Intermediates in the Recycling of 5-Methylthioribose to Methionine in Fruits

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ABSTRACT

The recycling of 5-methylthioribose (MTR) to methionine in avocado (Persea americana Mill, cv Hass) and tomato (Lycopersicon esculentum Mill, cv unknown) was examined. [14CH3]MTR was not metabolized in cell free extract from avocado fruit. Either [14CH3]MTR plus ATP or [14CH3]S-methylthioribose-1-phosphate (MTR-1-P) alone, however, were metabolized to new products by these extracts. MTR kinase activity has previously been detected in these fruit extracts. These data indicate that MTR must be converted to MTR-1-P by MTR kinase before further metabolism can occur. The products of MTR-1-P metabolism were tentatively identified as α-keto-γ-methylthiobutyric acid (α-KMB) and α-hydroxy-γ-methylthiobutyric acid (α-HMB) by chromatography in several solvent systems. [35S]α-KMB was found to be further metabolized to methionine and α-HMB by these extracts, whereas α-HMB was not. However, α-HMB inhibited the conversion of α-KMB to methionine. Both [1-14C]α-KMB and [U-14C]methionine, but not [U-14C]α-HMB, were converted to ethylene in tomato pericarp tissue. In addition, aminoethoxyvinylglycine inhibited the conversion of α-KMB to methionine. These data suggest that the recycling pathway leading to ethylene is MTR → MTR-1-P → α-KMB → methionine → S-adenosylmethionine → 1-aminocyclopropane-1-carboxylic acid → ethylene.

Methionine serves as a precursor of ethylene (15), SAM² and ACC being intermediates (6, 22). ACC synthase catalyzes the conversion of SAM to ACC and MTA (2). This reaction has been reported to be the rate-limiting step of ethylene biosynthesis in both vegetative and fruit tissues (6, 7, 12, 13, 22, 24).

In plants, MTA is degraded by a nucleosidic cleavage via MTA nucleosidase to MTR and adenine (11). Various moieties of MTA have been shown to be recycled into methionine in higher plant tissue. Adams and Yang (1) found that the methythio group of MTA was effectively recycled as a unit into methionine. Yung et al. (25) subsequently showed that, in apple tissue, the ribose portion of MTR was also incorporated into methionine. Recently, Wang et al. (20) demonstrated in tomato tissue that the ribose moiety of MTA was metabolized to form the four-carbon unit (2-aminobutrate) of methionine and suggested that ethylene was formed from the ribose portion of MTA via MTR, methionine, and ACC.

Kushad et al. (14) found that cell-free extracts from several fruit tissues contain MTR kinase activity. This enzyme catalyzes the ATP-dependent phosphorylation of MTR to MTR-1-P (9). The presence of this enzyme suggests that MTR-1-P also may be an essential intermediate in the recycling of MTA to methionine. The present study was undertaken to examine the fate of MTR-1-P.

MATERIALS AND METHODS

Plant Material. Unripe mature avocado fruits (Persea americana Mill, cv Hass) and tomato (Lycopersicon esculentum Mill, cv unknown) in the breaker stage were purchased from a local store.

Chemicals. [35S]Methionine (1,000 Ci/mmol), [U-14C]methionine (285 mCi/mmol), and [14CH3]SAM (58 mCi/mmol) were purchased from Amersham. [14CH3]MTR was prepared from [14CH3]SAM (16), [14CH3]MTR was obtained by acid hydrolysis of the corresponding [14CH3]MTA (16), and [14CH3]MTR-1-P was prepared by incubating [14CH3]MTA with partially purified calf liver MTA phosphotransferase as described by Ferro et al. (10). [35S]α-KMB and [U-14C]α-KMB were obtained according to a procedure described by Dixon (8) with α-KMB extracted from the reaction with diethyl ether. [U-14C]α-HMB was prepared from [U-14C]α-KMB using NaBH₄. [U-14C]α-KMB (2 µCi) was adjusted to pH 3 with 1 N acetic acid and was added to 2 mg NaBH₄ in 95% ethanol in a ratio of 1:1 (v/v). The mixture was incubated 10 min at room temperature and an aliquot was chromatographed in solvent system I to separate the product from the substrate. α-KMB and α-HMB were purchased from Sigma Chemical Co. AVG was a gift from Hoffman LaRoche.

Cell-Free Extract and Enzyme Assay. Fruit extracts were prepared by homogenization of the tissue in 0.2 M K-phosphate (pH 7.2), 3 mM DTT, 1% Triton-X-100 (v/v), and 3% PVP (w/v). The homogenate was passed through four layers of cheese cloth, and centrifuged for 20 min at 20,000g. The supernatant fluid was used as the cell free extract. MTR kinase activity was determined according to the procedure of Ferro et al. (9).

Chromatographic Procedures. Radioactive metabolites were separated and identified by four chromatographic systems. Descending paper chromatography was carried out in butanol:acetic acid:acetone:water (70:20:70:40, v/v) (solvent system I). TLC was performed on silica gels, F60 (EM Laboratories), with isobutanol:acetic acid:water (68:10:2, v/v) (solvent system II), ethyl acetate:ethanol:water (9:1.2, v/v) (solvent system III), and isobutyl alcohol:ethanol:water (68:20:10, v/v) (solvent system IV). All chromatograms were scanned with a Packard radiocromatogram scanner. For quantification of radioactivity, paper chromatograms were cut into 1-cm strips which were counted in a

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2 Abbreviations: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, 5'-methylthioribose-1-phosphate; α-KMB, α-keto-γ-methylthiobutyric acid; α-HMB, α-hydroxy-γ-methylthiobutyric acid; AVG, aminoethoxyvinylglycine (2-amino-4-[2'-aminoethoxy]trans-3-butenolic acid).
Beckman liquid scintillation counter.

Feeding Experiments. Plugs from breaker ripening stage of tomato pericarp tissue (1 cm in diameter) were excised with a cork borer and immediately rinsed in 2% KCl and blotted dry. Radioactive substrates were introduced into the plugs by the vacuum injection technique previously described (1). Two replicates of three plugs each were used for all experiments. The plugs were sealed in a 25-ml Erlenmeyer flask with a plastic bucket hanging through a rubber serum cap. Radioactive ethylene was adsorbed in 0.2 ml of 0.25 M Hg(ClO4)2 injected into the bucket. After 6 h, the buckets were placed in scintillation vials containing scintillation fluid and the radioactivity determined. Nonradioactive ethylene was measured by GC.

RESULTS

MTR Kinase Activity in Avocado Extracts. MTR kinase activity was determined from three morphological regions of the avocado fruit: the peel, outer exocarp, and inner exocarp. The corresponding enzyme activities are presented in Table I. The specific activity of the peel was lowest, being one-third that of the outer exocarp and one-half that of the inner exocarp. In subsequent experiments, therefore, the fruit was peeled and all

Table I. MTR Kinase Activity in Avocado Extract

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme Activity (pmol product formed/mg protein-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peel</td>
<td>508</td>
</tr>
<tr>
<td>Outer exocarp</td>
<td>1,805</td>
</tr>
<tr>
<td>Inner exocarp</td>
<td>1,080</td>
</tr>
</tbody>
</table>

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Metabolism of MTR in Avocado Extract. Inasmuch as MTR has been shown to be metabolized when fed to tomato pericarp tissue slices (20), the ability of avocado cell-free extracts to utilize MTR as a substrate was explored. Radiochromatogram scans revealed that [14CH3]MTR incubated with or without avocado extract for 8 h remained unaltered (Fig. 1A). The addition of 5 mM ATP to the reaction mixture, however, resulted in the formation of a new radioactive peak (Rf = 0.85, solvent system I) and a smaller peak at Rf = 0.70, which presumably was unreacted MTR (Fig. 1B). Chromatography of this same assay mixture in solvent system II (Fig. 2), however, demonstrated that the substrate [14CH3]MTR (Rf = 0.55) was completely degraded and that two new peaks at Rf 0.05 and 0.30 were formed. Chromatography in two other solvent systems (Table II) confirmed that two products were formed and that these products co-chromatographed with α-KMB and α-HMB. The inability to detect both products in solvent system I was due to the overlapping migration of MTR and α-KMB in this system. When α-
FIG. 3. Radiochromatogram scans of cell-free extracts of avocado fruit containing (A) no extract plus [14CH3]MTR-1-P and (B) extract plus [14CH3]MTR-1-P.

FIG. 4. Kinetics of product formation from MTR-1-P. (O), MTR-1-P; (☐), α-KMB; (●), α-HMB.

HMB and α-KMB were eluted and rechromatographed in the other three systems, they co-migrated with authentic samples of each of these compounds.

Metabolism of MTR-1-P in Avocado Extract. Because MTR was metabolized only when ATP was present and since MTR kinase activity has been found in these extracts, the ability of avocado extract to metabolize MTR-1-P was examined (Fig. 3). Incubation of [14CH3] MTR-1-P with avocado extract (in the absence of ATP) for 30 h yielded two radioactive products, α-KMB and α-HMB, as measured by scanning chromatograms developed in solvent systems I and II. MTR was not formed under these conditions. The metabolism of MTR-1-P to α-KMB and α-HMB by this extract, therefore, does not require the presence of ATP, indicating that MTR must first be activated to MTR-1-P via MTR kinase before it can be further metabolized.

To examine the kinetics of product formation from MTR-1-P, the reaction was stopped 6, 18, and 30 h after addition of the substrate and analyzed for MTR-1-P, α-KMB, and α-HMB as described in “Materials and Methods” (Fig. 4). MTR-1-P was metabolized to both α-KMB and α-HMB at approximately equal rates during the first 6 h. Thereafter, however, the rate of α-HMB accumulation exceeded that of α-KMB accumulation. By 30 h, only 11% of the MTR-1-P remained and of the two radioactive products formed, 70% was in the form of α-HMB. That the increase in α-HMB plus α-KMB is greater than the decrease in MTR-1-P between the 18 to 30 h of incubation may indicate the presence of an intermediate compound synthesized from MTR-1-P during the first 18 h and converted to α-KMB and α-HMB during the later stages of the incubation period.

Metabolism of α-KMB and α-HMB by Avocado Extract. Avocado extracts were incubated for 3 h with 1 mm asparagine and either [35S]α-KMB or [35S]α-HMB and analyzed for [35S] methionine, [35S]α-KMB, and [35S]α-HMB (Table III). More than 95% of the [35S] α-KMB was metabolized to methionine (63%) and α-HMB (32%), whereas [35S]α-HMB incubated with extract and asparagine remained unaltered. The addition of 2.5 mM α-HMB to the [35S]α-KMB reaction mixture resulted in a 26% decrease in the conversion of α-KMB to methionine and a concomitant increase in the level of [35S]α-KMB. The data
suggest that α-KMB is a precursor of both methionine and α-HMB, but that α-HMB cannot be further metabolized under these conditions. In addition, α-HMB appears to slightly inhibit the conversion of α-KMB to methionine.

**Ethylene Fixation from α-KMB, α-HMB, and Methionine.**

The ability of tomato pericarp tissue to metabolize [U-14C]α-KMB, [U-14C]methionine, and [U-14C]α-HMB to ethylene was examined (Table IV). Of the three compounds tested, only α-KMB and methionine served as substrates for the synthesis of ethylene; α-HMB was completely inactive. α-HMB at 10 mM did, however, inhibit the conversion of α-KMB to ethylene by 55%. These data are consistent with the data obtained with cell-free extracts which indicate that α-KMB, but not α-HMB, is a precursor of methionine and that α-HMB inhibits the conversion of α-KMB to methionine which is a precursor of ethylene.

**Effect of AVG on the Conversion of α-KMB to Ethylene.** AVG is an inhibitor of the enzymatic step leading from SAM to ACC and MTA. It has recently been shown that AVG inhibits the conversion of MTR to ethylene (20). If α-KMB is an intermediate between MTR and ACC, then AVG should also inhibit the conversion of α-KMB to ethylene. Therefore, tomato pericarp discs were infiltrated with α-KMB in the presence and absence of AVG, and the ethylene formed was measured (Table V). α-KMB at 1.0 and 2.0 mM increased ethylene production in a dose-dependent manner. The addition of 0.1 mM AVG almost completely inhibited ethylene production in these tissues in the presence of α-KMB, suggesting that α-KMB is metabolized to ethylene via SAM and ACC.

**DISCUSSION**

Wang et al. (20) have recently shown that ethylene is produced in tomato pericarp tissue from the ribose portion of MTA via MTR, methionine, and ACC. Collectively, the data presented here show that MTR-1-P and α-KMB are also intermediates in this recycling pathway. Based on these studies, this methionine salvage system and its relationship to ethylene biosynthesis is summarized in Figure 5.

The significance of MTR kinase activity in this recycling is illustrated by the inability of MTR to be further metabolized by cell extracts unless ATP is supplemented or until it is first activated to MTR-1-P. In mammalian tissue, MTA is directly converted to MTR-1-P by MTA phosphorolysis (21) and, therefore, the action of MTR kinase is not required. MTR kinase activity has also been found in Enterobacter aerogenes (9), an organism shown to recycle MTA to methionine via a similar pathway (18). Conversely, we have not detected MTR kinase activity in extracts from Escherichia coli. Interestingly, E. coli has been shown to export MTR directly into the medium where it accumulates (17). Thus, it would appear that the inability to form MTR-1-P via either MTA phosphorylase or the sequential action of MTA nucleosidase and MTR kinase renders the cell unable to further metabolize MTR.

α-KMB has been found to be an intermediate in the recycling pathway in animal (3, 4, 19) and bacterial (18) cells. It also has been shown to be converted to methionine in apple tissue (5). α-HMB has also been shown to be produced during the recycling of MTA in mammalian tissue (19). Our data, however, suggest that α-HMB is not a direct intermediate in this salvage pathway, but rather is synthesized from α-KMB in a side reaction not leading to ethylene synthesis. Although α-HMB does not appear to be an intermediate in the synthesis of ethylene from MTR-1-P, it does decrease ethylene synthesis, presumably by inhibiting the conversion of α-KMB to methionine.

According to the proposed recycling scheme, there is no new net synthesis of methionine. Rather, ethylene appears to be synthesized, after the first turn of the cycle, from the continual input of ATP. The balanced reaction of this scheme is:

\[ 2\text{ATP} \rightarrow \text{ADP} + \text{adenine} + 4\text{Pi} + 2\text{CO}_2 + \text{C}_2\text{H}_4 \]

Since ethylene has been shown to be synthesized from the ribose moiety of MTR (20) (presumably carbons 4' and 5'), which originated from the ribose moiety of ATP via SAM and MTA, the continuous generation of ATP could result in the continuous production of ethylene without the de novo synthesis of methionine in those systems in which this recycling pathway is operative.

**LITERATURE CITED**

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**FIG. 5. Proposed recycling pathway from MTR to methionine.**


