of glucose 6-phosphate to 5-keto-glucose 6-phosphate has been shown reversible, using rat testis cyclooalaloside, by Barnett et al. (3).

With 2.4 mM substrate present, the following compounds, at the mM concentration indicated, caused little or no inhibition of MI 1-phosphate synthase: glucose 1-phosphate, 2.66; UDP-glucose, 6.66; UDP-glucuronic acid, 2.66; UDP-xylene, 3.33; MI, 10.66; d-MI 1-phosphate, 2.49; glucuronic acid 1-phosphate, 6.66; galacturonic acid, 3.33 and fructose 1-phosphate, 2.66. The enzyme was inhibited 30% with 3.3 mM glucuronic acid, 48% with 6 mM D-2-deoxyglucose 6-phosphate, 38% with 5.8 mM mannose 6-phosphate and 21% with 2.66 mM

<table>
<thead>
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<th>Table I. Activities of Pathway Enzymes</th>
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<tr>
<td><strong>Enzyme</strong></td>
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<tr>
<td>Sugar nucleotide oxidation pathway</td>
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<tr>
<td>Phosphoglucomutase</td>
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<tr>
<td>l-UDP-Glucose pyrophosphorylase</td>
</tr>
<tr>
<td>l-UDP-Glucose dehydrogenase</td>
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<td>A. pseudoplatanus cambial tissue</td>
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<tr>
<td><strong>myo-Inositol oxidation pathway</strong></td>
</tr>
<tr>
<td>myo-Inositol 1-phosphate synthase</td>
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<tr>
<td>A. pseudoplatanus culture</td>
</tr>
<tr>
<td>myo-Inositol oxygenase</td>
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<tr>
<td>Glucuronokinase</td>
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<tr>
<td>UDP-Glucuronic acid pyrophosphorylase</td>
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* Ungerminated pollen.
* Germinated pollen.
* Taken for assay at the ammonium sulfate step of purification. Other activities reported in this table were determined on raw extracts of tissues.
* No difference was found in this value between germinated and ungerminated pollen, as reported earlier (18). Careful homogenization and protein extraction in this experiment may account for the discrepancy.
fructose 6-phosphate. Inhibition of MI 1-phosphate synthase by glucuronic is relatively weak when compared to the allosteric inhibition of UDP-glucose dehydrogenase by UDP-xylose (21). In Figure 1, kinetic data for sodium glucuronic inhibition are plotted according to Lineweaver and Burk and (insert) according to Dixon (11). Inhibition is competitive with a $K_i = 2.8 \text{ mM}$, calculated from the Dixon plot, compared to a $K_m$ for glucose 6-phosphate $= 0.18 \text{ mM}$.

Fructose 6-phosphate was found to be an effective inhibitor of Neurospora crassa MI 1-phosphate synthase by Mogyoros et al. (20). Mannose 6-phosphate also inhibited but at a much higher concentration. Both compounds inhibited A. pseudoplatanus synthase as determined by the radioisotope assay procedure. A degree of caution is needed, however, in accepting this observation without further study, since both glucose 6-phosphate isomerase and mannose 6-phosphate isomerase activities were detected in preparations of enzyme used in the present study. Unlabeled glucose 6-phosphate, formed by the action of these isomerases, would dilute the labeled substrate used in the assay and reduce the number of counts recovered as product, an effect indistinguishable from true inhibition by this assay.

It is interesting to note that among the compounds tested for inhibition, those with a substitution at carbon 1 showed little or no inhibition. Galacturonic acid, the carbon 4 epimer of glucuronic acid, also failed to inhibit. In a similar vein, Mogyoros et al. (20) found galactose 6-phosphate in contrast to fructose 6-phosphate, a very weak inhibitor. D-2-Deoxyglucose 6-phosphate, a substrate analog and a powerful competitive inhibitor of rat liver synthase (3), also inhibited the plant enzyme. Another analog, the phosphorylated derivative of the adduct of glucose and 3-aminoetriazole, acts as a non-competitive inhibitor (14).

Compared to the extensive feedback control which is found in the sugar nucleotide oxidation pathway, relatively little control appears in the MI oxidation pathway. Thus, the latter provides an alternative for biosynthesis of cell wall pectic substance, particularly under conditions where pentosyl and uronosyl nucleotides would exercise feedback control of the former pathway. The MI oxidation pathway also provides a biosynthetic route to pectin that no longer competes directly for UDP-glucose and glucose 1-phosphate, the hexosyl precursors of reserve and structural polysaccharides other than pectin. This may be one explanation for the evolution of this pathway and its predominance in cell wall pectin biosynthesis (4) as demonstrated in lily pollen (17), in spite of the relatively low activities of the first three enzymes.

Only control by metabolites of the two pathways involved in UDP-glucuronic acid biosynthesis have been considered here. Other kinds of control such as the recent report of in vivo regulation of MI biosynthesis in N. crassa by the NAD$^+$/NADH ratio (23) are beyond the scope of this discussion.

Acknowledgments—Data pertaining to the activity of MI 1-phosphate synthase in Lilium longiflorum pollen were determined by Dr. Claire-Lise Bourenfield. Pollen used in this study was gathered by graduate students through the courtesy of the Curtis Lehde Nurseries, Buffalo, New York. The excellent technical assistance of Miss Diane Bedgar is deeply appreciated.

LITERATURE CITED


