BIOSYNTHESIS OF α-LINOLENIC ACID BY DISRUPTED SPINACH CHLOROPLASTS[1]

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Received February 9, 1973

SUMMARY

A disrupted spinach chloroplast preparation readily synthesized [^{14}C]α-linolenate from [2-^{14}C]acetate under anaerobic conditions. It can be shown by degradation data that [^{14}C]oleate is not a precursor of [^{14}C]linolenate and that cis 7,10,13-hexadecatrienoic acid is the probable immediate precursor of the [^{14}C]linolenate.

INTRODUCTION

For some years, evidence has been submitted suggesting a sequential desaturation of stearic acid (as an activated derivative) to oleic, linoleic and finally to α-linolenic acid (1). While a number of enzymes have been described which convert stearyl CoA to oleyl CoA (2-4) and in turn oleyl CoA to linoleyl CoA (5-7), the direct demonstration of the formation of α-linolenic acid from linoleic acid by an enzyme preparation has not been made.

Recently, it was observed that intact chloroplasts from immature spinach leaves synthesized oleic, linoleic and linolenic acids from [^{14}C]acetate (8). In addition, cyanide inhibited the formation of oleic and linoleic acids without affecting α-linolenic acid synthesis. Disrupted chloroplasts formed only palmitic and stearic acids under similar conditions. In this communication, we shall report on a modified disrupted chloroplast system that readily forms ^{14}C-α-linolenic acid from [2-^{14}C]acetate. Evi-

[1]This work was supported by NSF Grant GB-19733X and NIH Grant GM-19213-01.

dence would suggest that the precursor of α-linolenic acid is cis 7,10,13-hexadecatrienoic acid and not oleic acid or linoleic acid.

MATERIALS AND METHODS

Intact chloroplasts were isolated from spinach purchased at the local market. The isolation medium consisted of 600 mM sorbitol, 60 mM $NaHCO_3$, 10 mM K_2HPO_4, 1 mM $MgCl_2$, 0.1% Na ascorbate, and 100 mM N-tris (Hydroxymethyl) methyl glycine and was adjusted to pH 7.9 with NaOH at room temperature. Mature spinach leaves were chopped in a blender with an equal volume of isolation medium. The mixture was filtered through miracloth and the resultant filtrate centrifuged for 45 sec at 3600g. The pelleted chloroplasts were brought to 1 mg/ml of chlorophyll with an appropriate volume of isolation medium. At this stage there were about 70-80% intact chloroplasts as determined by phase contrast microscopy. The chloroplast suspension was then disrupted in a French pressure cell at 15,000 psi to yield a pressate of stroma mixed with small fragments of lamellae. All the above procedures were conducted at 0°.

The reaction mixture for acetate incorporation consisted of 0.6 ml pressate (600 µg chlorophyll) and in µmoles: NADP, 0.5; NADH, 0.5; glucose-6-phosphate, 4.0; ATP, 2.0; dithiothreitol, 1.0; coenzyme A, 0.2; 0.15 units glucose-6-phosphate dehydrogenase and 0.5 mg Escherichia coli ACP; the final volume was 0.8 ml. The amount of [2-^{14}C]acetate in the reaction mixture was 1 µc (22.4 µc per µmole). The reactions were run in the dark at 13° for 1 hr and stopped by addition of 0.2 ml 10 N H_2SO_4.

Lipid extraction, methyl transesterification, gas-liquid and thin layer chromatography and scintillation counting were conducted as previously described (8). Chemical α-oxidation of linolenic acid (first converted to stearic acid by PtO_2/H_2 reduction) was carried out according to the procedure of Harris et al. (9) and reductive ozonolysis by the method of Stein and Nicolaidis (10).

RESULTS AND DISCUSSION

While the most prevalent fatty acid in spinach chloroplasts is α-linolenic acid, its synthesis and that of other polyunsaturated fatty acids by disrupted, fragmented chloroplast preparations has not been previously demonstrated. However, it has been shown that intact chloroplasts from immature spinach leaves did synthesize α-linolenic acid (8). Under the appropriate isolating conditions described above, it was possible to observe the synthesis of unsaturated fatty acids by pressates from mature spinach chloroplasts. A typical pattern of fatty acids synthesized from [2-^{14}C]acetate by the pressate is depicted in Figure 1. With different pressates, the percentage of labeled α-linolenic acid varied from a trace to 30 percent and appeared to depend upon the length of time the pressate was stored at 0°. Longer storage favored greater 18:3 formation, although there was a decrease in total [2-^{14}C]acetate incorporation. In addition, high concentrations of protein were important for high percentage yields of labeled unsaturated fatty acids. To date we have never been able to detect linolenic acid synthesis by stromal fractions alone, i.e. when pressates were centrifuged for 90 min at 100,000 x g the supernatant synthesized all the fatty acids observed in Figure 1 except linolenic acid.

The following evidence elucidates the structure and origin of α-linolenic acid formed by the chloroplast pressate.

(1) As illustrated in Figure 1, [^{14}C]methyl α-linolenate co-chromatographs with authentic methyl α-linolenate on a 10 foot HI-EFF-2BP column.

(2) The [^{14}C]product co-chromatographed on 7% silver nitrate impregnated silica G TLC plates with authenic methyl α-linolenate. Therefore, the [^{14}C]product contained three double bonds.

(3) The TLC purified [^{14}C]product again co-chromatographed with methyl α-linolenate on a HI-EFF-2BP column.

(4) After catalytic reduction with PtO_2/H_2, the [^{14}C]product co-chromatographed with methyl stearate. Thus the [^{14}C]product had a C_{18} chain length.

(5) Reductive ozonolysis of the [^{14}C]product gave a single [^{14}C]peak coincidental with methyl azelate semialdehyde, thus establishing that the first double bond in relation to the carboxylate carbon was in the 9,10 position. Furthermore, over 90% of the total [^{14}C] was associated with the methyl azelate semialdehyde peak. The short chain aldehydes and malonic dialdehyde had little if any [^{14}C] incorporation. Since this low recovery might have been related to the volatility of the products, further degrada-

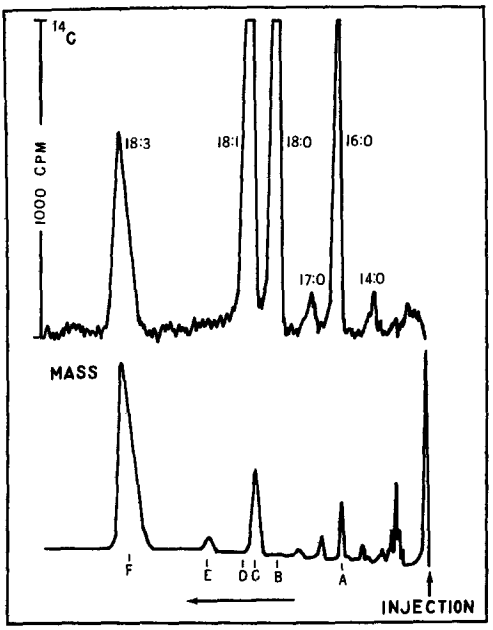

Figure 1. Gas liquid chromatography pattern of methyl esters of fatty acids synthesized by spinach chloroplast pressates from [2-^{14}C]-acetate. Chromatography was performed on a 10 ft chromosorb 15% HI-EFF-2BP column at 180°. (A) Palmitate, (B) Stearate, (C) Hexadecatrienoate, (D) Oleate, (E) Linoleate, (F) Linolenate.

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tion was carried out by chemical α-oxidation of the fully reduced [^{14}C]product, namely [^{14}C]stearate.

(6) As summarized in Table I, chemical α-oxidation of the [^{14}]stearate

Table I. Localization of [^{14}C]label in the carbon chain of linolenate and oleate synthesized from [2-^{14}C]acetate by chloroplast pressates. The respective acids were purified by $AgNO_3$-TLC and reduced to stearate with PtO_2/H_2 and subjected to chemical α-oxidation with $KMnO_4$ (9).

		Products of chemical α-oxidation Specific activity (DPM/μg)			
[^{14}C]Product	Experiment	18:0	17:0	16:0	15:0
Linolenate	1.	1.58	1.54	0.12	n.d.*
	2.	4.04	3.90	0.27	n.d.
Oleate	1.	2.08	1.99	1.67	1.71

*n.d. - not detected.

revealed that the [^{14}C] derived originally from the substrate, [2-^{14}C]acetate, was essentially located on the C_{17} of [^{14}C]stearate. In sharp contrast, the [^{14}C]stearate which was obtained by catalytic reduction from [^{14}C]oleate synthesized by the chloroplast pressate had its [^{14}C]label distributed along the C_{18} hydrocarbon chain. Reductive ozonolysis of [^{14}C]oleate obtained from [2-^{14}C]acetate by the pressate system had 55% of its [^{14}C] in n-nonanal and 45% in the methyl azelatesemialdehyde peak.

Clearly, then, the chloroplast pressate had synthesized α-linolenic acid by a chain elongation of one C_2 unit to a pre-existing precursor, presumably cis 7,10,13-hexadecatrienoic acid, an acid present in spinach chloroplasts.

To analyze this possibility further, advantage was taken of the effects of anaerobic conditions (N_2 phase), CN anion, FMN, and FAD on the biosynthesis of oleic acid by chloroplasts (8,11,12). Table II clearly shows that under

Table II. Pattern of incorporation of radioactivity into long chain fatty acids from [^{14}C]acetate by pressates of chloroplasts under different gas phases.

Incubating condition	Total DPM in lipids	Percent Radioactivity 16:0	18:0	18:1	18:3
air	22,170	29.5	26.8	13.0	30.7
O_2	22,380	30.5	24.4	24.3	20.9
N_2	17,760	35.3	35.3	0	29.4

Table III. Effect of inhibitors on the pattern of incorporation of radioactivity into long chain fatty acids from [^{14}C]acetate by pressates of chloroplasts.

Incubating Condition	Total DPM in lipid	Percent Radioactivity 16:0	17:0	18:0	18:1	18:3
control	522,200	18.5	4.8	46.8	26.3	2.9
0.1 mM KCN	595,500	12.8	5.7	75.1	4.3	1.4
0.1 mM FMN	529,100	16.8	4.0	74.2	0.4	4.0
0.1 mM FAD	512,100	16.7	3.3	76.0	0.4	2.5

aerobic conditons, [^{14}C]acetate was readily incorporated into 18:1(9) and 18:3 (9,12,15), while under anaerobic conditions, the synthesis of [^{14}C]oleate ceased but the synthesis of [^{14}C]18:3(9,12,15) was not inhibited. Table III summarizes the data on the effect of CN^-, FMN, and FAD on the synthesis of unsaturated fatty acids. Again, whereas these three compounds markedly inhibited oleate biosynthesis, no decrease in linolenate synthesis occurred. Finally it should be pointed out that in Figure 1, no formation of linoleic

acid, a possible precursor of linolenic acid, was observed.

In conclusion, the chloroplast pressate contains all the enzymes required for the flow of [^{14}C]acetate into long chain acids including the biosynthesis of oleate and linolenate. Since the formation of oleate can be inhibited under conditions where no linolenate inhibition was observed, since the degradation data fully support the inhibition studies by revealing a striking difference in the distribution of [^{14}C] in oleate and in linolenate, and since there was no indication of linoleic acid synthesis by the pressate system, we conclude that α-linolenate is formed by the disrupted chloroplast system by the elongation of endogeneous cis 7,10,13-hexadecatrienoic acid which remains unlabeled under these conditions. Palmitate cannot be considered as the C_{16} precursor since under anaerobic conditions, double bond systems cannot be introduced into the hydrocarbon chain (12). Whether or not this sequence of biosynthesis of linolenate occurs in the plant cell remains for further investigations to determine. These data do explain, however, the observations made earlier by Kannangara and Stumpf (8) with intact chloroplasts.

ACKNOWLEDGMENTS

The authors wish to acknowledge the helpful discussion of Dr. Jan Jaworski and technical assistance of Mrs. Barbara Clover.

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