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Study of the 3-Hydroxy Eicosanoyl-Coenzyme A Dehydratase and (*E*)-2,3 Enoyl-Coenzyme A Reductase Involved in Acyl-Coenzyme A Elongation in Etiolated Leek Seedlings[1]

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(*R,S*)-[1-^{14}C]3-Hydroxy eicosanoyl-coenzyme A (CoA) has been chemically synthesized to study the 3-hydroxy acyl-CoA dehydratase involved in the acyl-CoA elongase of etiolated leek (*Allium porrum* L.) seedling microsomes. 3-Hydroxy eicosanoyl-CoA (3-OH C20:0-CoA) dehydration led to the formation of (*E*)-2,3 eicosanoyl-CoA, which has been characterized. Our kinetic studies have determined the optimal conditions of the dehydration and also resolved the stereospecificity requirement of the dehydratase for (*R*)-3-OH C20:0-CoA. Isotopic dilution experiments showed that 3-hydroxy acyl-CoA dehydratase had a marked preference for (*R*)-3-OH C20:0-CoA. Moreover, the very-long-chain synthesis using (*R*)-3-OH C20:0-CoA isomer and [2-^{14}C]malonyl-CoA was higher than that using the (*S*) isomer, whatever the malonyl-CoA and the 3-OH C20:0-CoA concentrations. We have also used [1-^{14}C]3-OH C20:0-CoA to investigate the reductant requirement of the enoyl-CoA reductase of the acyl-CoA elongase complex. In the presence of NADPH, [1-^{14}C]3-OH C20:0-CoA conversion was stimulated. Aside from the product of dehydration, i.e. (*E*)-2,3 eicosanoyl-CoA, we detected eicosanoyl-CoA resulting from the reduction of (*E*)-2,3 eicosanoyl-CoA. When we replaced NADPH with NADH, the eicosanoyl-CoA was 8- to 10-fold less abundant. Finally, in the presence of malonyl-CoA and NADPH or NADH, [1-^{14}C]3-OH C20:0-CoA led to the synthesis of very-long-chain fatty acids. This synthesis was measured using [1-^{14}C]3-OH C20:0-CoA and malonyl-CoA or (*E*)-2,3 eicosanoyl-CoA and [2-^{14}C]malonyl-CoA. In both conditions and in the presence of NADPH, the acyl-CoA elongation activity was about 60 nmol mg^{-1} h^{-1}, which is the highest ever reported for a plant system.

In higher plants VLCFA result from elongation of acyl-CoA by malonyl-CoA in the presence of NADPH (Lessire et al., 1985a; Cassagne et al., 1994; Post-Beittenmiller, 1996). The elongation process is catalyzed by acyl-CoA elongase, an enzymatic complex of four different proteins (Bessoule et al., 1989). The overall elongation reaction involves four successive steps. The condensation of acyl-CoA with malonyl-CoA leading to the formation of 3-ketoacyl-CoA is catalyzed by KAS. This 3-ketoacyl-CoA is reduced to 3-hydroxyacyl-CoA by KCR. HCD is responsible for the dehydration of the 3-hydroxyacyl-CoA to form (*E*)-2,3 enoylacyl-CoA. The reduction of the resulting 2,3-enoyl-CoA is catalyzed by ECR.

Evidence in support of this pathway is provided by the identification of the chemical intermediates of the elongase reaction in leek (*Allium porrum* L.) leaves (Lessire et al., 1989) and *Lunaria annua* seeds (Fehling and Mukherjee, 1991), and very recently by the synthesis of VLCFA from 3-OH C20:0 (icosanoate)-CoA and (*E*)-2,3 C20:1 (eicosanoate)-CoA by microsomes of etiolated leek seedlings (Lessire et al., 1998). In particular, the KAS has been studied using membranes from leek leaves (Schneider et al., 1993) and developing rapeseeds (Fehling and Mukherjee, 1991; Domergue et al., 1996), as this enzyme is considered critical in determining the amount, chain length, and unsaturation of VLCFA synthesized by acyl-CoA elongase. Cloning the Arabidopsis *FAE1* gene (James et al., 1995) has led to isolation of the corresponding cDNAs, encoding KAS from different sources (Lassner et al., 1996; Barret et al., 1998), which have been expressed in yeast and in different plant tissues (Millar and Kunst, 1997; Todd et al., 1997).

Owing to the lack of a substrate (3-ketoacyl-CoA), KCR has never been studied. However, use of a maize *glossy8*

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Abbreviations: ECR, enoyl-CoA reductase; HCD, 3-hydroxyacyl-CoA dehydratase; KAS, 3-ketoacyl-CoA synthase; KCR, 3-ketoacyl-CoA reductase; 3-OH C20:0-CoA, 3-hydroxy eicosanoyl-CoA; VLCFA, very-long-chain fatty acids; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

mutant affected in wax synthesis has led to the cloning of a cDNA encoding for KCR, which could be involved in the acyl-CoA elongase complex (Xu et al., 1997). The corresponding cDNAs from leek, Arabidopsis, and barley have also been isolated, and the (*E*)-2,3 ECR activity has been demonstrated in leek and rapeseed membrane fractions (Fowler et al., 1995; Spinner et al., 1995).

Lessire et al. (1993) have investigated the presence of HCD in leek microsomes by measuring the reverse reaction using (*E*)-2,3 C16:1-CoA as the substrate. Adding antibodies raised against HCD purified from rat liver inhibited this activity and the overall fatty acid elongation. Immunoblotting experiments with these antibodies produced a protein with an apparent molecular mass of 65 kD.

Nevertheless, there is still a great deal that is unknown about the mechanism(s) of the partial reactions involved in acyl-CoA elongation. This may be due to the difficulties associated with measuring individual reactions caused by the lack of substrates and to the difficulties associated with purifying these membrane-bound proteins. For example, the reductant requirement for both reductases of the elongase is still debated (Cassagne et al., 1994). NADPH has been observed in numerous cases to be the "preferred" reductant, but NADH has also proven efficient for the overall elongation process (Agrawal and Stumpf, 1985; Murphy and Mukherjee, 1989; Taylor et al., 1992). Furthermore, the stereospecificity of the dehydration reaction has never been studied.

By using a chemically prepared (*R*,*S*) racemic mixture of [1-^{14}C]3-OH C20:0-CoA, we determined the stereospecificity of the (*R*,*S*)-3-hydroxy dehydratase from etiolated leek seedling microsomes and the reductant preference of the (*E*)-2,3 ECR.

MATERIALS AND METHODS

Materials

Leek (*Allium porrum* L.) seedlings (7 d old) were grown as reported previously (Lessire et al., 1985b). Leek seeds stored at 4°C overnight were sterilized by sodium hypochloride for 5 min and washed. The growth medium (5 g of agar-agar, 900 mL of water, and 100 mL of a nutritive solution containing 7.5 g of KCl, 6 g of $NaNO_3$, 2.5 g of $MgSO_4$, 0.95 g of $CaCl_2$, and 1.25 g of NaH_2PO_4 per liter) was heated to 100°C for 5 min. The vessels were sterilized for 2 h with sodium hypochloride. Seedling culture was performed in the dark at 25°C for 7 d. All chemicals were from Sigma. (*R*,*S*)-3-OH C20:0-CoA isomers and (*E*)-2,3 eicosanoyl-CoA were prepared and purified according to the method of Lucet-Levannier et al. (1995). The (*R*) and (*S*) isomers were synthesized by using an asymmetric aldolization reaction from (*S*)- or (*R*)-2-hydroxy-1,2,2-triphenylethyl acetate and (*Z*)-9,10-octadecanal, their purity being approximately 70% (Lucet-Levannier, 1995). [2-^{14}C]Malonyl-CoA (57 Ci mol^{-1}) came from NEN.

[1-^{14}C]3-OH C20:0-CoA Synthesis

[1-^{14}C]Ethyl 3-Hydroxy-Eicosanoate

[1-^{14}C]Acetate sodium salt (100 mCi) was dried at 140°C for 4 h under a vacuum. Triethyl phosphate (1.2 mL) was added under a nitrogen atmosphere and heated at 150°C for 4.5 h. [1-^{14}C]Ethyl acetate was distilled under a vacuum in two vials cooled to −20°C and mixed with 2 mL of dry tetrahydrofuran. The temperature was maintained at −80°C and 1.8 mL of 1 M lithium bis(trimethylsilyl)amide was added. After 45 min of incubation, 536 mg (2 mmol) of octadecanal in 4 mL of tetrahydrofuran was added and the solution was stirred at −65°C for 5.5 h. After hydrolysis with a solution of NH_4Cl, ethyl [1-^{14}C]3-hydroxy-eicosanoate was extracted with diethylether and washed with water.

We used petroleum ether:diethyl ether (80:20, v/v) to purify the product on silica gel, giving 59 mCi of ethyl [1-^{14}C]3-hydroxy-eicosanoate.

[1-^{14}C]3-Hydroxy Eicosanoic Acid

One milliliter of 1 M aqueous potassium hydroxide was added to 20 mCi of ethyl [1-^{14}C]3-hydroxy eicosanoate and dissolved in 4 mL of ethanol, and the solution was stirred for 15 h at room temperature. The reaction mixture was cooled in an ice-water bath and acidified with 1.5 mL of 1 N HCl. The acid was extracted with diethylether and washed with water. The ether extract was evaporated and the acid was dried under a vacuum.

[1-^{14}C]N-Succinimidyl-3-Hydroxy-Eicosanoate

N-Hydroxysuccinimide (46 mg, 0.4 mmol) was mixed with 4 mL of [1-^{14}C]3-hydroxy eicosanoic acid dissolved in anhydrous ethyl acetate. The mixture was stirred at 4°C for 10 min, then 82 mg (0.4 mmol) of dicyclohexylcarbodiimide in 1 mL of dry ethyl acetate was added. After stirring for 24 h at room temperature, the mixture was filtered to remove the dicyclohexylurea and the precipitate was washed with a small volume of ethyl acetate. The solution was evaporated to dryness and the solid residue was recrystallized in 5 mL of ethanol. This gave 13 mCi of product (radioactive yield, 65%).

[1-^{14}C]3-OH C20:0-CoA

Two milliliters of aqueous bicarbonate solution (26 mg, 0.308 mmol mL^{-1}) was added to 24 mg (0.03 mmol) of CoA *SH,2Na* under an argon atmosphere. The solution was stirred under argon for 10 min; then 1.6 mCi (13 mg, 0.03 mmol) of [1-^{14}C]*N*-succinimidyl-3-hydroxy-eicosanoate was dissolved in 3 mL of tetrahydrofuran and 6 mL of tetrahydrofuran was added. After stirring for 4 h at room temperature, 2 mL of 10 mM KH_2PO_4 was added and the pH was adjusted to 5.0 with 1 N HCl. The product was purified by preparative HPLC using a Nucleosil C_{18} 5-mm (250- × 10-mm) column and a mobile phase consisting of 10 mM KH_2PO_4 (A) and acetonitrile (B). The elution ran for 10

min from 60% of A to 55%; for 10 min from 55% of A to 45%; for 15 min from 45% of A to 15%; and then for 10 min to 60% of A using a flow rate of 3.8 mL/min. The products were detected by continious monitoring at 254 nm.

We obtained 0.32 mCi of product with a radiochemical purity better than 99%. The specific activity determined by MS (*DCI*/NH_3) on [1-^{14}C]*N*-succinimidyl-3-hydroxy-eicosanoate was 52 mCi mmol^{-1} (1.92 GBq mmol^{-1}).

Microsome Preparation

Seedlings (5.8 g) were ground in a mortar with 50 mL of 0.08 M Hepes buffer (pH 7.2) containing 10 mM β-mercaptoethanol and 0.32 M Suc. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 3,000*g* for 5 min. The supernatant was centrifuged at 12,000*g* for 20 min. The resulting pellet was discarded and the supernatant centrifuged at 150,000*g* for 15 min (CS 100, Hitachi, Tokyo, Japan). The supernatant was considered as the cytosolic fraction. The microsomal pellet was resuspended in 2 mL of 0.08 M Hepes buffer (pH 6.8) and centrifuged again at 150,000*g* for 15 min. The resulting pellet, resuspended in 2 mL of 0.08 M Hepes buffer (pH 6.8) and 10 mM β-mercaptoethanol, was used as the enzyme source.

[1-^{14}C]3-OH C20:0-CoA Dehydration Measurements

Routinely, 20 μg of microsomal proteins was incubated in the presence of 2 mM DTT, 1 mM $MgCl_2$, 150 μM Triton X-100, and 11 μM [1-^{14}C]3-OH C20:0-CoA in 0.08 M Hepes buffer (pH 6.8). The final volume was 0.1 mL and the reaction was carried out for 15 min at 30°C. The reaction was stopped by 0.1 mL of methanolic KOH/5N (CH_3OH:H_2O, 1:9) and the reaction mixture was heated at 80°C for 1 h. The fatty acids were extracted after acidification with 0.1 mL of H_2SO_4 (10 N) and layered on a HPLC plate (60F 254, Merck, Darmstadt, Germany). The plate was developed with hexane:diethyl ether:acetic acid (75:25:1, v/v) and then subjected to autoradiography. The spots corresponding to the 3-hydroxy and (*E*)-2,3 unsaturated fatty acids were scraped for radioactivity measurements. Control experiments (incubation time = 0) were carried out under the same conditions. Activities were measured as the difference between the percentages of [1-^{14}C]3-OH C20:0-CoA conversion in the assay and the control.

3-OH C20:0-CoA Elongation Measurements

Elongation activity was measured by using 9 μM [2-^{14}C]malonyl-CoA and 9 μM (*R*)- or (*S*)-3-OH C20:0-CoA in the presence of 2 mM DTT, 1 mM $MgCl_2$, 0.1 mM NADPH, 150 μM Triton X-100, and 60 μg of protein in 0.08 M Hepes buffer (pH 6.8). The final volume was 0.1 mL and the reaction was carried out for 1 h at 30°C. The reaction was then stopped and the fatty acids were extracted as described above. The radioactivity of the fatty acids was measured using a radioactivity spectrophotometer.

Permanganate-Periodate Oxidation

One milliliter of an oxidant solution of $NaIO_4$ (2.09%, w/w) and $KMnO_4$ (0.04%, w/w) in water and 1 mL of K_2CO_3 (0.25%, w/v) were added to 1 mL of fatty acid methyl ester dissolved in 1 mL of *tert*-butanol. The mixture was shaken at room temperature for 1 h. The mixture was then acidified with one drop of concentrated H_2SO_4, 0.5 mL of 2.4 M $Na_2S_2O_4$ was added, and the methyl esters were extracted by 4 mL of diethyl ether.

Lipid Separations

Acyl-CoA was characterized as reported previously (Juguelin and Cassagne, 1984) using TLC 60F 254 plates developed with butanol:water:acetic acid (5:2:3, v/v). Fatty acid methyl esters were analyzed using TLC plates impregnated with 10% (w/w) silver. The fatty acid methyl esters were prepared by the method reported by Lepage and Roy (1986) and separated according to chain length by radio-GLC using an autosystem (Perkin Elmer) equipped with a CP Sil 3% column (2 mm × 3 m) coupled to a radioactivity counter (model 894, Packard, Meriden, CT).

Protein Estimation

The protein content was measured according to the method of Bradford (1976), using BSA as a standard.

RESULTS AND DISCUSSION

Characterization and Properties of the Microsomal 3-OH C20:0-CoA Dehydratase

After incubation of leek microsomes with [1-^{14}C]3-OH C20:0-CoA, the reaction products were separated by TLC using butanol OH:CH_3COOH:H_2O (5:2:3, v/v) as developing solvent. The existence of a unique product migrating with a R_F of 0.5, which corresponded to long-chain acyl-CoAs, was detected by autoradiography. After saponification of this acyl-CoA fraction, the resulting fatty acids were layered onto TLC plates and further developed with hexane:diethyl ether:CH_3COOH (75:25:1, v/v). The autoradiography revealed two different components: a major product (R_F = 0.10) corresponding to the unreactant [1-^{14}C]3-OH C20:0-CoA fatty acid moiety and a second product with a R_F of 0.30. The second component was identified, as was the *trans*-2,3 eicosanoic acid, by a combination of silver nitrate TLC and GLC-MS (A. Knoll, personal communication). This result demonstrated the existence in etiolated leek seedling microsomes of a 3-OH acyl-CoA dehydratase activity able to synthesize (*E*)-2,3 C20:1-CoA from 3-OH C20:0-CoA.

The optimal conditions for dehydration were determined. 3-OH C20:0-CoA dehydration increased linearly during the first 20 min of the incubation and reached a plateau (Fig. 1A). The rate of (*E*)-2,3 C20:1-CoA synthesis from 3-OH C20:0-CoA increased proportionally as the microsomal protein increased, up to 25 μg/assay, and reached a maximum of 0.3 nmol/15 min (Fig. 1B). The

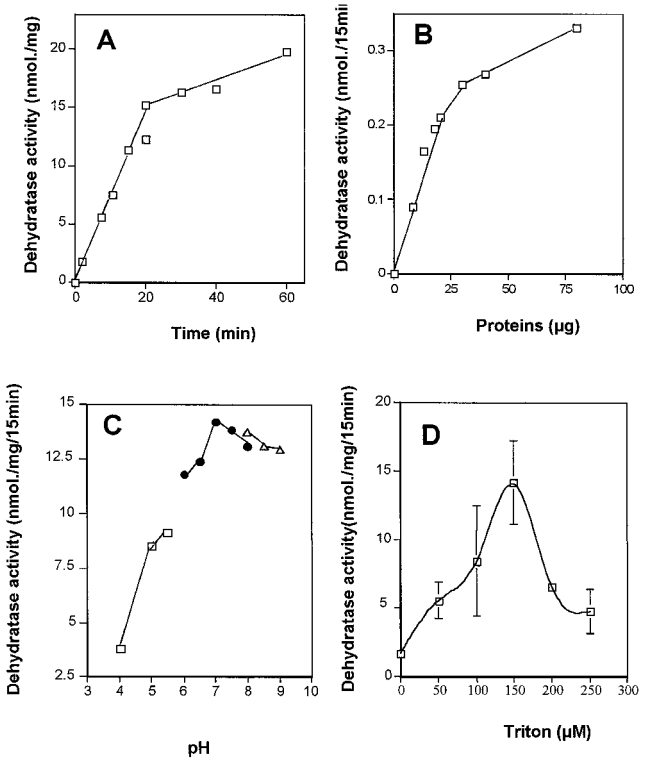

Figure 1. Kinetic studies of [1-^{14}C]3-OH eicosanoyl-CoA dehydratase. A, Dehydration time course; 20 μg of protein was incubated with 11 μM [1-^{14}C]3-OH C20:0-CoA at 30°C for different incubation times under the conditions specified in "Materials and Methods." B, Influence of protein amounts upon dehydratase activity; 11 μM [1-^{14}C]3-OH C20:0-CoA was incubated for 15 min with different amounts of microsomal proteins as described in "Materials and Methods." C, Influence of pH upon 3-OH C20:0-CoA dehydration; 20 μg of protein was incubated with 11 μM [1-^{14}C]3-OH C20:0-CoA for 15 min in 0.1 M acetate-citrate (□), 0.08 M Hepes (●), or 0.1 M Tris-Gly (△) buffers at different pH values. D, Influence of Triton X-100 upon dehydratase activity; [1-^{14}C]3-OH C20:0-CoA dehydration was measured in the presence of different concentrations of Triton X-100 under the conditions reported in "Materials and Methods." Results are expressed as the means of three independent experiments.

activity was also studied at different pH values (Fig. 1C); in contrast to the 3-OH dehydratase from rat liver, which presented three different optimal pH values (Bernert and Sprecher, 1979), suggesting the presence of different isozymes, the leek enzyme presented a single pH optimum at 7.0 to 7.5. The 3-OH acyl-CoA dehydratase activity was measured in the presence of increasing concentrations of Triton X-100 up to 250 μM (Fig. 1D). As with the rat liver dehydratase (Bernert and Sprecher, 1979) and the leek acyl-CoA elongase (Lessire et al., 1985a), the 3-OH acyl-CoA dehydratase activity was stimulated by addition of Triton X-100. Optimal activity was observed in the presence of 150 μM Triton X-100, which resulted in an 8-fold stimulation of the activity. All further experiments were done in the presence of 150 μM Triton X-100, using linear velocity conditions (20 μg of protein, 15 min of incubation).

Stereospecificity of the 3-OH C20:0-CoA Dehydratase

The dehydration of (*R*,*S*)-[1-^{14}C]3-OH C20:0-CoA was studied using isotopic dilutions with nonradioactive (*R*) and (*S*) isomers of 3-OH C20:0-CoA (Fig. 2). The addition to the dehydratase assay of increasing concentrations of the unlabeled (*S*) isomer resulted in only a slight decrease in the amount of labeled (*E*)-2,3 eicosanoyl-CoA product, suggesting that the amount of the product of dehydration was independent of the specific radioactivity of the substrate. In marked contrast, when the unlabeled (*R*) isomer was added, the amount of ^{14}C incorporated in the (*E*)-2,3 eicosanoyl-CoA decreased as a result of isotope dilution. These results demonstrate that leek microsomal 3-OH acyl-CoA dehydratase had a marked preference for the (*R*)-3-OH C20:0-CoA isomer.

We also evaluated the stereospecific requirement of the dehydratase by using (*R*) and (*S*) isomers of 3-OH C20:0-CoA as primers for fatty acid elongation and assaying elongase activity by incorporating radioactivity from malonyl-CoA. Microsomal proteins were incubated with either 9 μM (*R*)-3-OH C20:0-CoA or 9 μM (*S*)-3-OH C20:0-CoA in the presence of [2-^{14}C]malonyl-CoA, NADH, and NADPH. Regardless of the 3-hydroxy acyl-CoA isomer used in these assays, the rate of fatty acid elongation was identical for the first 10 min of the reaction (Fig. 3A), reflecting the fact that each isomer preparation was 30% racemic (Lucet-Levannier, 1995). Upon longer incubation, the (*R*) enantiomer continued to support fatty acid elongation for up to 40 min; then the rate tended toward a plateau. In contrast, when using the (*S*)-3-OH C20:0-CoA isomer, malonyl-CoA incorporation leveled off after 10 min.

The preference of the acyl-CoA elongase for (*R*)-3-hydroxy C20:0-CoA was observed regardless of the malonyl-CoA concentrations used in the assay (Fig. 3B). Fatty acid synthesis level was always 3- or 4-fold higher with the (*R*) than with the (*S*) isomer.

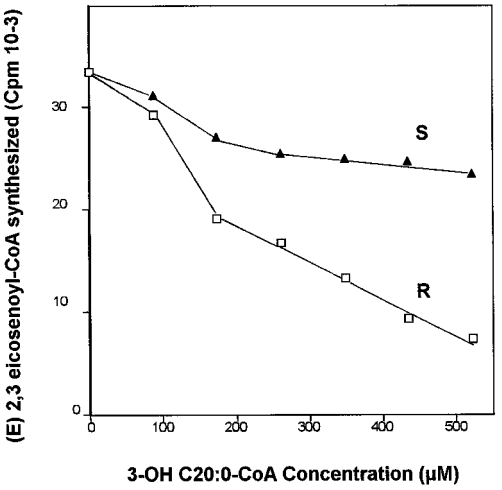

Figure 2. Effect of isotopic dilution by (*R*) and (*S*) isomers upon (*E*)-2,3 eicosanoyl-CoA synthesis. To the standard reaction mixture (20 μg of protein, 11 μM [1-^{14}C]3-OH C20:0-CoA) unlabeled (*R*)- or (*S*)-3-OH C20:0-CoA was added in the indicated concentrations. The reaction was run for 20 min at 30°C and the products were separated by TLC as indicated in "Materials and Methods.

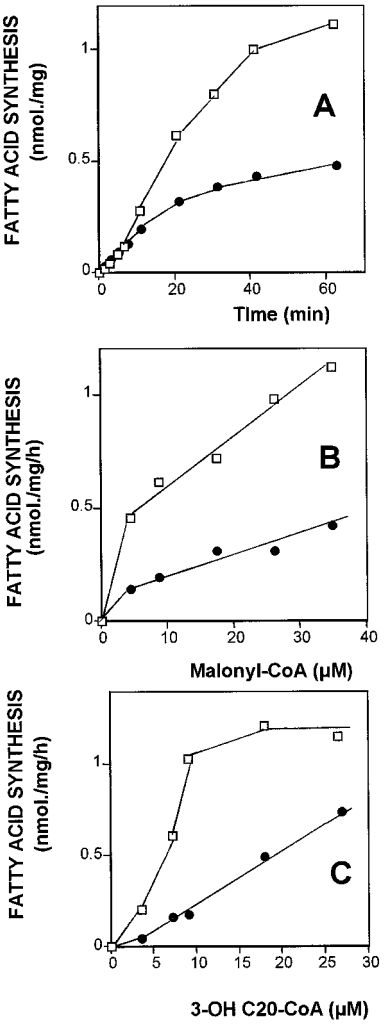

Figure 3. Kinetic studies of (*R*)- and (*S*)-3-OH eicosanoyl-CoA isomer elongation. The (*R*)- (□) and (*S*)- (●) 3-OH-C20:0 (9 μM) elongations were measured using initial velocity conditions. A, Time-course experiment; each isomer was incubated for various times in the presence of 20 μg of protein at 30°C, with 100 μM NADPH, 1 mM $MgCl_2$, 2 mM DTT, 0.15 mM Triton X-100, and 9 μM [2-^{14}C]malonyl-CoA in 0.08 M Hepes buffer, pH 6.8. The reaction was stopped after different incubation times and the radioactivity was measured in the fatty acid fraction as indicated in "Materials and Methods." B, Effect of malonyl-CoA concentration; 20 μg of protein was incubated for 20 min; the malonyl-CoA concentrations are indicated. C, Effect of 3-OH acyl-CoA concentration; same conditions as in A. Incubation time was 20 min, and acyl-CoA concentration varied as indicated.

Figure 3C shows the effect of increasing concentrations of the (*R*)- or (*S*)-3-hydroxy C20:0-CoA isomers on the acyl-CoA elongase activity. VLCFA synthesis was higher in the presence of the (*R*) than in that of the (*S*) enantiomer. These results show that (*R*)-3-hydroxy C20:0-CoA was a more efficient precursor for VLCFA synthesis than the (*S*) isomer, in agreement with the stereospecificity preference of 3-OH C20:0-CoA dehydratase.

Characterization of a Microsomal (*E*)-2,3 ECR

We identified the reductant cofactor required by the (*E*)-2,3 ECR component of the acyl-CoA elongase by incubating microsomal preparations with [1-^{14}C]3-OH C20:0-CoA in the presence of either NADH or NADPH, and analyzing the products (Table I). Analysis of the resulting fatty acids by TLC identified two products with R_F values of 0.30 and 0.35. In an animal-derived elongase system, the nature of these acyl products has been elucidated using GLC-MS analysis as the (*E*)-2,3 C20:1 and C20:0 fatty acids, respectively (A. Knoll, personal communication). The permanganate-oxidation degradation of the mixture of these two components confirmed this identification (Table I). In the control incubation in the absence of any reductant, no label was recovered in the fatty acid fraction resulting from the permanganate oxidation. This is the expected result if [1-^{14}C](*E*)-2,3 C20:1 is the unique product of dehydration of the [1-^{14}C]3-OH C20:0-CoA substrate (Table I). When NADH was added to the incubation, 12.3% of the labeled products were resistant to permanganate-oxidation degradation, indicating that in the presence of NADH (*E*)-2,3 C20:1-CoA could be partially reduced. This result is in good agreement with the TLC analysis showing that under these conditions 10.7% of the radioactivity was [1-^{14}C] C20:0-CoA and 89.3% was [1-^{14}C](*E*)-C20:1. In the presence of NADPH most of the radioactivity was resistant to permanganate oxidation. The TLC analysis shows that the main product was C20:0-CoA and that 89.5% of the 3-OH C20:0-CoA was converted to the saturated fatty acid.

We also demonstrated the NADPH preference of the (*E*)-2,3 ECR by measuring the disappearance of NADPH or NADH. The mean values of three independent experiments indicated that the rate of decrease of the A_{340} induced by the addition of 30 μM (*E*)-2,3 C20:1-CoA was 0.63 ± 0.28 nmol mg^{-1} min^{-1} in the presence of NADPH, whereas it was only 0.11 ± 0.04 nmol mg^{-1} min^{-1} in the presence of NADH.

Table I. *Effect of NADH and NADPH upon [1-^{14}C]3-OH C20:0-CoA metabolism*

See "Materials and Methods" for details of the experiment. Results are the means of two independent experiments.

Condition	POR[a]	(*E*)-2,3 C20:1-CoA Synthesis	Saturated Acyl-CoA Synthesis
	%	*nmol mg^{-1} h^{-1}*	
Control	0	54.9 (100)	ND[b]
+NADH	12.3	49.7 (89.3)	6.0 (10.7)
+NADPH	100	8.2 (10.5)	69.7 (89.5)

[a] POR, Radioactivity recovered in fatty acids after permanganate degradative oxidation. [b] ND, Not determined.

Table II. *Effect of malonyl-CoA upon [1^{14}C]3-OH C20:0-CoA dehydration and elongation*

The assays were carried out in the presence of 9 μM malonyl-CoA in the same conditions as in Table I. Fatty acids were analyzed by radio-GLC and reversed TLC as reported in "Materials and Methods."

Condition	(*E*)-2,3 C20:1-CoA Synthesis	Saturated Acyl-CoAs			
		Synthesis	Label distribution		
			C20:0	C22:0	C24:0
	nmol mg^{-1} h^{-1}			%	
Control	57.0 (100)	ND[a]	ND	ND	ND
+NADH	61.4 (88.3)	8.1 (11.7)	100	ND	ND
+NADPH	9.2 (10.2)	81.4 (89.8)	73	22	5

[a] ND, Not determined.

These results show that (a) in the presence of NADPH the (*E*)-2,3 C20:1-CoA resulting from the dehydration of 3-OH C20:0-CoA was reduced to C20:0-CoA; (b) (*E*)-2,3 ECR activity can be measured by quantification of the label recovered in C20:0-CoA; and (c) (*E*)-2,3 ECR has a marked preference for NADPH.

Finally, we assayed fatty acid elongation using as a primer either [1-^{14}C]3-OH C20:0-CoA or (*E*)-2,3 enoyl-C20:1-CoA in the presence of NADPH or NADH with unlabeled or [2-^{14}C]malonyl-CoA, respectively. Results with [1-^{14}C]3-OH C20:0-CoA as a primer are shown in Table II. In the absence of reductant (control), the primer substrate was only dehydrated, resulting in its conversion to (*E*)-2,3 C20:1-CoA, but was further metabolized to saturated acyl-CoAs. When NADPH was present in the incubation medium [1-^{14}C]3-OH C20:0-CoA was converted to (*E*)-2,3 C20:1-CoA and to C20:0-CoA, which subsequently underwent two rounds of additional elongation to form C22:0 and C24:0. Under these conditions approximately 48% of the initial substrate was used, and analysis of the labeled products showed that the presence of C20:0, C22:0, and C24:0 fatty acids represented 73%, 22%, and 5%, respectively, of the total label recovered in the saturated fatty acid fraction. In the presence of NADH, both (*E*)-2,3 C20:1-CoA and the saturated fatty acids, mainly C20:0, were detected, indicating that NADH could also be accepted as a reductant. However, it was less efficient than NADPH.

Regardless of the reductant used, the amount of label recovered in C20:0 was higher in the presence of malonyl-CoA than in its absence (Table I), perhaps because the ECR activity was 10-fold higher than the KAS activity. KAS activity had previously been estimated at 0.05 nmol mg^{-1} min^{-1} (Schneider et al., 1993), whereas the ECR rate measured in these experiments was at least 10-fold higher. To test this hypothesis, we studied the reduction and further elongation of (*E*)-2,3 C20:1-CoA by [2-^{14}C]malonyl-CoA using different concentrations of NADH and NADPH (Fig. 4). As expected, in the absence of a reductant, no elongation occurred because the enoyl-CoA substrate could not be reduced or condensed with [2-^{14}C]malonyl-CoA. In the presence of NADPH, radioactivity was incorporated from [2-^{14}C]malonyl-CoA in fatty acids. Radio-GLC analysis showed the presence of C22:0 and C24:0 (lignoceric acid) and that C22:0 was the main product. When NADH was used as the reductant, the rate of [2-^{14}C]malonyl-CoA incorporation into VLCFA was one-third that observed with NADPH, indicating that the preferred reductant of ECR was the latter. At a 100 μM concentration of reductants, the elongase activity was more than 3-fold higher in the presence of NADPH than in the presence of NADH. However, owing to the slopes of the curves, this difference tended to decrease at higher nucleotide concentrations, and at 1 mM concentration, the elongation activity was 2-fold higher in the presence of NADPH than in the presence of NADH.

We have demonstrated the presence of 3-OH acyl-CoA dehydratase and (*E*)-2,3 ECR in microsomes from etiolated leek seedlings. The 3-OH C20:0-CoA dehydratase activity was stimulated by Triton X-100. As has been reported for the rat liver elongation complex (Osei et al., 1989), these results suggest that access of 3-OH C20:0-CoA to the enzyme was facilitated by Triton X-100, perhaps due to the embedding of the dehydratase in the membrane. Under these conditions about 30% of the initial substrate was converted into the (*E*)-2,3 C20:1-CoA in 20 min, reflecting a specific activity of approximately 1 nmol min^{-1} mg^{-1}.

The dehydration reaction is stereospecific and prefers the (*R*)-3-OH C20:0-CoA isomer rather than the (*S*) isomer.

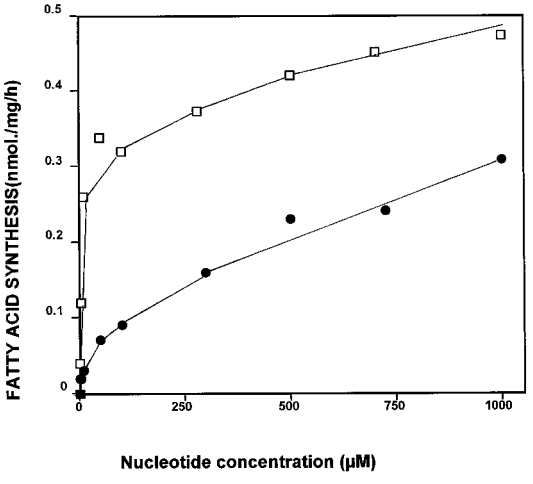

Figure 4. Effect of NADH and NADPH concentration upon (*E*)-2,3 eicosenoyl-CoA elongation. The 9 μM (*E*)-2,3 C20:1-CoA was incubated at 30°C for 60 min in the presence of 60 μg of protein and of NADPH (□) or NADH (●) at different concentrations: 1 mM $MgCl_2$, 2 mM DTT, 0.15 mM Triton X-100, and 9 μM [2-^{14}C]malonyl-CoA in 0.08 M Hepes buffer, pH 6.8. The reaction was stopped after different incubation times as indicated in "Materials and Methods."

When VLCFA synthesis was measured using (*S*)- or (*R*)-3-OH C20:0-CoA as precursors, the same preference for the (*R*) isomer was observed, suggesting that the dehydration step was responsible for the stereospecificity of acyl-CoA elongation. Moreover, by using [1-^{14}C]3-OH C20:0-CoA as a substrate, we have shown that (*E*)-2,3 ECR preferred NADPH as a reductant cofactor.

This study also demonstrates that in etiolated leek microsomes, (*E*)-2,3 C20:1-CoA resulting from the dehydration of 3-OH C20:0-CoA could, in the presence of NADPH, be further reduced to C20:0-CoA, and subsequently elongated to C22:0 and C24:0 fatty acids. Under these conditions the level of VLCFA synthesized from [1-^{14}C]3-OH C20:0-CoA in the presence of malonyl-CoA and NADPH is the highest yet reported for a plant system.

In conclusion, we have developed a specific isotopic assay for measuring the 3-hydroxy dehydratase and ECRs involved in VLCFA biosynthesis. This tool should prove very helpful in studying the structure of the acyl-CoA elongase complex.

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