Plant Physiol. (1993) 102: 1275–1278

An in Vivo Study of Substrate Specificities of Acyl-Lipid Desaturases and Acyltransferases in Lipid Synthesis in *Synechocystis* PCC6803[1]

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The cyanobacterium *Synechocystis* PCC6803 was fed heptanoic acid to study the substrate specificities of desaturases and acyltransferases in lipid synthesis. This aliphatic acid was elongated to C15, C17, and C19 fatty acids, which were incorporated into polar glycerolipids and desaturated. The double bonds were located at the Δ6, Δ9, Δ12, and ω3 positions of the fatty acids. This suggests that the Δ9 desaturase counts the carbon number from the carboxy terminus, whereas the so-called Δ15 desaturase counts from the methyl terminus. The counting mechanisms of the Δ6 and Δ12 desaturases are not fully understood. In the distribution of fatty acids at the *sn* positions of the glycerol moiety, the C17, C18, and C19 fatty acids were located at the *sn*-1 position, whereas the C15 and C16 fatty acids were located at the *sn*-2 position. This suggests that glycerol-3-phosphate acyltransferase specifically transfers heptadecanoic, octadecanoic, and nonadecanoic acids, whereas 1-acylglycerol-3-phosphate acyltransferase specifically transfers pentadecanoic and hexadecanoic acids.

Polar glycerolipids are characterized by two fatty acids at the *sn*-1 and *sn*-2 positions and by the head group at the *sn*-3 position of the glycerol moiety. Fatty acids are specified by the number of carbon atoms and by the positions of double bonds (or unsaturated bonds). The numbers before and after the colons indicate the numbers of the carbon atoms and the number of double bonds, respectively, and the numbers within parentheses indicate the Δ positions of the double bonds of the *cis* configuration counted from the carboxy terminus.

The major lipid constituents in cyanobacteria are monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and phosphatidylglycerol as in eukaryotic chloroplasts (Murata and Nishida, 1987). Cyanobacteria are classified into four groups according to their mode of fatty acid transfer and desaturation (Murata et al., 1992). Group 1 is characterized by the presence of only saturated and monounsaturated fatty acids, whereas groups 2, 3, and 4 also contain polyunsaturated fatty acids. The latter groups are also unique in their specific positional distribution because the C18 and C16 fatty acids are esterified to the *sn*-1 and *sn*-2 positions of the glycerol moiety, respectively.

Group 4, to which *Synechocystis* PCC6803 belongs, is characterized by desaturation that takes place at the Δ6, Δ9, Δ12, and Δ15 positions of the C18 fatty acids at the *sn*-1 position and by no desaturation of the C16 fatty acids at the *sn*-2 position (Wada and Murata, 1989; Murata et al., 1992). Because individual desaturases are responsible for the introduction of double bonds at specific fatty acid positions, it appears that there are four desaturases in *Synechocystis* PCC6803 that introduce double bonds at the Δ6, Δ9, Δ12, and Δ15 positions of the C18 fatty acids. The gene for the desaturase that is specific for the Δ12 position has previously been cloned (Wada et al., 1990).

Fatty acid synthesis in cyanobacteria is characterized by acyl-lipid desaturation in each desaturation reaction (Sato and Murata, 1982; Sato et al., 1986). By contrast, the first double bond in higher plants is introduced in the acyl-ACP form, and further desaturation takes place in the acyl-lipid form (Jaworski, 1987; Harwood, 1988). In both cases, however, the double bonds are located at exact positions. For example, in C18 fatty acids, the first double bond is introduced exclusively at the Δ9 position, and the others are introduced at the Δ12 and the Δ15 positions. Furthermore, in the cyanobacterial strains in groups 3 and 4, a double bond is introduced at the Δ6 position (Murata et al., 1992). This suggests that the desaturases can count the exact number of carbon atoms in the hydrocarbon chain and can introduce the double bond at a specific position.

Another interesting characteristic of lipid synthesis in cyanobacteria is the specific distribution of fatty acids at the *sn* positions of the glycerol moiety of glycerolipids. The C18 and C16 fatty acids are esterified at the *sn*-1 and the *sn*-2 positions in the cyanobacterial strains in groups 2, 3, and 4 (Murata et al., 1992). This suggests that the acyl-ACP:glycerol-3-P acyltransferase uses 18:0-ACP, specifically, as the substrate and that acyl-ACP:1-acylglycerol-3-P acyltransferase is specific to 16:0-ACP.

Previous studies have not explained the means by which individual desaturases are able to count carbon numbers for the introduction of double bonds at exact positions in the fatty acids or the precise means by which the acyltransferases for the *sn*-1 and *sn*-2 positions can discriminate the C18 and C16 fatty acids. To answer these questions, we fed heptanoic acid (C7:0) to *Synechocystis* PCC6803, a technique similar to that described by Howling et al. (1968), who used *Chlorella*

[1] This research was supported, in part, by Grants-in-Aid for Scientific Research on Priority Areas (04273102 and 04273103) from the Ministry of Education, Science, and Culture, Japan.

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Abbreviation: ACP, acyl-carrier protein.

vulgaris. Our results suggest that the Δ9 desaturase counts the carbon number from the carboxy terminus, whereas the so-called Δ15 desaturase counts the carbon number from the methyl terminus.

MATERIALS AND METHODS

Organism and Culture Conditions

Synechocystis PCC6803 was provided by Dr. T. Omata (Nagoya University), who had obtained it from the Pasteur Culture Collection. The cells were grown at 22°C under illumination of incandescent lamps at an intensity of 70 μE m^{-2} s^{-1}, with aeration of 1% CO_2 in air, as described previously (Ono and Murata, 1981). The culture medium was BG-11 (Stanier et al., 1971) supplemented with 20 mM Hepes-NaOH (pH 7.5) in the presence or absence of heptanoic acid or hexanoic acid at 20 nM. Cells at the exponential growth phase were collected by centrifugation at 2500*g* for 10 min at 4°C.

Extraction of Lipids and Fatty Acid Analysis

Lipids were extracted from the collected cells according to the method of Bligh and Dyer (1959). Analysis of the lipids was carried out according to the method of Sato and Murata (1988). The total lipids and the lipid classes, which were separated on precoated TLC plates (Merck 5721) developed with $CHCl_3$:CH_3OH:28% NH_4OH (65:35:5, v/v), were subjected to methanolysis with 5% HCl in methanol at 85°C for 2.5 h. The resultant methyl esters were analyzed with a gas-liquid chromatograph (GC-7A; Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionization detector. Fatty acid methyl esters were separated on a capillary column (50 m × 0.25 mm i.d., CPS-1; Quadrex Corp., New Haven, CT) coated with cyanopropylmethyl silicone at a thickness of 0.25 μm. Both the column and the flame ionization detector were maintained at 185°C. The relative amounts of fatty acid methyl esters were determined by comparing the areas under the peaks on the chromatogram using a data processor (C-R2AX; Shimadzu). The fatty acid methyl esters were identified by a gas chromatograph-mass spectrometer (JMS-DX-300; JEOL, Tokyo, Japan) equipped with a mass data analysis system (BJMS-3100; JEOL) using the same column as was used for the gas-liquid chromatography.

The double bond positions in the fatty acids were determined by GC-MS using the pyrrolidine method described by Andersson et al. (1975). A capillary column (30 m × 0.254 mm i.d., DB-1; J & W Scientific, Folsom, CA) was used and maintained at 260°C. The distribution of fatty acids at the *sn* position of the glycerol moiety of lipids was analyzed by selective hydrolysis at the *sn*-1 position with *Rhizopus delemar* lipase (Fischer et al., 1973).

RESULTS AND DISCUSSION

We first fed 20 nM propionic acid (C3:0) to *Synechocystis* PCC6803. This fatty acid suppressed growth and was not efficiently incorporated into glycerolipids. Then, 20 nM heptanoic acid (C7:0) was used as a precursor of the fatty acids having odd carbon numbers. This acid did not disturb the growth of the cyanobacterium, nor did a supplement of hexanoic acid (C6:0, 20 nM). The composition of lipid classes was not affected by supplementation of the culture medium with C6:0 and C7:0.

Table I shows the effect of feeding C7:0 to *Synechocystis* PCC6803 on the fatty acid composition of glycerolipids. When the cells were grown at 22°C in the inorganic medium BG-11, the major fatty acids were 16:0, 16:1, 18:0, 18:1(9), 18:2(9, 12), 18:2(6, 9), 18:3(6, 9, 12), 18:3(9, 12, 15), and 18:4(6, 9, 12, 15). Supplementation of an even-numbered aliphatic acid, C6:0, did not alter the fatty acid composition. When an odd-numbered aliphatic acid, C7:0, was supplemented, the following new fatty acids also appeared: 15:0, 15:1(9), 17:0, 17:1(9), 17:2(9, 12), 17:2(6, 9), 17:3(6, 9, 12), 17:3(9, 12, 14), 17:4(6, 9, 12, 14), 19:0, 19:1(9), 19:2(9, 12), 19:2(6, 9), 19:3(6, 9, 12), 19:3(9, 12, 16), and 19:4(6, 9, 12, 16). The three double bonds were observed at the same Δ positions, i.e. at the Δ6, Δ9, and Δ12 positions in the C15, C16, C17, C18, and C19 fatty acids. In contrast, the fourth double bond appeared at the Δ14, Δ15, and Δ16 positions,

Table I. *The effect of supplementation with hexanoic acid (C6:0) and heptanoic acid (C7:0) in the culture medium on the composition of fatty acids of total lipids in Synechocystis PCC6803*

The concentration of C6:0 and C7:0 in the culture medium was 20 nM. The values for the percentage of fatty acids are the averages of three independent experiments. t, Trace amount, <0.5%.

Fatty Acid	Mol% of Total Fatty Acids		
	None	C6:0 added	C7:0 added
14:0	t	1	t
15:0	0	0	5 (±1)
15:1 (9)	0	0	t
16:0	54 (±3)	54 (±2)	47 (±1)
16:1 (9)	4 (±1)	4 (±1)	3 (±1)
17:0	0	0	5 (±1)
17:1 (9)	0	0	10 (±2)
17:2 (6, 9)	0	0	t
17:2 (9, 12)	0	0	5 (±1)
17:3 (6, 9, 12)	0	0	3 (±0)
17:3 (9, 12, 14)	0	0	1 (±1)
17:4 (6, 9, 12, 14)	0	0	t
18:0	1 (±0)	t	1 (±0)
18:1 (9)	2 (±0)	2 (±1)	1 (±0)
18:2 (6, 9)	t	t	t
18:2 (9, 12)	6 (±1)	6 (±1)	3 (±1)
18:3 (6, 9, 12)	20 (±2)	21 (±1)	5 (±1)
18:3 (9, 12, 15)	6 (±1)	5 (±2)	2 (±1)
18:4 (6, 9, 12, 15)	7 (±1)	7 (±1)	3 (±0)
19:0	0	0	1
19:1 (9)	0	0	1
19:2 (6, 9)	0	0	t
19:2 (9, 12)	0	0	2
19:3 (6, 9, 12)	0	0	1
19:3 (9, 12, 16)	0	0	1
19:4 (6, 9, 12, 16)	0	0	t

Table II. *The distribution of fatty acids at the sn positions of monogalactosyl diacylglycerol from Synechocystis PCC6803 grown in the presence of 20 nM heptanoic acid (C7:0)*

Fatty Acid	Mol% of Total Fatty Acids		
	sn-1	*sn*-2	Total
14:0	0	t[a]	t
15:0	0	5	6
15:1 (9)	0	t	t
16:0	3	43	46
16:1 (9)	3	t	3
17:0	2	1	3
17:1 (9)	11	t	11
17:2 (6, 9)	t	0	t
17:2 (9, 12)	6	0	6
17:3 (6, 9, 12)	4	0	4
17:3 (9, 12, 14)	t	0	t
17:4 (6, 9, 12, 14)	t	0	t
18:0	t	0	t
18:1 (9)	t	0	t
18:2 (6, 9)	t	0	t
18:2 (9, 12)	2	0	2
18:3 (6, 9, 12)	7	0	7
18:3 (9, 12, 15)	t	0	t
18:4 (6, 9, 12, 15)	4	0	4
19:0	1	0	1
19:1 (9)	1	0	1
19:2 (6, 9)	1	0	1
19:2 (9, 12)	2	0	2
19:3 (6, 9, 12)	1	0	1
19:3 (9, 12, 16)	1	0	1
19:4 (6, 9, 12, 16)	t	0	t

[a] t, Trace amount, <0.5%.

in the C17, C18, and C19 fatty acids, respectively. This indicates that the last double bond is located at the ω3 position.

These observations suggest that the Δ9 desaturase counts the carbon number from the carboxy terminus and that the so-called Δ15 desaturase counts the carbon number from the methyl terminus. Therefore, we refer to the latter desaturase as "ω3 desaturase." It is clear that the Δ6 and the Δ12 desaturases do not count the carbon number from the methyl terminus. Therefore, the Δ12 desaturase should be called neither ω6 desaturase nor n-6 desaturase. However, it is still unclear whether the Δ6 and the Δ12 desaturases can count the carbon number from the carboxy terminus or from the double bond at the Δ9 position.

Howling et al. (1968) fed radiolabeled C15:0, C17:0, and C19:0 fatty acids to *C. vulgaris* and observed that double bonds were introduced at the Δ9 and Δ12 positions. Although the introduction of the first double bond at the Δ9 position in *C. vulgaris* may have taken place in 18:0-ACP, it is likely that the stearoyl-ACP desaturase also counts the carbon number from the carboxy terminus. The second double bond is located at the Δ12 position in *C. vulgaris*, as it is in *Synechocystis* PCC6803.

Gurr (1971) fed to *C. vulgaris* radiolabeled C15:1, C16:1, C17:1, C18:1, and C19:1 with double bonds at different positions and observed that the fatty acid with a double bond at either Δ9 or ω9 position was desaturated to yield a methylene-interrupted diunsaturated fatty acid. This result may suggest that the desaturase responsible for the second unsaturation can count the carbon number from the first double bond. However, it is striking that monounsaturated fatty acids were not desaturated in *C. vulgaris* unless the positions of the double bonds were located at either Δ9 or ω9 positions (Gurr, 1971).

The distribution of fatty acids at the *sn* position of the glycerol moiety of lipids from cyanobacterial cells grown in inorganic medium is unique, i.e. the C18 and the C16 fatty acids are esterified to the *sn*-1 and the *sn*-2 positions, respectively (Murata and Nishida, 1987; Wada and Murata, 1989, 1990). Table II presents the distribution of fatty acids in monogalactosyl diacylglycerol when the cyanobacterium was grown in the presence of C7:0. The C17, C18, and C19 fatty acids were esterified at the *sn*-1 position with a minor contribution of C16 acids, whereas the C15 and C16 fatty acids were esterified at the *sn*-2 position with a minor contribution of the C17 acids.

These observations suggest the degree of specificities of the acyltransferases. The acyl-ACP:glycerol-3-P acyltransferase is specific to the C17, C18, and C19 fatty acids, whereas the acyl-ACP:1-acylglycerol-3-P acyltransferase is specific to the C14, C15, and C16 fatty acids. It should be noted that the specificities of acyltransferases toward the chain length of the fatty acids showed some flexibility.

ACKNOWLEDGMENTS

The positions of double bonds in the fatty acid methyl esters were determined by GC-MS at the Center for Analytical Instruments of the National Institute for Basic Biology.

Received February 16, 1993; accepted April 15, 1993.

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