S-Adenosyl-l-methionine: Loganic Acid Methyltransferase

A CARBOXYL-ALKYLATING ENZYME FROM VINCA ROSEA*

(Received for publication, September 29, 1972)

K. MADHAVA MADYASTHA,† ROCCO GUARNACCIA,§ CLAIRE BAXTER, AND CARMINE J. COSCIA
From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

SUMMARY

A partially purified enzyme from vinca rosea was shown to catalyze the transfer of the methyl group of S-adenosyl-l-methionine to loganic acid forming loganin, an intermediate in the biosynthesis of indole alkaloids of this plant. Cell-free extracts, prepared in the presence of polyvinyl pyrrolidone, Na2S2O5, and dithiothreitol (DTT), were subjected to acetone precipitation, ammonium sulfate fractionation, and DEAE-cellulose column chromatography. In assays with such preparations, DTT enhanced methyltransferase activity in contrast to mercaptoethanol, reduced glutathione, or cysteine. A combination of NADPH and FAD could partially replace DTT with the crude cell-free extract. Additional evidence for an active role of reduced sulfhydryl groups of the enzyme was obtained by inhibition studies with p-chloromercuribenzoate, iodoacetamide, and N-methylmaleimide. A divalent metal cation dependency was not detected. The apparent $K_m$ for loganic acid was 12.5 $\mu M$. Substrate specificity studies revealed that the rates of methylation of loganic and secologanic acids were comparable. Under the same assay conditions no significant methylation of 7-deoxyloganic, 7-epiloganic, or geniposidic acids was observed. Since the occurrence and biosynthesis of loganic and secologanic acids have also been demonstrated in V. rosea, both acids are thus implicated in the biosynthesis of the indole alkaloids.

The biosynthesis of the complex indole alkaloids from tryptophan and mevalonic acid involves a number of intricate rearrangements, and, although some mechanistic aspects of the pathway have been elucidated by in vivo tracer experiments (3), the method has limitations. One problem is the occasional incorporation of labeled compounds which are not authentic intermediates. In such circumstances clarification may depend upon examination of the enzymes involved in individual steps (3).

The iridoid glucoside, loganic acid, has been implicated in the biosynthesis of indole alkaloids in the higher plant, Vinca rosea, by demonstration of its occurrence and in vivo conversion to its methyl ester loganin (Fig. 1) (1). The latter was the first such monoterpenoid glucoside precursor of indole alkaloids detected in this plant (2). Batterby and co-workers (4), however, have reported that 7-deoxyloganin also fulfills the criteria of an indole alkaloid intermediate in V. rosea. These results prompted a more careful inspection of the stages at which methylation can occur in this pathway.

We now report on the partial purification of an enzyme which transfers the methyl group of S-adenosyl-l-methionine to the carboxyl group of loganic acid. Preliminary communications of parts of these results have appeared (5, 6).

EXPERIMENTAL PROCEDURE

Materials

Chemicals used were of the highest quality commercially available. All solvents were redistilled. Unlabeled S-adenosyl-L-methionine and dithiothreitol were purchased from Sigma, S-adenosyl-L-[methyl-14C]methionine was from Amersham-Searle. Dowex 1 X8 (200 to 400 mesh, formate form), Bio-Gel P-10, and Polyclar AT were purchased from Bio-Rad Labs. Silica Gel GF-254 (E. Merck, Darmstadt) was used routinely for analytical and preparative thin layer chromatography. The iridoid glucosides quench ultraviolet light-induced fluorescence of fluor in this silica gel.

The iridoid glucosides quench ultraviolet light-induced fluorescence of fluor in this silica gel.

* Part VI in a series on "Monoterpene Biosynthesis." For Part V, see Reference 1. This study was supported by National Science Foundation Grant GB 17957 and a Career Development Award of the National Institutes of Health (to C. J. C.).
† Present address, Department of Biochemistry, Indian Institute of Science, Bangalore 12, India.
Substrates and Products—Loganic acid was isolated from seeds of *Strychnos nux vornica* by previously reported techniques (7). Loganic acid was prepared by methylation of loganic acid with diazomethane.

Secologanin and secologanin were isolated from V. rosea as previously described (1). Geniposidic acid is a new iridoid glucoside which we have extracted from *Genipa americana* plants and characterized as its methyl ester geniposide (7a).

7-Deoxyloganin acid and 7-deoxyloganin were prepared by barium hydroxide saponification of their corresponding tetraacetates. 7-Deoxyloganin tetraacetate was obtained by catalytic hydrogenation of three different acetylated iridoid glucosides, geniposide pentacetate, asperuloside tetraacetate, and gardenoside pentaacetate (8, 9).

In a typical experiment, 300 mg of the acetylated glucoside dissolved in 60 ml of methanol was subjected to 45 p.s.i. of hydrogen gas in the presence of 50 mg of PtO₂ at 25°. The mixture was shaken until hydrogen uptake ceased. After filtration and solvent removal, the 7-deoxyloganin tetraacetate (or 7-deoxyloganin acid tetraacetate where asperuloside tetraacetate was the starting material) was purified by preparative thin layer chromatography on silica gel, developing with benzene-hexane-methanol (4:5:4:5:1, v/v/v) to detect 7-deoxyloganin. The reaction mixture was brought to pH 5 to 6 with 1 N H₂SO₄, and the BaSO₄ precipitate was removed by centrifugation. Free glucosides were purified by preparative thin layer chromatography in the above solvent systems. In the case of the acid, anion exchange chromatography on Dowex 1-formate form was employed (7). 7-Deoxyloganin was purified by recrystallization (m.p. 182–185°).

7-Epiloganin pentaacetate (m.p. 144–146° [α]₂₅° ~ 119.8°) was prepared according to the method of Batterby et al. (10) and hydrolyzed with barium hydroxide as described above to afford 7-euploganin and 7-epiloganin acid.

Satisfactory elemental analyses were obtained for the polyaacetate methyl esters of all iridoid glucosides utilized.

Plant Material—V. rosea seeds (variety 3187, Atlee-Burpee Seed Co.), were germinated in the dark at 30-32° on filter paper placed over a 4-cm layer of wet vermiculite. Etiolated seedlings were collected 4 to 14 days after onset of germination with optimal transmethylase activity occurring in 6- to 8-day-old seedlings. Initial studies with tender leaves and shoots from 4- to 8-month-old plants (5) afforded preparations with about one-fourth the transmethylase specific activity of seedlings. The preparations described in this paper were from whole seedlings unless otherwise indicated.

Methods

Preparation of Subcellular Fractions—Plant material was ground to a fine powder in liquid nitrogen and extracted with 2 to 3 volumes of 0.2 M phosphate buffer (pH 7.2), containing 0.01 M sodium metabisulfite and 1 mM DTT.¹ To this slurry, Polyclat AT (tissue:Polyclat AT (4:1, w/w)) was added, and the extract was squeezed through two layers of silk cloth. After centrifugation of the homogenate at 10,000 × g for 20 min, the supernate was fractionated on a column of Bio-Gel P-10 (approximately 50 ml of gel per 15 ml of plant extract) equilibrated with 0.1 M phosphate buffer (pH 7.2) containing 1 mM DTT. With this buffer as eluant, the first protein-containing filtrate (1 void volume) was collected. To this, 3 volumes of acetone were added at -15°. After decanting the acetone, the precipitate was freed of residual acetone by evaporation in vacuo and dissolved in 0.1 M phosphate buffer (pH 7.2) containing 1 mM DTT. Denatured protein was removed by centrifugation, and the clear supernatant was used for enzymatic assay (Table I). This preparation retained activity for only a few days.

The supernatant was further fractionated by addition of solid ammonium sulfate. The 30 to 65% ammonium sulfate precipitate was taken up in 0.1 M phosphate buffer containing 1 mM DTT and desalted by passage through a Bio-Gel P-10 column equilibrated with 5 mM phosphate buffer (pH 7.2) containing 1 mM DTT. This eluant was applied to a DEAE-cellulose column equilibrated with 5 mM phosphate buffer (pH 7.3) containing 1 mM DTT. The protein was eluted from the column discontinuously with increasing concentrations (5 mM to 1 M) of phosphate buffer containing 1 mM DTT (see Fig. 2). Such preparations were highly labile retaining activity for only a few days. Protein concentrations were measured by the method of Lowry et al. (11), with bovine serum albumin as standard.

Assay of Loganic Acid Methyltransferase—Unless otherwise specified, assay mixtures contained in a final volume of 0.5 ml: phosphate buffer (pH 7.2), 30 μmoles; loganic acid, 3.0 μmoles; S-adenosyl-L-[methyl-14C]methionine, 200 nmoles (140,000 dpm); DTT, 4 μmoles; and enzyme. The mixture was incubated at 32–33° for 3 hours.

At the end of the incubation period, 1 ml of methanol containing 0.5 μg of loganin was added to the mixture. Upon centrifugation, the supernatant was subjected to preparative thin layer chromatography on silica gel eluting with chloroform-methanol (7:3, v/v) or chloroform-methanol-water (65:25:4, v/v/v). The loganin band was removed from the plate, extracted with methanol, and filtered, and the filtrate was evaporated to dryness. The residue was taken up in water, and the mixture was brought to a pH of 8 to 9 and applied to a column of Dowex 1-formate form. Upon elution with 3 ml of water, a neutral fraction was obtained and subjected to descending paper chromatography (Whatman No. 1 developed with 1-butanol-acetic acid-water (15:25:4, v/v/v)). The radioactive loganin spot was located using a Packard Radiogrammatograph Scanner (model 7201), cut out, and counted in a Packard liquid scintillation counter (model 3380) (65% efficiency). Elimination of any of the above steps afforded relatively high control values (≥10%).

In a few initial experiments, the identity of the reaction product as [14C]loganin was further established by adding 10 μg of unlabeled loganin to the incubation mixture, subjecting it to preparative thin layer chromatography, and crystallizing to constant specific activity. For example, in one experiment, the specific activity of loganin in the third, fourth, and fifth recrystallization was 590, 567, and 570 dpm per mg, respectively (2.22 × 10³ dpm per mmole). The recrystallized [14C]loganin was acetylated and recrystallized to constant activity as loganin pentaacetate (356, 354, and 339 dpm per mg in the second, third, third,
Table I
Purification of loganic acid methyltransferase

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein mg</th>
<th>Specific activity dpm loganin formed/mg protein/hr</th>
<th>Apparent recovery of activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>240</td>
<td>5,457</td>
<td>100</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>110</td>
<td>13,230</td>
<td>112</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation, 30-65%</td>
<td>37.5</td>
<td>14,175</td>
<td>40.7</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>4.2</td>
<td>28,490</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Fig. 2. Chromatographic separation of methyltransferase on DEAE-cellulose.

Table II
Inhibition of loganic acid transmethylase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Relative activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>N-Methylmaleimide</td>
<td>1 X 10^-3</td>
<td>32.6</td>
</tr>
<tr>
<td></td>
<td>4 X 10^-4</td>
<td>61.6</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1 X 10^-3</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>4 X 10^-4</td>
<td>32</td>
</tr>
<tr>
<td>p-Chloromercurbenzoate</td>
<td>4 X 10^-4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>4 X 10^-6</td>
<td>0</td>
</tr>
<tr>
<td>KCN</td>
<td>1 X 10^-3</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>1 X 10^-4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1 X 10^-6</td>
<td>32</td>
</tr>
</tbody>
</table>

"In this assay enzyme and inhibitor were preincubated for 20 min, and DTT was omitted from the incubation mixture.

"Where reversal of inhibition was attempted, 4.0 μmoles of DTT was preincubated with the enzyme at 32-33°C for 4 min, and then inhibitor and substrates were added.

RESULTS

Cell-free preparations of V. maxima seedlings are capable of methylating loganic acid in the presence of S-adenosyl-L-methionine and a reducing agent. As seen in Fig. 3, this transmethylase activity is linear with respect to protein concentration. In these studies, optimal conversion observed was 40 nmoles of loganin per hour per mg of protein. The presence of strong reducing agents was required during initial extraction of seedlings and in all subsequent procedures. In assaying of ammonium sulfate precipitated protein, optimal activity was exhibited at the relatively high concentration of 8 mM dithiothreitol (Fig. 4).

The Sulfhydryl Group Requirement—Further purification on DEAE-cellulose afforded a highly labile preparation which was used to demonstrate sulfhydryl group involvement in transmethylase activity. As seen in Table II, both reversible as well as irreversible sulfhydryl group inhibitors reduce methyltransferase activity. Complete inactivation of enzyme was achieved in an experiment using ammonium sulfate-precipitated enzyme. As indicated (Table II), omission of DTT from the incubation mixture and longer preincubation of inhibitor with enzyme resulted in loss of all activity. Observation of incomplete inactivation in other experiments suggests that DTT added to the incubation mixture may regenerate free sulfhydryl groups from disulfide bridges and thereby reactivate some enzyme. As disulfides, it is unlikely that they would have reacted with inhibitor during the preincubation.

The mode of inhibition by CN^- ion is speculative. It is known that CN^- ion can open disulfide bridges by forming thiocyanates, a reaction which does not occur with sulfhydryl groups.
FIG. 5. A, rate of loganin formation versus loganic acid concentration. The standard assay mixture contained S-adenosyl-L-methionine (SAM) (130,000 dpm); loganic acid as indicated, and enzyme (ammonium sulfate precipitated). B, determination of the $K_m$ value of methyltransferase for loganic acid. The standard assay mixture was used containing 200 nmoles of S-adenosyl-L-methionine.

If this were the mechanism operative in the present case, the disulfide bridge opened by CN$^-$ ion is not identical with that reduced by DTT. This conclusion is based on the results of the last experiment in Table II revealing that preincubation with DTT which should reduce accessible disulfide bridges did not prevent CN$^-$ inhibition.

Effect of Divalent Cations—A number of transmethylases require divalent metal ions (12, 13), but with our preparation enzyme activity was neither enhanced by 2 mM Mg$^{2+}$ or 0.5 mM Mn$^{2+}$ nor inhibited by 1 mM EDTA.

Kinetic Studies—Substrate saturation was observed for loganic acid (Fig. 5A), and a linear Lineweaver-Burk plot (Fig. 5B) was obtained. From the latter, an apparent $K_m$ value of 12.5 mM for loganic acid was calculated. The possibility of inhibition by excess substrate, i.e. homotropic regulation, is suggested by Fig. 5 and will be investigated when the enzyme is further purified and stabilized.

Substrate Specificity Studies—In these experiments, standard incubation mixtures were used with 3 pmoles of the indicated acid (Table III). In most assays ammonium sulfate precipitated protein was utilized. Instead of the chromatographic assay, 10 mg of carrier ester was added, and the compound was purified by preparative thin layer chromatography. All glucosides except 7-epiloganin were acetylated, and the resultant polyacetate was recrystallized to constant radioactivity. Where no transmethylase activity was observed, a single recrystallization usually afforded a radioactive-free acetylated derivative. Methyl palmitate was separated by preparative thin layer chromatography and counted directly. 7-Epiloganin was assayed chromatographically in the same manner as loganin.

### Table III

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>RELATIVE RATE OF CONVERSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loganic Acid</td>
<td>100</td>
</tr>
<tr>
<td>Secologanic Acid</td>
<td>90</td>
</tr>
<tr>
<td>7 - Deoxyloganic Acid</td>
<td>0</td>
</tr>
<tr>
<td>Geniposidic Acid</td>
<td>0</td>
</tr>
<tr>
<td>7 - Epiloganic Acid</td>
<td>0</td>
</tr>
<tr>
<td>CH$_3$(CH$<em>2$)$</em>{14}$ - COOH</td>
<td>0</td>
</tr>
</tbody>
</table>

DISCUSSION

The S-adenosyl-L-methionine-dependent transmethylase isolated from V. rosea catalyzes a relatively uncommon type of O-methylation in converting loganic acid to its methyl ester. Several other carboxyl group methyltransferases have been reported and they may be classified into two groups depending on whether their substrates are macromolecular or of low molecular weight.

In higher plants, pectin methyl esters are synthesized by a particulate enzyme preparation at the macromolecular level (although some contradictory evidence exists) (13-15). Free carboxyl groups of proteins are reported to be methylated in certain mammalian tissues, but the inherent difficulty of product identification has made these results somewhat tenuous (16).

Carboxyl methylation of magnesium protoporphyrin IX appears to be a key step in chlorophyll synthesis in plants (17, 18). Recently, fatty acids were proven to be enzymically methylated in Mycobacterium phlei (19). A variety of other naturally occurring methyl esters which include those of terpenoid origin, e.g. juvenile hormones (20) and methyl jasmonate (21), as well as...
of aromatic derivation (22) may be synthesized in the same manner.

Loganic acid transmethylase is a typical O-methyltransferase in its requirement for reducing agents and its sensitivity to sulfhydryl reagents. These characteristics may arise from readily accessible sulfhydryl groups on the enzyme which are subjected to facile oxidation by enzymes released upon homogenization of the plant tissue. Cell-free extracts prepared from leaves with sodium metabisulfate and DTT were nearly four times more active than extracts prepared in the presence of metabisulfite alone. The relatively strong reducing agent, DTT (reduction-oxidation potential, −0.33 volt), could be partially replaced by equimolar amounts of monothiols such as mercaptoethanol or reduced cysteine (reduction-oxidation potential, −0.22 volt) in the extraction.

The need for reducing equivalents in the enzyme assay, however, could not be satisfied by monothiols (5). The presence of mercaptoethanol, reduced glutathione, or cysteine did not give values above those observed in the absence of reducing agents. This is not entirely understandable. Clearly some increase in activity would be expected even though comparable activities would necessitate concentrations of monothiol far greater than that of DTT.

Interestingly, either reduced lipoic acid or a combination of FAD and NADPH (5 mM each) partially substituted for DTT with the crude cell-free extract (6). Subsequent purification by acetone treatment and ammonium sulfate precipitation provides slight purification and loss of the FAD NADPH enzyme reactivation ability. Hastings has isolated a NADH dehydrogenase from photobacteria, which reduces FMN which in turn is believed to reduce a disulfide bridge on luciferase of those organisms (23).

Thus far, examination of more purified preparations has been hampered by increased lability of the enzyme as the purification proceeds. It appears, however, that less reducing equivalents of DTT are necessary in assays with more purified preparations such as the DEAE-cellulose eluate, suggesting the removal of oxidizing enzymes.

Substrate specificity studies implicate loganic and secologanic acids in this enzymatic reaction while excluding 7-deoxyloganic and 7-epiloganic acids. This suggests the enzyme may have a critical binding site for a hydroxyl at C-7 to which secologanic acid can also attach since its hydrated aldehyde group can rotate freely. The orientation of the glucosyl moiety is completely different for each epimer. Thus either the glucosyl residue also has a linking site or 7-epiloganic acid cannot fit into the active site due to interaction of its glucosyl group with amino acid residues. Inouye et al. have found in Daphniphyllum macropodum that, although 7-epiloganin is utilized in the biosynthesis of asperuloside, it must first be epimerized to loganin with loss of the C-7 hydrogen (24).

These results demonstrate that loganic acid methyltransferase will not alkylate 7-deoxyloganic acid. Our attempts to detect 7-deoxyloganin or 7-deoxyloganic acid either by isolation in large scale extraction or by isotope dilution experiments, wherein loganic acid was labeled by [2-14C]mevalonate to the extent of 1%, have been unsuccessful. Furthermore, we have found loganic and secologanic acid in V. rosea at concentrations much higher than that of loganin. Loganic acid constitutes 1% of the seed weight, and as germination proceeds in the first week its concentration decreases, whereas that of loganin and indole alkaloids increases. Simultaneously, de novo synthesis of loganic acid is occurring (25), and transmethylase activity in the seedling becomes optimal. It is clear then that, during this active period of indole alkaloid synthesis (3), loganic acid turnover is proceeding in a comparable fashion.

Acknowledgments—The excellent technical assistance of E. Tegtmeyer is acknowledged. We thank Professor J. M. Robbitt for a generous gift of asperuloside.

REFERENCES


* E. Tegtmeyer and C. J. Coscia, unpublished observations.
S-Adenosyl-l-methionine:Loganic Acid Methyltransferase: A CARBOXYL-ALKYLATING ENZYME FROM VINCA ROSEA
K. Madhava Madyastha, Rocco Guarnaccia, Claire Baxter and Carmine J. Coscia


Access the most updated version of this article at http://www.jbc.org/content/248/7/2497

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/7/2497.full.html#ref-list-1