Peroxisomal Metabolism of Propionic Acid and Isobutyric Acid in Plants*

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The subcellular sites of branched-chain amino acid metabolism in plants have been controversial, particularly with respect to valine catabolism. Potential enzymes for some steps in the valine catabolic pathway are clearly present in both mitochondria and peroxisomes, but the metabolic functions of these isoforms are not clear. The present study examined the possible function of these enzymes in metabolism of isobutyryl-CoA and propionyl-CoA, intermediates in the metabolism of valine and of odd-chain and branched-chain fatty acids. Using 13C NMR, accumulation of β-hydroxypropionate from [2-13C]propionate was observed in seedlings of Arabidopsis thaliana and a range of other plants, including both monocots and dicots. Examination of coding sequences and subcellular targeting elements indicated that the completed genome of A. thaliana likely codes for all the enzymes necessary to convert valine to propionyl-CoA in mitochondria. However, Arabidopsis mitochondria may lack some of the key enzymes for metabolism of propionyl-CoA. Known peroxisomal enzymes may convert propionyl-CoA to β-hydroxypropionate by a modified β-oxidation pathway. The chyl1–3 mutation, creating a defect in a peroxisomal hydroxyacyl-CoA hydrolase, abolished the accumulation of β-hydroxyisobutyrate from exogenous isobutyrate, but not the accumulation of β-hydroxypropionate from exogenous propionate. The chyl1–3 mutant also displayed a dramatically increased sensitivity to the toxic effects of excess propionate and isobutyrate but not of valine. 13C NMR analysis of Arabidopsis seedlings exposed to [U-13C]valine did not show an accumulation of β-hydroxypropionate. No evidence was observed for a modified β-oxidation of valine. 13C NMR analysis showed that valine was converted to leucine through the production of α-ketoisovalerate and isopropanoylmalate. These data suggest that peroxisomal enzymes for a modified β-oxidation of isobutyryl-CoA and propionyl-CoA could function for metabolism of substrates other than valine.

Propionate, in the form of propionyl-CoA, is produced from a number of metabolic precursors in higher eukaryotes. It is the final product of odd-chain fatty acid β-oxidation (1). It is also produced during the catabolism of several amino acids, including isoleucine, methionine, and valine (1, 2). Propionyl-CoA is also a final product of metabolism of the branched acid, pyrroline-5-carboxylate, derived from the degradation of chlorophyll (3). Aside from a basic understanding of metabolic biochemistry, the anabolic and catabolic pathways for propionyl-CoA are also of considerable importance in metabolic engineering of polyhydroxyalkanoates in plants, especially in the production of mixed polyhydroxyalkanoate polymers that have relied on the use of propionyl-CoA as a metabolic intermediate (4, 5). Several pathways have been confirmed for the catabolism of propionyl-CoA (6–8). Bacteria and yeast utilize a 2-methylcitrate pathway with reactions analogous to those of the tricarboxylic acid cycle and glyoxylate cycle (6). Mammals use a well-established biotin and B12-dependent pathway for conversion of propionyl-CoA to succinyl-CoA (7, 8).

Although plants have the same capacity described above to produce propionyl-CoA, their catabolism of propionyl-CoA is not clearly understood, and there are several conflicting reports regarding enzyme activities for different pathways. Examination of plant genomes does not reveal the presence of obvious orthologues corresponding to enzymes from either the bacterial or mammalian pathways. Plant genomes do code for a number of biotin-dependent carboxylases but none with a high level of homology to known propionyl-CoA carboxylases. Nevertheless, propionyl-CoA carboxylase activity has been demonstrated in plant extracts (9), raising some confusion about this enzyme. In the absence of a specific propionyl-CoA carboxylase, this reaction could be catalyzed by either an acetyl-CoA carboxylase or a methylcrotonyl-CoA carboxylase as a side reaction or as a secondary function of such an enzyme. Two separate isoforms of acetyl-CoA carboxylase are present in higher plants (10) as well as methylcrotonyl-CoA carboxylase (9). A problem would then arise as to the subsequent fate of methylmalonyl-CoA derived from carboxylation of propionyl-CoA in the absence of enzymes that could further metabolize methylmalonyl-CoA such as a racemase and a mutase, which appear to be absent in plants. Furthermore, whether B12 is involved in plant metabolism is also not clear. Although plants are generally considered to lack B12 (11–13), there are reports in the literature of B12 and other corrin compounds from plant sources (14, 15).

Several other possible pathways have been proposed for the metabolism of propionyl-CoA in various organisms. These include conjugation to glyoxylate to produce either hydroxyglutarate (16) or 3-methylmalate (17), and conversion to acrylyl-CoA and β-hydroxypropionyl-CoA by enzymes similar to those used in a modified β-oxidation pathway for valine catabolism (2). Several studies have reported the production of β-hydroxypropionate from either acrylate or propionate by a modified β-oxidation pathway in either whole plants or isolated...
peroxisomes (2, 12, 13). Although this provides significant evidence for such a pathway, it is still not clear whether this is the primary pathway or the only functional pathway for metabolism of propionyl-CoA in plants. Furthermore, it is not clear whether this modified β-oxidation pathway occurs exclusively in peroxisomes or if it is also in mitochondria. This is an important question as propionyl-CoA can be produced by multiple pathways that are expected to occur in both organelles.

With the completed genome sequence of Arabidopsis thaliana and genome databases of numerous other plant species, it is now possible to consider not only the pathways for catabolism but also the genes involved and the subcellular localization of the corresponding enzymes. Examination of genes coding for key enzymes of a modified β-oxidation pathway for propionyl-CoA suggests that the catabolic disposal of propionyl-CoA may be exclusively peroxisomal in plants and that β-hydroxypropionyl-CoA may be the final product of this metabolism. This concept is consistent with previous reports of β-hydroxypropionate production in plants (2, 13).

In the present study, we have confirmed the accumulation of β-hydroxypropionate in A. thaliana and other plant species using [2-13C]propionate by 13C NMR spectroscopy. The peroxisomal production of β-hydroxyisobutyrate from exogenous isobutyrate was also examined. Plant genomes code for both mitochondrial and peroxisomal forms of β-hydroxyacyl-CoA dehydrogenase, a key enzyme in this pathway, which also hydrolyzes branched-chain hydroxyacyl-CoA esters (18–21). It has been suggested that these peroxisomal hydrolyses may be functionally important for valine catabolism. The present study shows that β-hydroxypropionate is produced from exogenous propionate but not from propionyl-CoA derived from valine and that mutation of a peroxisomal hydroxyacyl-CoA dehydrogenase results in increased sensitivity to propionate but not to valine. This mutation also abolished the accumulation of β-hydroxyisobutyrate from exogenous isobutyrate. 13C NMR studies of A. thaliana seedlings treated with [U-13C]valine showed that the major pathway for valine metabolism was not through a modified β-oxidation pathway, but through the conversion to leucine.

These data support a pathway in peroxisomes for the β-oxidation of isobutryl-CoA and propionyl-CoA from metabolic sources other than valine. A better understanding of this pathway may be valuable for the manipulation of this metabolism in the engineered synthesis of polyhydroxyalkanoates containing these acids (4, 5).

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased from Sigma-Aldrich unless otherwise noted. 13C-Labeled compounds were purchased from Cambridge Isotopes. β-Hydroxypropionate was synthesized according to Herter et al. (22). β-Hydroxyisobutyrate was synthesized according to Rougraff et al. (23). Malonate semialdehyde was synthesized according to Menon et al. (24). chy1–3 seeds were generously provided by Dr. Bonnie Bartel, Rice University.

**Cell Culture and Seedling Growth Conditions for NMR Preparation**—Landsberg erecta green cell cultures were grown in 400-ml cultures with Gamborg basal salts (25) Research Products International Corp.) treated or untreated with 0.1 mM unlabeled propionate. After 6 days of growth, cultures were filtered and resuspended in 50 ml of medium with or without 1 mM [2-13C]propionate for the specified time. Cells were then centrifuged at 1200 × g for 30 min, treated with perichloric acid (Fisher Scientific, 5% final concentration) to stop metabolism and placed at –80 °C prior to NMR analysis. Wild-type A. thaliana ecotype Columbia (Col-0) and chy1–3 mutant seedlings were also used. Approximately 300–400 seedlings were surface sterilized for 30 s with 70% EtOH, then with 10% bleach for 30 min, then rinsed at least four times with sterile water. Seeds were dried on filter paper in a sterile hood then sprinkled on plates using one-half-strength Murashige-Skoog medium (MS) (26) and 1% (w/v) sucrose solidified with 0.8% (w/v) agar (Fisher Scientific, 5% final concentration) to stop metabolism and then transferred to growth chambers. Seedlings were grown under 16-h light/8-h dark photoperiods at 21 °C–23 °C. After 4 days, seedlings were removed from the plates and placed in 50 ml of liquid, one-half-strength MS media for 10–24 h with or without 2 mM [2-13C]propionate or [U-13C]valine. Seedlings were then rinsed with sterile water, ground to a fine powder in liquid nitrogen, resuspended in 5 ml of 5% perichloric acid, and stored at –80 °C prior to NMR analysis. Lactuca sativa (lettuce), Pisum sativum (pea), Triticum aestivum (wheat), and Zea mays (corn) seeds were all produced from Burpee®. Seeds were surface-sterilized with 0.1% bleach and then rinsed several times with sterile water. Seedlings were dark-grown on towelettes saturated with water for up to 6 days before being placed in MS liquid media for 24 h with [2-13C]propionate. Seedlings were prepared as above; however, before they were ground in liquid nitrogen, the seed coat was removed.

All frozen samples were thawed to room temperature and neutralized with 10 m KOH. Samples were centrifuged at 1200 × g for 15–30 min at 4 °C. The supernatant was lyophilized and resuspended in 1–2 ml of 100% D2O. Samples were syringe-filtered or centrifuged at maximum speed for 10 min before being placed in a 200-× 5-mm 535-PP NMR tube. 13C spectra were acquired at 125.77 MHz with a deuterium lock on a Bruker AVANCE™ at 500 MHz with a 5-mm TXI probe. Spectra were obtained with a 30° pulse angle, relaxation time of 2 s, and 1024 scans, and the spectra were Fourier-transformed using 1-Hz line broadening. Chemical shifts were calibrated to propionate C2 at 30.8 ppm (6) or to 2,2-dimethyl-2-silapentane-5-sulfonate. Heteronuclear multiple quantum correlation (HMQC) spectra were measured in the 1H-detected mode via multiple quantum coherence with proton decoupling in the 13C domain, using a program obtained from Bruker.

**End Point Assay for Quantitation of β-Hydroxyisobutyrate**—Wild-type Col-0 and chy1–3 seedlings were grown as above, however at day 4, seedlings were removed from the agar plates and placed in 10 ml of liquid Gamborg basal salt media containing neutralized 1 mM isobutryate. After 10 h, seedlings were thoroughly rinsed with distilled water and ground to a fine

2The abbreviations used are: MS, Murashige-Skoog medium; BCKDH, branched-chain α-keto acid dehydrogenase; HMQC, heteronuclear multiple quantum correlation; IBA, indole-3-butyric acid.
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FIGURE 1. Proposed pathways for metabolism of valine and propionyl-CoA in mitochondria and peroxisomes in plants. Enzymes 1–7 can be found in Table 1. A, 2-isopropylmalate synthase; B, 3-isopropylmalate dehydratase; C, 3-isopropylmalate dehydrogenase.

powder in liquid nitrogen; they were weighed and resuspended in 5 ml of 5% perchloric acid, then placed at −80 °C prior to NMR analysis. Frozen samples were thawed to room temperature, neutralized with 10 M KOH, and spun down at 1200 × g for 30 min. The supernatant was then lyophilized before being resuspended in 2 ml of sterile H2O. Any undissolved plant debris was removed by centrifuging the sample for 5 min at maximum speed. The assay conditions were based on Rougraff et al. (23) and are as follows: 0.67 M Tris(hydroxymethyl)aminomethane, 3.3 mM MgSO4, 1.7 mM EDTA, 0.13 M hydrazine sulfate, 1 mM NAD+, 50–100 μl of extract, and 9.6 μg/ml hydroxyisobutyrate dehydrogenase to a final volume of 1.0 ml. A no-enzyme and no-NAD+ negative control was used for background. Each reaction was initiated with 20 μl of enzyme after baseline was reached (~15 min). The production of NADH was measured at 340 nm on a Varian Cary UV-visible spectrophotometer with a circulating water jacket at 37 °C.

Valine, Isobutyrinate, and Propionate Growth Response—Wild-type Col-0 and chy1–3 seedlings were surface-sterilized as above and placed on one-half-strength MS plates containing 1% sucrose and 0.8% agar, with valine, isobutyrate, or propionate as indicated. Seedlings were placed at 4 °C for 2 days and then moved to growth chambers at 21–23 °C for 7 days. Seedlings were then removed from the agar and the primary roots were measured against untreated seedlings.

RESULTS

dee flowers. The absence of enzymes for other, more common pathways of propionyl-CoA metabolism has lead to the suggestion that plants metabolize propionyl-CoA by a modified β-oxidation pathway (2, 12, 13). This pathway would be highly analogous to the mitochondrial catabolism of isobutyryl-CoA derived from valine as shown in Fig. 1. Table 1 summarizes A. thaliana genes encoding possible enzymes in this pathway and the putative subcellular localizations of the corresponding gene products. These include all the enzymes necessary to convert valine to propionyl-CoA in mitochondria. However, it is not clear whether the same enzymes that convert isobutyryl-CoA to propionyl-CoA can also convert propionyl-CoA to acetyl-CoA in mitochondria as shown in Fig. 1. Of particular importance here is the apparent lack of a mitochondrial acyl-CoA dehydrogenase (Fig. 1, step 3) that may oxidize propionyl-CoA to acetyl-CoA (27). The only known mitochondrial acyl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase, appears to be highly specific for the branched-chain acyl-group derived from valine (28).

There are also enzymes with putative peroxisomal localization that could catalyze a specific subset of this pathway. These include numerous acyl-CoA oxidases (Fig. 1, step 3), enoyl-CoA hydratases (step 4), and hydroxyacyl-CoA hydrolases (step 5; see Table 1). However, the early enzymes of valine degradation (branched-chain amino transferase, step 1 and BCKDH, step 2) do not appear to have peroxisomal localization signals (Table 1). Neither do the enzymes that catalyze the final steps in this pathway, hydroxyisobutyrate dehydrogenase (step 6) and methylmalonate semialdehyde dehydrogenase (step 7). The predicted A. thaliana proteins for these two enzymes each contain sequences that could clearly serve for mitochondrial targeting and lack known peroxisomal targeting sequences (29). Therefore, analysis of the A. thaliana genome raises questions regarding the mitochondrial catabolism of propionyl-CoA and supports a truncated pathway for propionyl-CoA in peroxisomes that could serve primarily in the detoxification of acyl-CoA with β-hydroxypropionate as an end product. This would be consistent with the previous reports of β-hydroxypropionate production in isolated peroxisomes (2, 13) and the observed accumulation of [2,13C]hydroxypropionate described in the present study.

Metabolism of [2,13C]Propionate in A. thaliana Seedlings and Suspension Cell Cultures—Previous studies of propionate metabolism in higher plants utilized a number of different plant species but not the model plant A. thaliana. To determine if propionate is metabolized via a β-oxidation pathway in A. thaliana, we performed carbon NMR analyses of A. thaliana seedlings and suspension cell cultures exposed to [2,13C]propionate. When A. thaliana seedlings (4 days days after imbition) were incubated with [2,13C]propionate in liquid MS medium with the addition of [2,13C]propionate and analyzed by 13C NMR, a unique spectral peak at 40.1 ppm accumulated, corresponding to β-hydroxypropionate C2 (Fig. 2B). This peak was not observed in natural abundance spectra of control
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TABLE 1
Genes coding for putative enzymes for catabolism of valine and propionyl-CoA in A. thaliana

| Enzyme                                      | Locus name | Localization | Reference |
|---------------------------------------------|------------|--------------|-----------|
| 1 Branched-chain aminotransferase           | At1g10060  | M            | 46, 47    |
| BCKDH E1a                                   | At1g21400  | M            | 48        |
| BCKDH E1β                                   | At1g09300  | M            | 48        |
| BCKDH E2                                   | At3g13450  | M            | 48        |
| BCKDH E3                                   | At3g06850  | M            | 48        |
| 2 Acyl-CoA dehydrogenase/oxidase            | At3g17240  | M            | 49        |
| Isovaleryl-CoA dehydrogenase                | At3g045300 | M            | 28        |
| ACX1                                       | At3g16760  | P (1)        | 29, 37, 41|
| ACX2                                       | At3g65110  | P (1)        | 29, 37, 41|
| ACX3                                       | At3g06290  | P (2)        | 29, 38, 39, 41|
| ACX4                                       | At3g51840  | P (1)        | 29, 40, 41|
| ACX5                                       | At3g35690  | P (1)        | 29, 41    |
| ACX6                                       | At3g08310  | P (1)        | 29, 41    |
| 3 ATP binding/acyl-CoA dehydrogenase        | At3g06810  | P (1)        | 29        |
| Enoyl-CoA hydratase                         | At3g24360  | M            | 21        |
|                                            | At4g16120  | P (1)        | 21        |
|                                            | At4g29010  | P (1)        | 29, 43    |
|                                            | At3g06860  | P (1)        | 29, 43    |
|                                            | At3g43280  | P (1)        | 29, 42    |
|                                            | At4g07600  | NCS          |          |
| 4 Hydroxyacyl-CoA hydrolase                 | At3g31810  | M            | 21        |
|                                            | At3g60510  | M            | 21        |
|                                            | At2g05660  | P (1)        | 29, 21    |
|                                            | At2g03650  | P (1)        | 29, 21    |
|                                            | At5g69400  | P (1)        | 29, 21    |
| 5 Hydroxyacid dehydrogenase                | At3g29030  | M            | 21        |
|                                            | At3g29120  | M            | 21        |
|                                            | At1g17650  | M            | 44        |
| 6 (Methyl) malonate semialdehyde dehydrogenase | At3g14170  | M            | 44        |

ª Mitochondrial predicted localizations (M) are based on prediction scores from PREDOTAR (50) and TargetP (51) or based on the cited literature. Type 1 peroxisomal predicted localizations (P (1)) are most often based on the presence of C-terminal SKL or AKL sequences. Type 2 peroxisomal predicted localizations (P (2)) are based on the conserved motif as described in the AraPerox database (29).

ª ACX, acyl-CoA oxidase.

ª NCS, no clear signal was found for the prediction of a mitochondrial or peroxisomal localization.

samples (Fig. 2A) and was persistent in samples incubated with the labeled propionate for various time periods ranging from several hours to over 24 h. A dramatic and persistent accumulation of β-hydroxypropionate, as a spectral peak at 40.1 ppm, was also observed in A. thaliana suspension cell cultures. As with seedlings, this was the only major peak observed to accumulate. This peak arose after several hours of incubation with [2-13C]propionate and persisted at relatively high signal strengths for time periods greater than 24 h. (Fig. 3). Several unique peaks with weaker signal strengths appeared at specific time points, but with much less abundance compared with hydroxypropionate. Comparison of these peaks to those produced by relevant standard compounds indicated that none of these peaks correspond to those of other metabolites in this pathway such as acrylate or malonate which could potentially catalyze the hydrolysis of hydroxypropionyl-CoA in peroxisomes (21, 30). The dramatic accumulation of [2-13C]hydroxypropionate that was observed in wild-type plants was also observed in the chy1 mutant (Fig. 2C). In contrast, the metabolism of exogenous isobutyrate was dramatically altered in the chy1–3 seedlings. When wild-type A. thaliana seedlings were incubated with exogenous isobutyrate, an accumulation of β-hydroxyisobutyrate could be observed using an enzyme end-point assay known to be specific for β-hydroxyisobutyrate (23) (Fig. 5). Only very low concentrations of β-hydroxyisobutyrate were present in seedlings without the addition of exogenous isobutyrate.

Effect of chy1 Mutation on Metabolism of Propionate and Isobutyrate—Although NMR analysis revealed a dramatic accumulation of β-hydroxypropionate from exogenous propionate in wild-type seedlings, it is not clear whether this was the result of mitochondrial or peroxisomal metabolism, or both. To address this question, identical NMR analyses were performed using [2-13C]propionate with the well characterized chy1–3 mutant A. thaliana line. The CHY1 gene codes for a peroxisomal form of β-hydroxyacyl-CoA hydrolase, which could potentially catalyze the hydrolysis of hydroxypropionyl-CoA in peroxisomes (21, 30). The dramatic accumulation of [2-13C]hydroxypropionate that was observed in wild-type plants was also observed in the chy1 mutant (Fig. 2C). In contrast, the metabolism of exogenous isobutyrate was dramatically altered in chy1–3 seedlings. When wild-type A. thaliana seedlings were incubated with exogenous isobutyrate, an accumulation of β-hydroxyisobutyrate was abolished in the chy1–3 mutant, suggesting that these reactions occurred in the
Peroxisomes (Fig. 5). That the accumulation of hydroxypropionate was not so dramatically affected suggests that different enzymes may be involved in the hydrolysis of hydroxypropionyl-CoA versus hydroxyisobutyryl-CoA. Furthermore, assuming that exogenous isobutyrate is activated to its CoA thioester, these data indicate that metabolism of isobutyryl-CoA in plants can occur in peroxisomes, producing \( \beta \)-hydroxyisobutyrate. This peroxisomal metabolism may utilize similar enzymes as that for isobutyryl-CoA derived from valine in mitochondria, but may end with the production of hydroxyisobutyrate, resulting in its accumulation.

**Metabolism of [2-\( ^{13} \)C]Propionate in Different Plants**—To determine if metabolism of [2-\( ^{13} \)C]propionate in *A. thaliana* seedlings and cell cultures is consistent with that in other plants, we performed similar \( ^{13} \)C NMR analyses using seedlings from a variety of species, including both monocots and dicots. Fig. 6 shows carbon NMR spectra of metabolites produced from [2-\( ^{13} \)C]propionate in a range of plants, including *L. sativa* (lettuce) *P. sativum* (pea), *T. aestivum* (wheat), and *Z. mays* (corn). Each showed a dramatic accumulation of \( \beta \)-hydroxypropionate (unique peak at 40.1 ppm), which was very similar to that observed in *A. thaliana*, and a notable absence of any other accumulating species as compared with natural abundance spectra. Thus, there appeared to be no major differences in the metabolism of exogenous propionate in these varied plant species as observed by carbon NMR analysis.

**Metabolism of [U-\( ^{13} \)C]Valine in *A. thaliana* Seedlings**—The major known pathway for valine catabolism is a modifi ed \( \beta \)-oxidation pathway producing propionyl-CoA (31). We considered whether propionyl-CoA produced from mitochondrial metabolism of valine would also lead to an accumulation of \( \beta \)-hydroxypropionate. Incubation of *A. thaliana* seedlings with uniformly labeled [\( ^{13} \)C]valine failed to reveal the production of [\( ^{13} \)C]hydroxypropionate as measured by both one-dimensional carbon NMR and HMOC analysis. [\( ^{13} \)C]Hydroxypropionate was not produced from valine under a variety of conditions, including various concentrations of valine and various times of incubation with the \( ^{13} \)C label. Instead, the major product observed from the metabolism of [U-\( ^{13} \)C]valine in growing *A. thaliana* seedlings was leucine. The label from [U-\( ^{13} \)C]valine was incorporated into carbons 3–6 of leucine, observed as unique spectral peaks with shifts at 42.6, 26.7, 24.7, and 23.6 ppm, respectively (Fig. 7). These data are consistent with the conversion of valine to \( \alpha \)-ketoisovalerate by the branched-chain transaminase, and then entry into the known pathway for leucine biosynthesis (Fig. 1). This pathway would result in exactly the labeling pattern observed as shown in Fig. 8.

**Effects of Exogenous Isobutyrate, Propionate, and Valine on Seedling Growth of Wild-type and chy1–3 *A. thaliana***—Wild-type *A. thaliana* seedlings have been shown to display a characteristic sensitivity to valine during germination and seedling growth.
growth (32). In the present study, *A. thaliana* also displayed a similar sensitivity to propionate and isobutyrate. This toxicity occurred with concentrations as low as 100 μM and was most readily measured as a morphological effect on root length (Fig. 9). We considered that these effects are most likely related to the known toxicity of methylacrylyl-CoA and acrylyl-CoA, and that these compounds might increase to toxic levels when isobutyrate, propionate, or valine, are presented in excess (21, 30). Because the end-point assays presented above suggest that the metabolism of exogenous isobutyrate occurred primarily in peroxisomes and was dependent on the function of CHY1, we compared the sensitivity of wild-type and chy1–3 seedlings to isobutyrate, propionate, and valine. The chy1–3 mutant exhibited the same level of sensitivity to valine as wild-type seedlings. These data suggest that the toxicity of isobutyrate and propionate may be related to the peroxisomal production of methylacrylyl-CoA and acrylyl-CoA, respectively, and that the disposal of these compounds depends on CHY1. Furthermore, peroxisomal CHY1 appears to play no role in the toxicity of

**FIGURE 4.** HMQC analysis of standard β-hydroxypropionate and *A. thaliana* seedlings with [2-13C]propionate. A, 4-day-old wild-type seedlings were incubated with [2-13C]propionate for 24 h in liquid MS medium before performing HMQC analysis. B, standard β-hydroxypropionate was analyzed by HMQC using the same protocol as for seedlings.

**FIGURE 5.** β-Hydroxyisobutyrate accumulation in wild-type and chy1–3 *A. thaliana* seedlings. Four-day-old wild-type and chy1–3 seedlings were incubated with 2 mM isobutyrate for 24 h. Accumulation of β-HIBA was measured as described under “Experimental Procedures” and expressed as nanomoles/mg wet weight. Error bars indicate the standard error of the mean.
valine, consistent with the theory that valine metabolism occurs primarily in mitochondria.

DISCUSSION

The availability of a complete and well annotated genome data base for *A. thaliana* allows for the identification of genes with putative function in various metabolic pathways (27). This information also provides clues regarding the likely subcellular location of the corresponding enzymes. With regard to propionate metabolism, this capability is valuable considering there are pathways for the production of propionyl-CoA in both the mitochondria and peroxisomes. Examination of the *A. thaliana* genome shows that all of the enzymes required for conversion of valine to propionyl-CoA may reside in the mitochondria. The problem that arises is the subsequent fate of the propionyl-CoA produced in mitochondria. Although propionyl-CoA could possibly be converted to acetyl-CoA by a similar pathway as to that of isobutyryl-CoA, this may not be the case for mitochondrial propionyl-CoA. A primary obstacle for this pathway is the apparent lack of a mitochondrial acyl-CoA dehydrogenase functional for oxidation of propionyl-CoA (27). An isovaleryl-CoA dehydrogenase has been characterized from several plant species, including *A. thaliana* (28, 33, 34). However, this enzyme has been shown to be highly specific for branched-chain acyl groups and was inactive with short-chain acyl-CoA substrates like butyryl-CoA and propionyl-CoA (28). A mitochondrial enoyl-CoA hydratase specifically capable of producing hydroxypropionyl-CoA has also not been clearly identified, however mitochondrial enoyl-CoA hydratase activity has been reported (35). Putative mitochondrial enzymes exist that could potentially catalyze subsequent steps, including hydroxyacyl-CoA hydrolases, hydroxyacid dehydrogenases, and a methylmalonate/malonate semialdehyde dehydrogenase (Table 1). However, the substrate specificities and kinetic parameters for these enzymes have not yet been determined. The genes listed in Table 1 may support the complete metabolism of valine to propionyl-CoA in mitochondria but not necessarily the conversion of propionyl-CoA to acetyl-CoA as shown in Fig. 1. Alternatively, it is also possible that propionyl-CoA could exit the mitochondrion by conversion to propionyl-carnitine and enter other pathways for further degradation. Acyl-carnitine carrier proteins have recently been identified in *A. thaliana* (36).

**FIGURE 6. Production of β-hydroxypropionate in various plants.** Seedlings were dark grown on towelettes saturated with sterile water for ~6 days. They were then incubated with 1 mM [2-13C]propionate in MS medium for 24 h and then analyzed by 13C NMR as described under “Experimental Procedures.” A, Pea; B, lettuce; C, wheat; D, corn.
There are also, clearly, genes coding for a specific subset of this pathway in the peroxisomes (Fig. 1 and Table 1). Identification of gene products with likely peroxisomal localization has been greatly advanced through the recognition of type 1 and type 2 peroxisomal targeting sequences (30). There are multiple isoforms of acyl-CoA oxidase (ACX1 through ACX6) with clear peroxisomal targeting sequences. The expression and activities of these enzymes have been partially characterized (37–41). Multiple homologues also exist for enoyl-CoA hydratases and hydroxyacyl-CoA hydrolases (Fig. 1, step 5) with clear peroxisomal targeting sequences (21, 27, 42, 43). However, a β-hydroxyacid dehydrogenase (step 6) homologue is not present in the A. thaliana genome that contains a clear type 1 or type 2 peroxisomal targeting signal (Fig. 1, step 6). There also appears to be no peroxisomal homologue of methylmalonate semialdehyde dehydrogenase (step 7), the enzyme believed to convert malonate semialdehyde to acetyl-CoA (44). It is plausible that hydroxypropionate could be oxidized by an as yet unidentified peroxisomal dehydrogenase. However, in the absence of a functional malonate semialdehyde dehydrogenase, peroxisomal oxidation of hydroxypropionate to malonate semialdehyde should be deleterious without any other means of disposal of this potentially toxic compound. Therefore, it appears that peroxisomal catabolism of propionate could be expected to produce β-hydroxypropionate as an end product rather than acetyl-CoA.

The observed accumulation of [2-13C]hydroxypropionate from [2-13C]propionate is consistent with the genetic features described above. No evidence was found for the production of acetate from [2-13C]propionate as was previously reported for lima bean leaves and stems (13). This may be a difference between the plant materials studied: whole A. thaliana seedlings versus lima bean leaves and stems. It may also be attributed to the metabolism of malonate semialdehyde dehydrogenase, peroxisomal oxidation of hydroxypropionate to malonate semialdehyde should be deleterious without any other means of disposal of this potentially toxic compound. Therefore, it appears that peroxisomal catabolism of propionate could be expected to produce β-hydroxypropionate as an end product rather than acetyl-CoA.

Accumulation of β-hydroxyisobutyrate from exogenous isobutyrate is also consistent with this metabolic model. That the production of hydroxyisobutyrate was abolished in the chy1 mutant conclusively shows that this pathway occurs in peroxisomes. The data presented in this study suggest that there might be multiple metabolic sources of isobutyryl-CoA in plants. Isobutyryl-CoA is expected to be an early product of valine catabolism and, as such, should be produced in the mitochondria. However, this study also provides evidence for a role of the peroxisomal enzyme CHY1 in metabolism of isobutyryl-CoA. Possible sources of both isobutyryl-CoA and propionyl-CoA in peroxisomes may include branched-chain fatty acids such as phytic acid. There is evidence for peroxisomal metabolism of phytic acid in mammalian systems, but little is known of this pathway in plants. Although there have been preliminary reports of branched-chain amino acid degradation in plant peroxisomes (2, 13), this still remains to be conclusively shown. In light of this, it is particularly noteworthy that the
chy1–3 mutant displayed increased sensitivity to isobutyrate but not valine, suggesting that this enzyme may not have a central role in valine degradation. However, the best evidence for a role in peroxisomes in valine catabolism is the observation of reduced production of $^{14}$CO$_2$ from [U-$^{14}$C]valine in a chy1 mutant A. thaliana (30). This observation remains difficult to explain in light of the probable mitochondrial localization of key enzymes such as branched-chain amino transferase and BCKDH that produce isobutyryl-CoA from valine. Nevertheless, certain enzyme activities of the valine catabolic pathway may be present in both mitochondria and peroxisomes for metabolism of isobutyryl-CoA derived from compounds other than valine.

No evidence for the production of hydroxypropionate from valine was observed in this study. Surprisingly, the major product observed from the metabolism of [U-$^{13}$C]valine was leucine. The pattern of incorporation of labeled carbon into leucine was exactly as one would expect for the conversion of [U-$^{13}$C]valine to $\beta$-ketoisovalerate and entry into the known pathway for leucine biosynthesis (Fig. 8). This may be the result of gene expression during seedling growth favoring amino acid biosynthesis rather than degradation. $\beta$-Ketoisovalerate is an intermediate shared by the expected pathways for valine degradation and for valine and leucine biosynthesis. If the enzymes for valine and leucine biosynthesis were highly expressed during seedling growth, then exogenous valine may produce $\beta$-ketoisovalerate and enter into this biosynthetic pathway. It is also possible that other pathways, such as a $\beta$-oxidation, may be fully functional, but may have occurred too rapidly to observe the accumulation of metabolic intermediates. Therefore, one cannot conclude that leucine was the only product produced from [U-$^{13}$C]valine. Nevertheless, from this study it is clear that leucine is a significant product of valine metabolism in A. thaliana seedlings.
Therefore, whether propionyl-CoA is produced from valine in mitochondria of plants still remains a major question.

The increased sensitivity of the chy1 mutant to excess propionate and isobutyrate represents new insight into the previously reported phenotypes of this mutant. Mutations in this gene are known to produce defects in peroxisomal β-oxidation and in responses to indole butyric acid (IBA) and 2,4-dichlorophenoxybutyric acid (21, 30). These effects are believed to result from increased production of methylacrylyl-CoA, a toxic metabolite of valine. Altered responses to IBA are also believed to be the result of defects in β-oxidation caused by increased production of methylacrylyl-CoA (21, 30). IBA is converted to acrylyl-CoA and methylacrylyl-CoA on 3-oxidation (30). The increased sensitivity of the chy1 mutant to excess propionate and isobutyrate is also likely due to the increased production of acrylyl-CoA and methylacyl-CoA, respectively.

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