Molecular-biological analysis of fatty acid desaturase in relation to temperature response in cyanobacteria

ラン藻の温度適応に関わる脂肪酸不飽和化酵素の分子生物学的解析

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Abstract

The molecular-biological aspects of fatty acid desaturase in relation to temperature response are studied in the cyanobacterium, Synechocystis PCC6803.

The previous biochemical and molecular biological studies on the fatty acid desaturase in animals, yeast, bacteria, higher plants, and cyanobacteria are reviewed in Chapter 1. The studies on lipids, fatty acids and the physiological roles of unsaturated fatty acids in cyanobacteria are also described in Chapter 1.

The temperature-induced changes in the fatty acid composition of the cyanobacterium, Synechocystis PCC6803, are studied in Chapter 2. The experimental results suggest that this cyanobacterium desaturates the C_{18} acids esterified to the sn-1 position of glycerol moiety to compensate the decrease in membrane fluidity at low temperature.

Two desaturation mutants are isolated from the cyanobacterium, Synechocystis PCC6803, and characterized in Chapter 3. The mutants, designated Fad6 and Fad12, are defective in the desaturation of fatty acids at Δ^6 and Δ^{12} positions of C_{18} acids, respectively.

The gene, which complements Fad12, has been isolated from a genomic library of the cyanobacterium, Synechocystis PCC6803, and analyzed in Chapter 4. The sequence analysis of the gene (designated desA) shows that it includes an open-reading frame of 1053 bp which codes for 351 amino acids and corresponds to a relative molecular mass of 40494 daltons. The results of

transformation of Anacystis nidulans R2-SPc with desA suggest that the gene encodes Δ^{12} desaturase.

The changes in the membrane constituents by mutation of a fatty acid desaturase (Δ^{12} desaturase) and by transformation with desA gene in Synechocystis PCC6803 are studied in Chapter 5. It is shown that the fatty acid desaturase may be located both in thylakoid and the cytoplasmic membranes, and that the fatty acid desaturation takes place also in both types of membranes in Synechocystis PCC6803.

The thermal properties of photosynthetic activities of a desaturation mutant (Fad12) and a transformant of Fad12 of Synechocystis PCC6803 are described in Chapter 6. The experimental results suggest that the desaturation at the Δ^{12} position of fatty acids in glycerolipids does not alter the thermal properties of photosynthetic activities.

Abbreviations

Lipids

DG, diacylglycerol

GlcDG, monoglucosyl diacylglycerol

GalDG, monogalactosyl diacylglycerol

Gal_2DG, digalactosyl diacylglycerol

PA, phosphatidic acid

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PI, phosphatidylinositol

SQDG, sulfoquinovosyl diacylglycerol

Fatty acids

Fatty acids are represented by numbers of carbon atoms and double bonds, before and after a colon, respectively, and the positions of double bonds counted from the carboxy terminus are indicated by numbers in superscript as follows:

14:0, myristic acid

$14:1^{\Delta 9}$, myristoleic acid

16:0, palmitic acid

$16:1^{\Delta 9}$, palmitoleic acid

$16:2^{\Delta 9,12}$, $\Delta^{9,12}$-hexadecadienoic acid

17:0, marganic acid

18:0, stearic acid

$18:1^{\Delta 9}$, oleic acid

$18:1^{\Delta 11}$, cis-vaccenoic acid

$18:2^{\Delta 6,9}$, $\Delta^{6,9}$-octadecadienoic acid

$18:2^{\Delta 9,12}$, linoleic acid

$18:3^{\Delta 9,12,15}$, α-linolenic acid

$18:3^{\Delta 6,9,12}$, γ-linolenic acid

$18:4^{\Delta 6,9,12,15}$, $\Delta^{6,9,12,15}$-octadecatetraenoic acid

Lipid molecular species

Lipid molecular species are expressed by a combination of abbreviated forms of fatty acids, e. g., 18:0/16:0, sn-1-stearoyl-sn-2-palmitoyl species

Others

ACP, acyl carrier protein

CDP, citidine 5'-diphosphate

CoA, coenzyme A

DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea

GLC, gas-liquid chromatography

GC-MS, gas chromatography-mass spectrometry

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC, high performance liquid chromatography

PSI, photosystem I

PSII, photosystem II

TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

TLC, thin-layer chromatography

Chapter 1

General introduction

I. Glycerolipids and fatty acids in cyanobacteria

(1) Glycerolipids

The cyanobacteria contain four major glycerolipids: MGDG, DGDG, SQDG and PG, and a minor component, GlcDG (Murata and Nishida 1987). The molecular structures of these lipids are presented in Fig. 1. The level of MGDG is 50-60% of the total glycerolipids, and DGDG, SQDG and PG each amounts to 10-20%, whereas GlcDG amounts to only about 1% (Sato and Murata 1982a).

The glycerolipid composition of thylakoid, cytoplasmic and outer membranes has been compared in Anacystis nidulans (Omata and Murata 1983). No significant difference among the three types of membranes is observed. However, the glycerolipid content relative to the total dry weight was 19%, 57% and 3% in the thylakoid, cytoplasmic and outer membranes, respectively (Omata and Murata 1983).

(2) Fatty acids

The fatty acids so far known to be present in cyanobacteria are 16:0, $16:1^{\Delta 9}$, $16:2^{\Delta 9,12}$, 18:0, $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$, $18:3^{\Delta 6,9,12}$, $18:3^{\Delta 9,12,15}$ and $18:4^{\Delta 6,9,12,15}$ (Murata and Nishida 1987). Some species, such as A. nidulans, contain $18:1^{\Delta 11}$ (Sato et al. 1979).

Kenyon (1972) and Kenyon et al. (1972) classified the cyanobacteria into four groups with respect to the composition and metabolism of fatty acids. According to this classification, the strains in the first group contain only saturated and

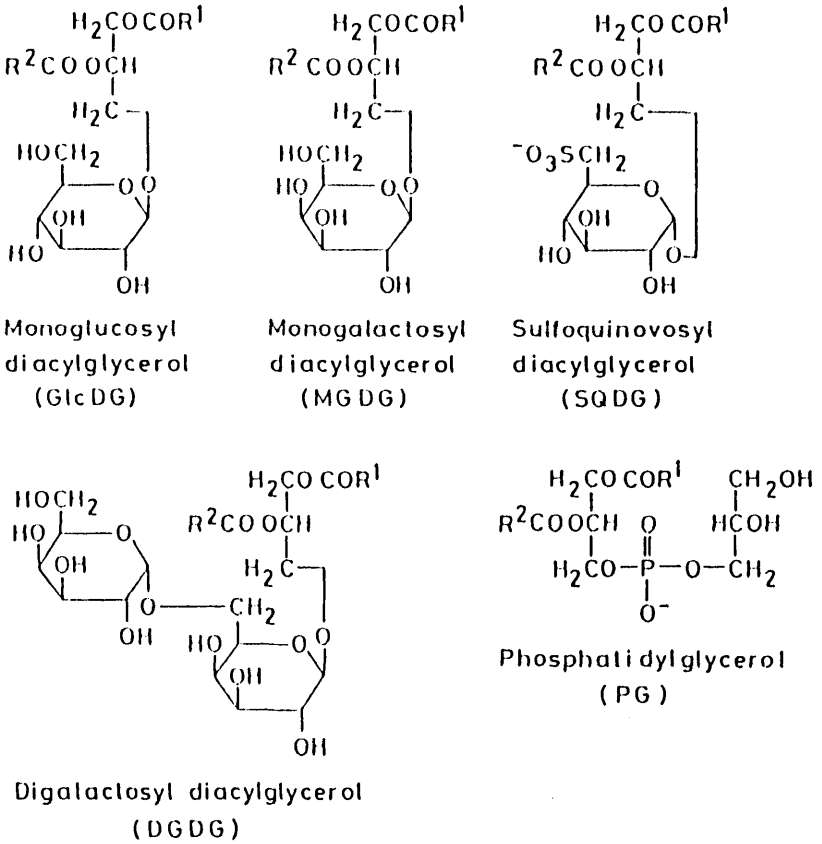


Fig. 1. Glycerolipids of cyanobacteria (blue-green algae).

monounsaturated fatty acids, whereas those in other groups contain, in addition, polyunsaturated fatty acids characteristic of each group. The second group is characterized by the presence of $18:3^{\Delta 9,12,15}$, the third group by $18:3^{\Delta 6,9,12}$, the fourth group by $18:4^{\Delta 6,9,12,15}$. Unicellular strains of cyanobacteria belong to first and third groups (Kenyon 1972). Filamentous cyanobacteria are distributed to all four groups (Kenyon et al. 1972, Oren et al. 1985)

The fatty acid composition of individual lipids from A. nidulans and Anabaena variabilis was studied (Nichols et al. 1965, Sato et al. 1979). The fatty acid composition of MGDG is similar to that of DGDG, and the fatty acid composition of SQDG is similar to that of PG in each alga. In A. nidulans, the ratio of the monounsaturated to saturated acids is higher in MGDG and DGDG than in SQDG and PG (Nichols et al. 1965, Hirayama, 1967, Sato et al. 1979). In A. variabilis, MGDG and DGDG contain greater proportions of polyunsaturated fatty acids than SQDG and PG (Sato et al. 1979). This tendency was also demonstrated in A. cylindrica, Nostoc calcicola, Tolypothrix tenuis (Zepke et al. 1978), Oscillatoria splendida and Spilulina platensis (Nichols and Wood 1968). As will be discussed in Chapters 2 and 3, the fatty acid composition depends markedly on growth temperature.

Positional distribution of fatty acids at the sn-1 and sn-2 positions of glycerol moiety can be determined by selective enzymatic hydrolysis of the ester linkage at the sn-1 position with Rhizopus delemar lipase (Fischer et al. 1973). Two types of the positional distribution are known. In A. nidulans, most of

the monounsaturated fatty acids are esterified at the sn-1 position and most of 16:0 is at the sn-2 position in all lipid classes (Sato et al. 1979). In the filamentous cyanobacteria such as A. variabilis (Sato et al. 1979), A. cylindrica, O. chalybea, N. calcicola, and T. tenuis (Zepke et al. 1978), the C_{18} acids are esterified to the sn-1 position and the C_{16} acids to the sn-2 position in all lipid classes.

Molecular species of glycerolipids are characterized by the combination of the acyl groups at the sn-1 and sn-2 positions, and by the polar substituent group at the sn-3 position of glycerol moiety. The major molecular species in all the lipid classes of A. nidulans are of a sn-1-16:1$^{\Delta 9}$-sn-2-16:0 type, and those in all lipid classes of A. cylindrica, A. variabilis, O. chalybea, N. calcicola and T. tenuis are of a sn-1-C_{18}-sn-2-16:0 type (Sato and Murata 1982b, Zepke et al. 1978).

II. Physiological roles of unsaturated fatty acids in cyanobacteria

A number of organisms can regulate the fatty acid composition of their membrane lipids in response to a change in ambient temperature (Russell 1984, Thompson 1980). Sato and Murata have reported the low temperature-induced desaturation of fatty acids in the cyanobacterium, A. variabilis (Sato and Murata 1980, 1981). When the growth temeprature is shifted from 38^{o}C to 22^{o}C, the rapid and transient introduction of a cis double bond into 16:0 and the slow desaturation of C_{18} acids take place. The

rapid change in levels of unsaturation of the C_{16} acids after the temperature shift does not require de novo synthesis of fatty acids (Sato and Murata 1981). The mass-spectrometric analysis of ^{13}C-enriched MGDG indicates that 16:0 is desaturated in the lipid-bound form and not replaced by previously synthesized $16:1^{\Delta 9}$ (Sato et al. 1986).

Ono and Murata have proposed the mechanism for the chilling injury of A. nidulans based on the results of the phase transition temperature which are determined by various methods and chilling susceptibility of the intact cells (1981a, 1981b, 1982). At chilling temperature, the lipids of the cytoplasmic membranes enter the phase separation state, which induces leakage of electrolytes from the cytoplasm to the outer medium because of increased permeability of the cytoplasmic membranes to small molecules and ions. The loss of electrolytes in the cytoplasm results in irreversible inactivation of photosynthesis. This mechanism is supported by the hydrogenation of plasma membrane (Vigh et al. 1985a).

The effect of unsaturation and chain length of constituent fatty acids on the thermotropic phase behavior of glycerolipids has been intensively studied in artificially synthesized bilayer membranes (Silvius 1982). An increase in unsaturation and decrease in the chain length of esterified fatty acids are known to lower the phase transtion temperature of membrane lipids (Chapman 1975). Thus, the changes in fatty acid composition with growth temperature, as described above, can be regarded as an adaptive response to changes in the ambient

temperature (Ono and Murata 1982, Murata et al. 1984). The roles of the unsaturated fatty acids may be to regulate the membrane fluidity for the proper functioning of biological membranes and to keep the membranes in liquid crystalline state at the environmental temperature.

III. Biosynthesis of glycerolipids in higher plants and cyanobacteria

(1) Higher plants

The fatty acid synthesis in plant cells takes place exclusively in plastids (Stumpf 1980, 1987). The final product of the fatty acid synthesis is palmitoyl-ACP, a part of which is elongated to stearoyl-ACP and subsequently desaturated to oleoyl-ACP (Jaworski 1987, Stumpf 1987). Although stearic acid is desaturated to oleic acid in the ACP-bound form within plastids, all the other desaturation take place in a lipid-bound form (Jaworski 1987, Harwood 1988). The oleic acid is desaturated to linoleic acid and further to linolenic acid in all the lipid classes in both plastids and cytoplasm. The palmitic acid, on the other hand, is desaturated only in plastids in which that bound to the sn-2 position of PG is desaturated to Δ^{3t}-hexadecenoic acid and that bound to the sn-2 position of MGDG to $\Delta^{7,10,13}$-hexadecatrienoic acid.

A scheme for the lipid biosynthesis in higher-plant leaves proposed by Roughan and Slack (1982) is shown in Fig. 2.
The first step of glycerolipid biosynthesis in higher-plant

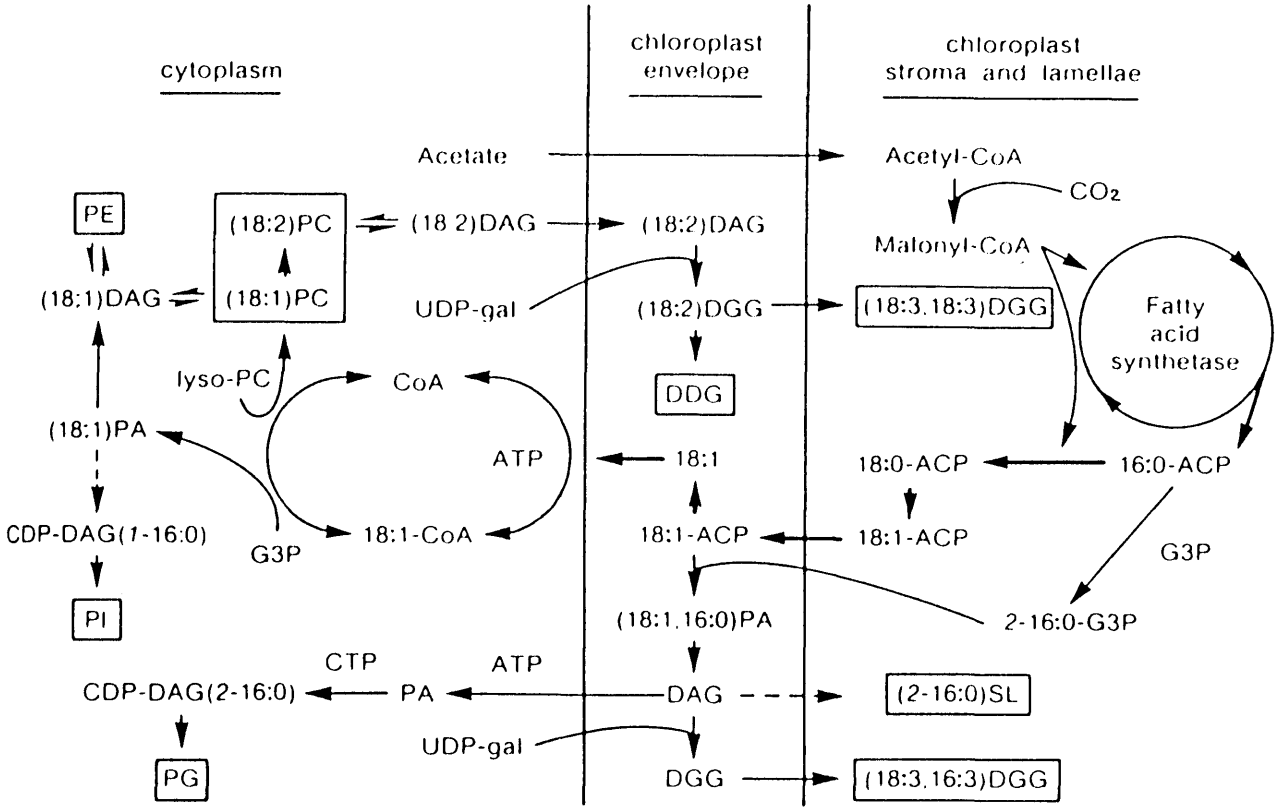


Fig. 2. A scheme for the lipid biosynthesis in higher plants according to Roughan and Slack (1982). G3P, glycerol 3-phosphate; UDP, uridine 5'-diphosphate.

cells is formation of PA by stepwise estrifications of glycerol 3-phosphate with fatty acids. Two pathways are operative in synthesis of PA in plant tissues (Roughan and Slack 1982, 1984), i. e., the cytoplasmic (or eukaryotic) pathway and the plastidial (or prokaryotic) pathway. The former activity is associated mainly with endoplasmic reticulum (Moore 1984, Sauer and Robinson 1985), and the latter activity is located on the plastid envelopes (Andrews et al. 1985, Block et al. 1983, Dorne et al. 1982).

In the cytoplasmic pathway, fatty acids bound to ACP, which are synthesized in plastids, are released from ACP in stroma. The free fatty acids are converted to acyl-CoA on the outer envelope membrane and exported to cytoplasm (Joyard and Douce 1987). Then, they are transferred into glycerol 3-phosphate and lysophosphatidic acid to yeild PA in the endoplasmic reticulum (Roughan and Slack 1982). In this pathway, either oleic acid, palmitic acid or, in some cases, stearic acid is esterified at the sn-1 position of PA and only oleic acid is esterified to sn-2 position of PA (Roughan 1987). The PA is converted into metabolic intermediates, DG and CDP-DG. PC and PE are synthesized from the DG, and PI and PG are synthesized from the CDP-DG (Moore 1982, 1984, 1987). The oleic acid esterified to sn-1 and 2 positions of PC, but not palmitic acid or stearic acid, is desaturated to linoleic acid and further to linolenic acid, then the phosphocholine group is removed from PC (Slack et al. 1977, Williams et al. 1976). The resultant DG is transported back to the plastid envelope and used for synthesis of glycolipids.

In the plastidial pathway, the fatty acids bound to ACP are

transferred to glycerol 3-phosphate and lysophosphatidic acid to yield PA on plastid envelope. In this pathway, either oleic acid, palmitic acid or, in some cases, stearic acid is esterified at the sn-1 position of PA same as the cytoplasmic pathway. However, at the sn-2 position of PA, only palmitic acid is esterified in the plastidial pathway (Roughan 1987). The resultant PA is used for synthesis of MGDG, DGDG, SQDG and PG (Andrews and Mudd 1985, Joyard et al. 1986, Kleppinger-Sparace and Mudd 1987, Roughan et al. 1980, Sparace and Mudd 1982). The fatty acids bound to these lipids are desaturated on the envelope (Joyard et al. 1980, Siebertz and Heinz 1977, Siebertz et al. 1980).

The two pathways for synthesis of PA are clearly distinguished by their specificity of fatty acid esterification at the sn-2 position of the glycerol moiety (Roughan 1987). The relative contribution of the cytoplasmic and plastidial pathways in the biosynthesis of glycerolipids depends on the plant species (Heinz and Roughan 1983, Roughan and Slack 1984). It is possible to estimate the relative contribution of each pathway by the proportions of C_{18} and C_{16} fatty acids at the sn-2 position of the glycerol moiety.

(2) Cyanobacteria

Nichols et al. (1965) first studied the lipid metabolism in cyanobacteria, A. nidulans and A. variabilis. They found that the label was incorporated into MGDG, DGDG, SQDG and PG, when the cells were incubated with [^{14}C]acetate. They also found that exogenously added radioactive 16:0, 18:0 and 18:1$^{\Delta 9}$ were

desaturated to $16:1^{\Delta 9}$, $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ respectively, in A. variabilis. Feige et al. (1980) conducted $H^{14}CO_3$ pulse-labeling experiments in 30 species of cyanobacteria, and observed that ^{14}C is first incorporated into GlcDG, then radioactivity appears in MGDG. They proposed that GlcDG is a precursor of MGDG in the biosynthesis of glycolipids in the cyanobacteria. Sato and Murata (1982a) showed by pulse-labeling experiments that in A. variabilis the conversion from GlcDG to MGDG results from epimerization of glucose to galactose but not by replacement of glucose by galactose. They further demonstrated that DGDG is produced by transfer of galactose from unidentified galactose carrier to MGDG (Sato and Murata 1982a). SQDG and PG are also rapidly lebeled, suggesting that these lipids are directly synthesized, but not via GlcDG (Sato and Murata 1982a).

Sato and Murata (1982c) also observed in A. variabilis a membrane-associated activity of uridine 5'-diphosphate-glucose:diacylglycerol glucosyltransferase which synthesizes GlcDG by transfer of glucose from uridine 5'-diphosphate-glucose to diacylglycerol. The GlcDG synthesis activity seems to be located in both the thylakoid and plasma membranes of A. nidulans (Omata and Murata 1986).

The activities of glycerol 3-phosphate acyltransferase and 1-acylglycerol 3-phosphate acyltransferase, the enzymes involved in the first steps of glycerolipid synthesis, have been characterized in crude extracts of A. variabilis (Lem and Stumpf 1984b). These enzymes from A. variabilis use acyl-ACP, but not acyl-CoA as the substrate. These observations indicate that the

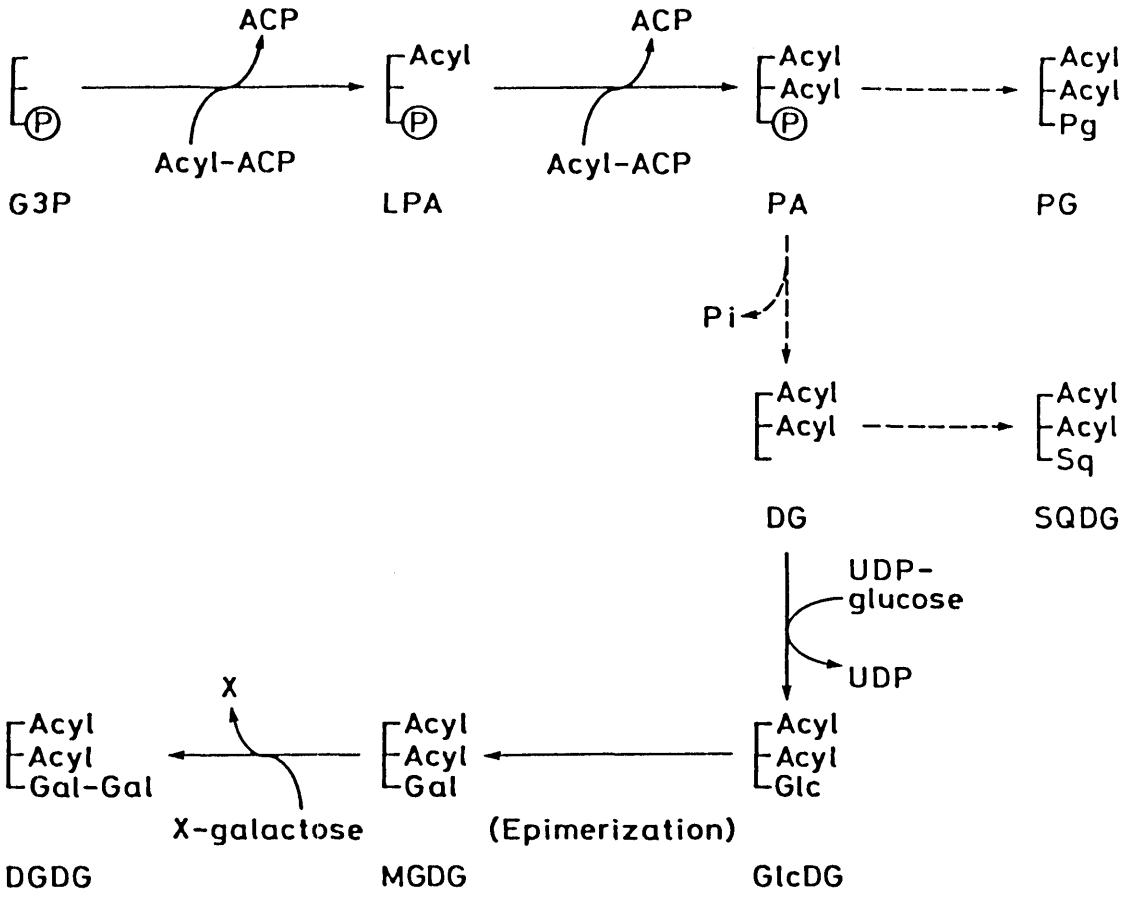


Fig. 3. A scheme for glycerolipid biosynthesis in cyanobacteria. The reactions indicated by broken arrows have not yet been experimentally demonstrated. G3P, glycerol 3-phosphate; LPA, 1-acyl-glycerol 3-phosphate or lysophosphatidic acid, Pg, phosphoglycerol; Sq, sulfoquinovose; Glc, glucose; Gal, galactose; X, unidentified galactose carrier.

precursor of the glycerolipids is PA, which is converted to glyco-, sulfo-, and phospholipids in this cyanobacterium.

A tentative scheme for glycerolipid synthesis in cyanobacteria, based on limited information, is proposed in Fig. 3.

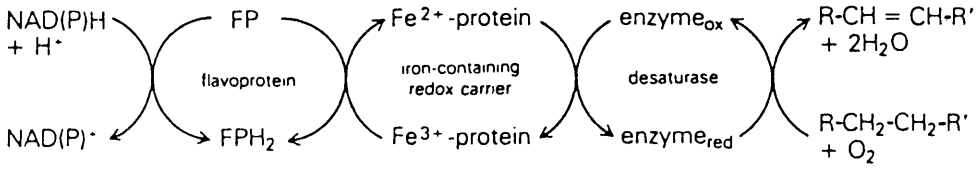
IV. Biochemistry and molecular biology of fatty acid desaturase

(1) Animal

Unsaturated fatty acids are synthesized by the oxidative desaturation which is catalyzed by desaturase associated with cytochrome $\underline{b}_5$ and cytochrome $\underline{b}_5$ reductase in the endoplasmic reticulum. The desaturation requires oxygen and NADH or NADPH, and can occur only through the acyl-CoA derivatives before the acyl groups are incorporated into phospholipids (Holloway 1983). The cis double bonds can be introduced at the Δ^4, Δ^5, Δ^6, Δ^8 and Δ^9 positions, but not beyond the Δ^9 position.

The stearoyl-CoA desaturase (Δ^9 desaturase) system has been extensively studied. This system in microsomes of rat liver is composed of three components: cytochrome $\underline{b}_5$, cytochrome $\underline{b}_5$ reductase and desaturase (cyanide-sensitive oxidase) (Enoch et al. 1976, Holloway 1971, Sreekrishna et al. 1981, Strittmatter et al. 1974, Thiede et al. 1986). The pathway for electron transport coupled to desaturation of acyl chains is shown in Fig. 4. These three components have been isolated and purified to homogeneity (Strittmatter 1974). They are all hydrophobic, membrane-bound proteins. Phospholipids are required to reconstitute the activity. Other necessary components of the complete system are

General scheme



(1) Animal	NADH (NADPH)	cytochrome $\underline{b}_5$ reductase	cytochrome $\underline{b}_5$	acyl-CoA
(2) Yeast	NADH (NADPH)	?	cytochrome $\underline{b}_5$	stearoyl-CoA phospholipids
(3) Bacteria	NADPH	flavoprotein	$\underline{b}$-type cytochrome or iron-protein	palmitoyl-CoA stearoyl-CoA
(4) Higher plant	NADPH (NADH)	?	ferredoxin (?)	stearoyl-ACP glycerolipids
(5) Cyanobacteria	?	?	?	glycerolipids

Fig. 4. Electron transport coupled to desaturation of fatty acids. ox, oxidized; red, reduced; R, alkyl chain; R', alkyl chain.

NADH, oxygen and the acyl-CoA substrate. The enzyme activity is enhanced in the rat liver, when the rats are fed by fat-free foods after starvation or insulin is administered to the diabetic rats (Joshi and Aranda 1979, Prasad and Joshi 1979).

The stearoyl-CoA desaturase of rat liver is a single polypeptide of 41400 daltons containing 62% nonpolar amino acid residues and one atom of non-heme iron (originally estimated as 53 kDa) (Strittmatter et al. 1974, Thiede et al. 1986). The enzyme activity is inhibited by cyanide and iron chelator but not carbon monoxide. The modification of the arginyl residues by butanedione showed that arginyl residue may be a part of the binding domain for the CoA moiety of the substrate (Enoch and Strittmatter 1978). The modification of tyrosyl residues by acetylation or nitration is a companied by loss of the iron prosthetic group and simultaneous loss of stearoyl-CoA desaturase activity (Enoch and Strittmatter 1978). This suggests that tyrosyl residues are involved in the binding of non-heme iron. The stearoyl-CoA desaturase has also been isolated from chicken liver by Prasad and Joshi (1979) and antibody against the purified enzyme was prepared (Prasad et al. 1980). The molecular weight (33600) of chicken liver enzyme is considerably smaller than that of rat liver enzyme. The chicken enzyme is inhibited by cyanide, as the rat liver enzyme. It is suggested that the chicken enzyme is localized on the cytoplasmic surface of the endoplasmic reticulum and that the active site of desaturase is exposed to the cytoplasm, since the activity in microsomal vesicles is inhibited by the anti-chicken desaturase antibody and is lost by

proteolysis with trypsin but not with subtilisin and chymotrypsin (Prasad et al. 1980).

The level of the translatable mRNA for rat liver stearoyl-CoA desarurase is elevated 50-fold by refeeding fat-free foods after starvation (Thiede and Strittmatter 1985). The cDNA for rat liver stearoyl-CoA desaturase has been isolated by the differential hybridization method using the mRNAs extracted from the rat liver cells before and after refeeding fat-free foods. It has been sequenced. It includes an open-reading frame of 1074 bp coding for 358 amino acid residues (Thiede et al. 1986). The genomic DNA and cDNA clones for the mouse stearoyl-CoA desaturase has also been isolated from the 3T3-L1 adipocytes (Ntambi et al. 1988). When the 3T3-L1 preadipocytes diffentiate to adipocytes, the transcription of the gene for stearoyl-CoA desaturase is elevated. The predicted amino acid sequence (355 residues) exhibits 92% homology to that of the rat liver desaturase. The gene contains 6 exons and 5 introns with all intron-exon junctions conforming to the GT/AG splicing rule. As determined by S1 nuclease mapping and primer extension analysis, the transcriptional initiation site is located 152 upstream from the initiation ATG codon. The "TATA" box is located 30 bp upstream of the Cap site. A typical "CCAAT" box is not present in the 5'-flanking region. The elements with homology (75%) to the putative fat-specific transcriptional element FSE2 (Phillips et al. 1986) and core consensus sequences for cAMP and glucocorticoid regulatory elements (Ryden and Beeman 1989) are located at the upstream region from the transcriptional

initiation site.

The Δ^6 desaturase has been solubilized and purified from rat liver microsome by Okayasu et al. (1981). The purified enzyme is a single polypeptide of 66 kDa. The amino acid residues amounting to 41% are nonpolar. The desaturation of linoleoyl-CoA in the reconstituted system is inhibited by cyanide and iron chelator. The Δ^6 desaturase contains one atom of non-heme iron. These properties are similar to those of stearoyl-CoA desaturase.

The Δ^5 desaturase, which desaturates eicosatrienoyl-CoA to arachidonoyl-CoA, has been partially purified from rat liver microsomes (Pugh and Kates 1979). The activity depends on oxygen and either NADH or NADPH, and is inhibited by cyanide but not by carbon monoxide.

The cytochrome $\underline{b}_5$ reductase has been solubilized and purified from calf liver (Spatz and Strittmatter 1973) and rabbit liver (Mihara and Sato 1975). The enzyme of calf liver contains 391 amino acid residues with a relative molecular mass of 43 kDa, and is amphipathic protein which is composed of hydrophilic and hydrophobic domains (Tajima et al. 1979). The protein is synthesized on the free polysomes and is bound to the endoplasmic reticulum, the Golgi and the mitochondrion (Meldolesi et al. 1980). From immunological studies and peptide mapping it appears that the proteins distributed in various subcellular locations are identical (Meldolesi et al. 1980).

The cytochrome $\underline{b}_5$ has been solubilized and purified from a number of animals. All of cytochrome $\underline{b}_5$ contains 133 amino acid residues and with a relative molecular mass of 16 kDa (Fleming et

al. 1978, Kondo et al. 1979, Ozols and Gerard 1977a, 1977b, Ozols and Heinemann 1982). It is a amphipathic enzyme like cytochrome b_5 reductase (Tajima et al. 1979). The amino acid sequence of this enzyme isolated from a variety of animals has been compared and it is found that the sequence of the hydrophobic domain is highly conserved in pig (Ozols and Gerard 1977a), horse (Ozols and Gerard 1977b), cow (Fleming et al. 1978), rabbit (Kondo et al. 1979) and rat (Ozols and Heinemann 1982). The cytochrome b_5 is synthesized on the free polysomes (Okada et al. 1982, Rachubinski et al. 1980). Although it does not contain the leader sequence, it is found in the membrane fraction of endoplasmic reticulum in the highest concentration. The mechanism for the distribution of the enzyme has not been elucidated.

(2) Yeasts

In the desaturase system in yeasts, saturated fatty acids are desaturated to monounsaturated fatty acids in the form of CoA derivatives. Further desaturation to polyunsaturated fatty acids occurs while the fatty acids are bound to phosphatidylcholine (Pugh and Kates 1975, Talamo et al. 1973). Desaturation of phospholipid-linked oleic acid in Torulopisis utilis (Talamo et al. 1973) and Candida lipolytica (Pugh and Kates 1973) has been demonstrated in the cell-free microsomal systems. The yeast contains cytochrome b_5 which is necessary for the desaturation at the Δ^9 position (Ohba et al. 1979).

A mutant of Saccharomyces cerevisiae (ole1) which requires unsaturated fatty acids has been isolated by Resnick and Mortimer

(1966). This mutant is incapable of desaturating exogenous added saturated fatty acids, and is apparently defective in the Δ^9 desaturase activity. Stukey et al. (1989) have recently isolated the OLE1 gene, which complements the ole1 mutant, from the genomic library of S. cerevisiae. Although this gene may encodes the stearoyl-CoA desaturase, its sequence has not been determined.

(3) Bacteria

In bacteria, unsaturated fatty acids are synthesized by two types of pathway (Fulco 1983). The first pathway, which is found only in bacteria and is extensively studied in Escherichia coli, does not require oxygen and is called "anaerobic pathway". In this pathway, the double bond is introduced during the fatty acid synthesis by 3,4-dehydration of β-hydroxydecanoyl-ACP which is catalyzed by hydroxydecanoyl-ACP dehydrase. The resultant $10{:}1^{\Delta 3}$ is elongated to $16{:}1^{\Delta 9}$ and $18{:}1^{\Delta 11}$ by condensing enzymes, β-ketoacyl-ACP synthetase I and II (Rosenfeld et al. 1973, Garwin et al. 1980).

The second pathway, in which unsaturated fatty acids are synthesized by desaturase is found in certain bacteria. The bacterial desaturase system has not been characterized as well as animal systems. The cell-free system of fatty acid desaturation is prepared from Mycobacterium smegterium in a stable form (Fulco and Bloch 1964). In this system palmitoyl-CoA and stearoyl-CoA are desaturated to the corresponding Δ^9 derivatives in the presence of NADPH, FAD, Fe^{2+} and oxygen. NADH can not replace

NADPH nor can ferric replace ferrous iron. It thus appears that a flavoprotein and a non-heme iron are involved in this desaturation reaction (Fulco and Bloch 1964). The desaturation system in M. phlei is basically similar to that in M. smegmatis except that no ferrous iron requirement is demonstrable (Kashiwabara and Sato 1973).

(4) Higher plant

In higher plants, unsaturated fatty acids are synthesized by the oxidative desaturation in endoplasmic reticula and chloroplasts. The double bonds introduced are located at Δ^6, Δ^9, Δ^{12} and Δ^{15} positions of C_{18} fatty acids, and at Δ^3, Δ^7, Δ^{10} and Δ^{13} positions of C_{16} fatty acids. All these double bonds are of cis configuration. Only in PG a double bond of trans configuration is introduced at the Δ^3 position of 16:0.

In plants, stearoyl-ACP, but not stearoyl-CoA as in animals and yeasts, is used as a substrate by the Δ^9 desaturase (stearoyl-ACP desaturase). McKeon and Stumpf has partially purified this enzyme from maturing safflower seeds (1982). The enzyme is soluble and exists as a dimer with a relative molecular mass of 68 kDa. It is distributed in proplastids or the chloroplast stroma. It requires molecular oxygen and NADPH with ferredoxin which can acts as the electron carrier. Its enzymatic properties have been characterized (Stumpf 1981). This desaturase is specific to acyl-ACP. Stearoyl-CoA and palmitoyl-ACP are only 5% and 1% as active as stearoyl-ACP. Although the Kms for palmitoyl-ACP and stearoyl-ACP are 0.51 μM and 0.38 μM, respectively, the

Vmax for the latter is more than 100 times as high as that for the former. It appears that the ACP moiety and the chain length are important for the desaturation activity.

The polyunsaturated fatty acids of plants are synthesized by membrane-bound desaturases (Harwood 1988). Fatty acids bound to glycerolipids, but not to acyl-CoA or acyl-ACP, are desaturated in these reactions. These types of desaturase in plants are termed the plant-type desaturases. They are distributed in the endoplasmic reticulum (Demandre et al. 1986, Murphy et al. 1984, Slack et al. 1976, 1979) and in the chloroplast (Jones and Harwood 1980). However, none of them has been isolated because of the difficulties in their solubilization and the *in vitro* reconstitution of desaturation reaction system. The genes for the desaturation reaction of fatty acids have not been isolated from higher plants.

Cytochrome b_5 and cytochrome b_5 reductase have been purified from potato microsome using zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Relative molecular masses of cytochrome b_5 and cytochrome b_5 reductase are 16.7 kDa and 44 kDa, respectively (Bonnerot et al. 1985, Galle et al. 1984). The latter enzyme shows a visible absorption spectrum typical of a flavoprotein. However, whether these enzymes are involved in the desaturation of fatty acids in plants is still in question.

Browse, Somerville and their colleagues have isolated a series of mutants of *Arabidopsis thaliana* which are defective in the desaturation of particular position of fatty acids (Browse et

al. 1985, 1986, 1989, Kunst et al. 1989, Somerville et al. 1987). In order to elucidate the function of unsaturated fatty acids, they investigated the changes due to the mutation in the chloroplast structure, the photosynthetic characteristic and the thermal tolerance (Hugly et al. 1989, Kunst et al. 1989, McCourt et al. 1987). But they have not found any significant difference between wild type and the mutants. It is expected that the genes for the desaturation of fatty acids will be isolated from Arabidopsis using the complementation analysis of these mutants.

(5) Cyanobacteria

The physiological study of desaturation of fatty acids in cyanobacteria has been extensively done in A. variabilis (Murata and Nishida 1987). According to Sato and Murata (1982b) the desaturation of fatty acids takes place in GlcDG, MGDG, SQDG and PG, and DGDG is synthesized by transfer of galactose from an unidentified galactose carrier to MGDG. It is suggested that in the early stage of glycerolipid synthesis in cyanobacteria only saturated fatty acids are esterified to glycerol moiety and subsequently desaturated to mono- and polyunsaturated fatty acids while bound to the glycerolipids, in contrast to the case that in higher plants stearic acid is desaturated to oleic acid in the ACP-bound form (Sato et al. 1986). This is supported by in vitro experiments of Lem and Stumpf (1984a) as well as Stapleton and Jaworski (1984), in which only saturated fatty acids are synthesized from [^{14}C]acetate in the extract of A. variabilis and neither added stearoyl-ACP nor stearoyl-CoA is desaturated.

However, the enzymes and the genes for the fatty acid desaturation in cyanobacteria has not been studied.

V. Aim of the present study

Cyanobacteria respond to low temperature by desaturating fatty acids of membrane lipids to compensate the decrease in membrane fluidity (Murata and Nishida 1987). A number of other organisms use the same strategy to protect the cells against the low-temperature effect (Cossins and Sinensky 1984, Russell 1984, Thompson 1980). However, the enzymes involved in the fatty acid desaturation in plants have long been in question.

The plant-type desaturases introduce a double bond into fatty acids bound to glycerolipids. In animal, in contrast, all the desaturations of fatty acids take place before they are bound to glycerolipids. None of the plant-type desaturases has been isolated from algae or plants, because the solubilization of the enzymes and reconstitution of their activity _in vitro_ are difficult.

The aim of the present study is to isolate the genes for the plant-type desaturases from the cyanobacterium, _Synechocystis_ PCC6803 and to study the temperature-induced regulation of the expression of these genes in order to understand the molecular mechanism of temperature acclimation in cyanobacteria.

Chapter 2

Temperature-induced changes in the fatty acid composition of the cyanobacterium, Synechocystis PCC6803

Summary

Changes in glycerolipid and fatty acid composition with a change in growth temperature were studied in the cyanobacterium, Synechocystis PCC6803. Under isothermal growth conditions, temperature did not significantly affect the composition of the various classes of lipids, but a decrease in temperature altered the degree of unsaturation of C_{18} acids at the sn-1 position, but not that of C_{16} acids at the sn-2 position of the glycerol moiety in each class of lipids. When the growth temperature was shifted from 38°C to 22°C, the desaturation of C_{18} acids, but not that of C_{16} acids, was stimulated. The desaturation of fatty acids occurred only in the light and was inhibited by chloramphenicol, rifampicin and 3-(3,4-dichlorophenyl)-1,1-dimethylurea, but not by cerulenin, an inhibitor for fatty acid synthesis. These findings suggest that desaturase activities are induced after a shift from a higher to a lower temperature, and that the desaturation of fatty acids is connected with the reactions involved in photosynthetic electron transport.

Introduction

A number of organisms can regulate the fatty acid composition of their membrane lipids in response to a change in ambient temperature (Russel 1984, Thompson 1980). Such temperature-induced changes in fatty acid composition are explained in terms of the regulation of membrane fluidity that is necessary for the proper functioning of biological membranes (Cossins 1984).

Prokaryotic algae, cyanobacteria, resemble the chloroplasts of eukaryotic plants with respect to lipid and fatty acid composition (Murata and Nishida 1987) and membrane structure (Stanier et al. 1971). Thus, the cyanobacteria may be regarded as a model system for elucidation of the effects of temperature on membrane lipids in the chloroplast. Sato and Murata (1980, 1981) studied the desaturations of fatty acids induced by low temperature in the cyanobacterium Anabaena variabilis, and they showed that the desaturation acts to compensate for the decrease in membrane fluidity that results from the decrease in temperature. They suggested that desaturase activity is induced after a shift to lower temperatures (Sato and Murata 1981). However, the enzymes involved in the desaturation of fatty acids remain to be identified.

The cyanobacterium, Synechocystis PCC6803, is a transformable strain and, therefore, it offers a molecular-genetic approach to the resolution of the role of the desaturation of fatty acids in the acclimation to changes in temperature. However, the effects of temperature on the lipid and fatty acid composition of this alga have not been reported. Kenyon (1972) reported that this cyanobacterium contains 16:0, $16:1^{\Delta 9}$, $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$ and a unique fatty acid, $18:3^{\Delta 6,9,12}$, as major fatty acids. This fatty acid composition is very different from that of A. variabilis and, therefore, the nature of the changes in fatty acids in response to temperature may differ between Synechocystis PCC6803 and A. variabilis.

In the present study, we examined the effect of temperature

on the lipid and fatty acid composition of Synechocystis PCC6803 under isothermal growth conditions and after a downward shift in temperature. In this cyanobacterium the C_{18} fatty acids were desaturated at the sn-1 position of the glycerol moiety to compensate for the decrease in membrane fluidity at low temperature.

Materials and Methods

Organism and culture conditions---Synechocystis PCC6803 was kindly provided by Dr. T. Omata of the Institute for Physical and Chemical Research, who obtained it originally from the Pasteur Culture Collection. The cells were grown photoautotrophically in BG-11 (Stanier et al. 1971) supplemented with 20 mM HEPES-NaOH (pH 7.5) under illumination by incandescent lamps (70 $\mu E\ m^{-2}s^{-1}$) with aeration by 1.0% CO_2 in air, as described previously (Ono and Murata 1981a). For isothermal growth experiments, the cells were grown at a constant temperature for more than 7 days prior to use. Cultures in the exponential phase of growth were used for experiments. For temperature-shift experiments, cultures in the exponential phase were transferred from 38 to 22°C according to the method described previously (Sato and Murata 1981). At a designated time after the downward shift in temperature, a portion of culture was withdrawn, and the cells were collected by centrifugation at 2500 x g for 10 min at 4°C. The effects of light, chloramphenicol, rifampicin, cerulenin, and DCMU on changes in lipid and fatty acid composition after the shift in temperature were investigated by the previously described method

(Sato and Murata 1981).

Extraction of lipids and analysis of fatty acids---Lipids were extracted from the collected cells by the method of Bligh and Dyer (1959). Analysis of lipids was carried out according to the method of Sato and Murata (1988). The total lipid and the various classes of lipids, which were fractionated 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 (Shimadzu, GC-4A or GC-14A) equipped with a hydrogen flame-ionization detector. Fatty acid methyl esters were separated on a glass column (2.0 m x 4.0 mm internal diameter) packed with 15% diethyleneglycol succinate on Chromosorb W or on a capillary column (Quadrex, CPS-1, 50 m x 0.25 mm internal diameter) coated with cyanopropylmethyl silicone at a thickness of 0.25 μm. Temperatures of the column and the flame-ionization detector were 180°C and 260°C, respectively. The relative amounts of fatty acid methyl esters were determined by comparison of areas under the peaks on the chromatogram, as calculated with a data processor (Shimadzu, C-R3A). The fatty acid methyl esters were identified by a gas chromatograph-mass spectrometer (JEOL, JMS-DX-300) equipped with a mass data-analysis system (JEOL, BJMS-3100) and the same columns as used for GLC.

Positions of double bonds in fatty acids were determined by GC-MS according to the procedure (the pyrrolidine method) of Andersson et al. (1975) on a column (1.0 m x 2.5 mm internal

diameter) packed with 3% OV-1 (Gasukuro Kogyo) at a column temperature of 230°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 by Rhizopus delemar lipase (Fischer et al. 1973).

Analysis of molecular species---Analysis of the molecular species of MGDG was carried out by a modified version of the method of Lynch et al. (1983). The isolated MGDG was dissolved in methanol/water (95:5, v/v), and separated into molecular species by HPLC (JASCO, Trirotor-III), on a system equipped with a reversed-phase column (TOSOH, ODS 120T, 30 cm x 7.6 mm internal diameter). The solvent system and flow rate were methanol/water (95:5, v/v) and 1.0 ml min^{-1}, respectively. The elution of molecular species was monitored at 208 nm with a UV detector (JASCO, UVIDEC-100-III). For the identification and quantitative determination of molecular species, fractions corresponding to the peaks were collected. After methanolysis, as described above, the amount of each molecular species was calculated from levels of its constituent fatty acids which were determined by gas-liquid chromatography, as described above.

Results

Isothermal growth conditions---Synechocystis PCC6803 contained four major glycerolipids, MGDG, DGDG, SQDG and PG, as is the case in other cyanobacteria (Murata and Nishida 1987). The composition of lipid classes was not significantly affected by growth temperature (Table 1). The relative level of MGDG was a

Table 1 Lipid composition of *Synechocystis* PCC6803 grown at four different temperatures

Lipid	Growth temperature			
	38°C	34°C	28°C	22°C
	(mol %)			
MGDG	53	53	53	54
DGDG	16	19	22	18
SQDG	19	19	15	15
PG	12	9	10	13

little more than 50% of the total glycerolipids, and relative levels of DGDG, SQDG and PG ranged from about 10% to about 20%.

The fatty acid composition of total lipids isolated from cells grown at different temperatures is shown in Table 2. 16:0, $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 6,9,12}$ were the major fatty acids. The proportion of 16:0 was greater than 50%, regardless of growth temperature. These results are in agreement with those for the same cyanobacterium reported by Kenyon (1972). $18:3^{\Delta 9,12,15}$ and $18:4^{\Delta 6,9,12,15}$ were present only in cells grown at 28°C and 22°C, but they were not found in cells grown at 38°C and 34°C. The ratio of C_{18} to C_{16} acids was relatively constant. Significant changes in response to growth temperature were observed in the C_{18} acids. With decreasing temperature of cultures, relative levels of $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ decreased while those of $18:3^{\Delta 9,12,15}$, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ increased. In contrast, the levels of the various C_{16} acids stayed almost constant.

$18:4^{\Delta 6,9,12,15}$ has been reported to be present in some filamentous cyanobacteria, Tolypothrix tenuis, Phormidium sp. (Kenyon et al. 1972), Oscillatoria splendida and Pseudoanabaena sp. (Loura et al. 1987), although the positions of the double bonds in this fatty acid have not been reported. Based on the results of our analysis by gas chromatography-mass spectrometry, the double bonds in $18:4^{\Delta 6,9,12,15}$ from Synechocystis PCC6803 appear to be located at the $\Delta^{6,9,12,15}$.

In order to determine which lipids respond to changes in growth temperature by changes in the degree of unsaturation of

Table 2 Fatty acid composition of total lipids from Synechocystis PCC6803 grown at four different temperatures

Fatty acid	Growth temperature			
	38°C	34°C	28°C	22°C
	(mol %)			
14:0	t	t	t	t
16:0	58	54	52	51
$16:1^{\Delta 9}$	2	4	3	3
17:0	1	1	1	t
18:0	2	t	t	t
$18:1^{\Delta 9}$	9	7	3	2
$18:2^{\Delta 9,12}$	15	14	9	6
$18:3^{\Delta 9,12,15}$	0	0	3	8
$18:3^{\Delta 6,9,12}$	13	20	26	21
$18:4^{\Delta 6,9,12,15}$	t	t	3	8

t, trace amount (less than 0.4%)

their fatty acids, we examined the fatty acid compositions of individual classes of lipids from the cells grown at 38°C and 22°C (Table 3). At both temperatures, the fatty acid compositions of MGDG and DGDG were similar. The three major fatty acids, 16:0, $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$, were found in all four classes of lipids, whereas $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ were restricted to MGDG and DGDG and $18:3^{\Delta 9,12,15}$ was found only in SQDG and PG. This type of distribution of fatty acids into the various classes of lipids is similar to that found in O. splendida and Pseudoanabaena sp. (Loura et al. 1987). The composition of C_{18} acids depended on growth temperature in all classes of lipids, while that of C_{16} acids was less affected by growth temperature, except in the case of SQDG. In MGDG and DGDG, the relative levels of $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ at 22°C were higher than those at 38°C, and in SQDG and PG, the relative level of $18:3^{\Delta 9,12,15}$ at 22°C was higher than that at 38°C. In SQDG, the relative level of 16:0 was higher than that in the other lipids and decreased with a decrease in growth temperature from 38°C to 22°C.

The positional distributions of fatty acids in the glycerol moieties of lipids in cells grown at 38°C and 22°C are shown in Table 4. All C_{18} acids were located exclusively at the sn-1 position. The small amount of $16:1^{\Delta 9}$ was also located at the sn-1 position. Palmitate was almost exclusively esterified to the sn-2 position. This distribution of fatty acids is similar to that found in filamentous cyanobacteria, such as A. variabilis (Sato et al. 1979), Anabaena cylindrica, Oscillatoria chalybea, Nostoc calcicola and Tolypothrix tenuis (Zepke et al. 1978). Note that

Table 3 Fatty acid composition of lipid classes from *Synechocystis* PCC6803 grown at 38°C and 22°C

Fatty acid	Lipid							
	MGDG		DGDG		SQDG		PG	
	38°C	22°C	38°C	22°C	38°C	22°C	38°C	22°C
	(mol %)							
14:0	t	t	1	1	1	1	2	1
16:0	55	50	57	51	76	63	55	51
$16:1^{\Delta 9}$	3	3	2	4	2	4	1	1
17:0	1	t	1	t	1	1	t	t
18:0	2	t	2	1	4	t	6	1
$18:1^{\Delta 9}$	9	2	6	2	9	5	14	2
$18:2^{\Delta 9,12}$	14	5	14	6	6	10	20	5
$18:3^{\Delta 9,12,15}$	0	0	0	1	0	13	t	35
$18:3^{\Delta 6,9,12}$	16	29	17	24	1	2	1	2
$18:4^{\Delta 6,9,12,15}$	t	10	0	10	0	t	0	1

t, trace amount (less than 0.4%)

Table 4a Positional distribution of fatty acids at the sn-1 and sn-2 positions of the glycerol moiety of lipids from Synechocystis PCC6803 grown at 38°C and 22°C

Fatty acid	MGDG				DGDG			
	38°C		22°C		38°C		22°C	
	1*	2*	1*	2*	1*	2*	1*	2*
	(mol %)							
14:0	0	1	t	t	2	2	0	1
16:0	7	49	7	47	4	47	8	47
$16:1^{\Delta 9}$	3	0	2	1	3	0	2	1
17:0	1	0	t	t	t	0	t	t
18:0	1	0	t	t	2	1	1	1
$18:1^{\Delta 9}$	6	0	0	1	5	t	1	t
$18:2^{\Delta 9,12}$	17	t	4	t	17	t	4	0
$18:3^{\Delta 9,12,15}$	0	t	1	t	0	0	2	0
$18:3^{\Delta 6,9,12}$	15	t	26	t	17	t	23	0
$18:4^{\Delta 6,9,12,15}$	t	t	10	t	t	t	9	0

t, trace amount (less than 0.4%).

*The numbers 1 and 2 stand for the sn-1 and sn-2 positions of the glycerol moiety, respectively.

Table 4b Positional distribution of fatty acids at the sn-1 and sn-2 positions of the glycerol moiety of lipids from Synechocystis PCC6803 grown at 38°C and 22°C

	SQDG				PG			
Fatty acid	38°C		22°C		38°C		22°C	
	1*	2*	1*	2*	1*	2*	1*	2*
	(mol %)							
14:0	0	1	0	1	0	2	0	1
16:0	33	46	17	46	9	45	9	46
$16:1^{\Delta 9}$	3	0	4	t	2	0	1	t
17:0	t	0	1	t	t	t	t	t
18:0	2	1	1	2	3	1	1	1
$18:1^{\Delta 9}$	6	1	5	t	11	2	3	1
$18:2^{\Delta 9,12}$	6	t	8	t	24	t	5	t
$18:3^{\Delta 9,12,15}$	t	t	13	t	1	t	29	0
$18:3^{\Delta 6,9,12}$	t	t	1	t	t	t	2	0
$18:4^{\Delta 6,9,12,15}$	t	t	t	0	t	t	t	0

t, trace amount (less than 0.4%).

*The numbers 1 and 2 stand for the sn-1 and sn-2 positions of the glycerol moiety, respectively.

the major changes in fatty acid composition with growth temperature occurred in the C_{18} acids at the sn-1 position in all classes of lipids.

The molecular species separated by HPLC were identified by GLC of their constituent fatty acids. Table 5 shows the molecular species composition of MGDG from the cells grown at 38°C and 22°C. The major molecular species of MGDG were 16:0/16:0, $18:1^{\Delta 9}$/16:0, $18:2^{\Delta 9,12}$/16:0 and $18:3^{\Delta 6,9,12}$/16:0, and $18:4^{\Delta 6,9,12,15}$/16:0 was detected only in the cells grown at 22°C. Levels of 16:0/16:0, $18:1^{\Delta 9}$/16:0 and $18:2^{\Delta 9,12}$/16:0 were higher at 38°C than at 22°C, while levels of $18:3^{\Delta 6,9,12}$/16:0 and $18:4^{\Delta 6,9,12,15}$/16:0 were higher at 22°C than at 38°C. At 22°C, $18:3^{\Delta 6,9,12}$/16:0 accounted for almost 70% of the total molecular species. The fatty acid composition of MGDG, calculated from the results in Table 5, coincided closely with that determined directly, as shown in Table 3.

Shift in temperature from 38°C to 22°C---Changes in the total fatty acids and in the packed cell volume of a unit volume of culture after a shift in temperature from 38°C to 22°C are shown in Figure 5. The amount of total fatty acids and the packed cell volume ceased to increase during the first 10 h after the downward shift in temperature, indicating that the cells were not growing during the time when the analysis was conducted.

The lipid composition was not affected for 24 h after the downward shift in temperature from 38°C to 22°C (data not shown). Changes in the fatty acid composition of the total lipids after the shift in temperature are shown in Figure 6. After the shift

Table 5 Molecular species composition of MGDG from Synechocystis PCC6803 grown at 38°C and 22°C

Molecular species	Growth temperature	
	38°C	22°C
	(mol %)	
16:0/16:0	13	2
$16:1^{\Delta 9}$/16:0	7	3
18:0/16:0	2	t
$18:1^{\Delta 9}$/16:0	15	3
$18:2^{\Delta 9,12}$/16:0	34	8
$18:3^{\Delta 9,12,15}$/16:0	0	t
$18:3^{\Delta 6,9,12}$/16:0	29	69
$18:4^{\Delta 6,9,12,15}$/16:0	0	14

t, trace amount (less than 0.4%)

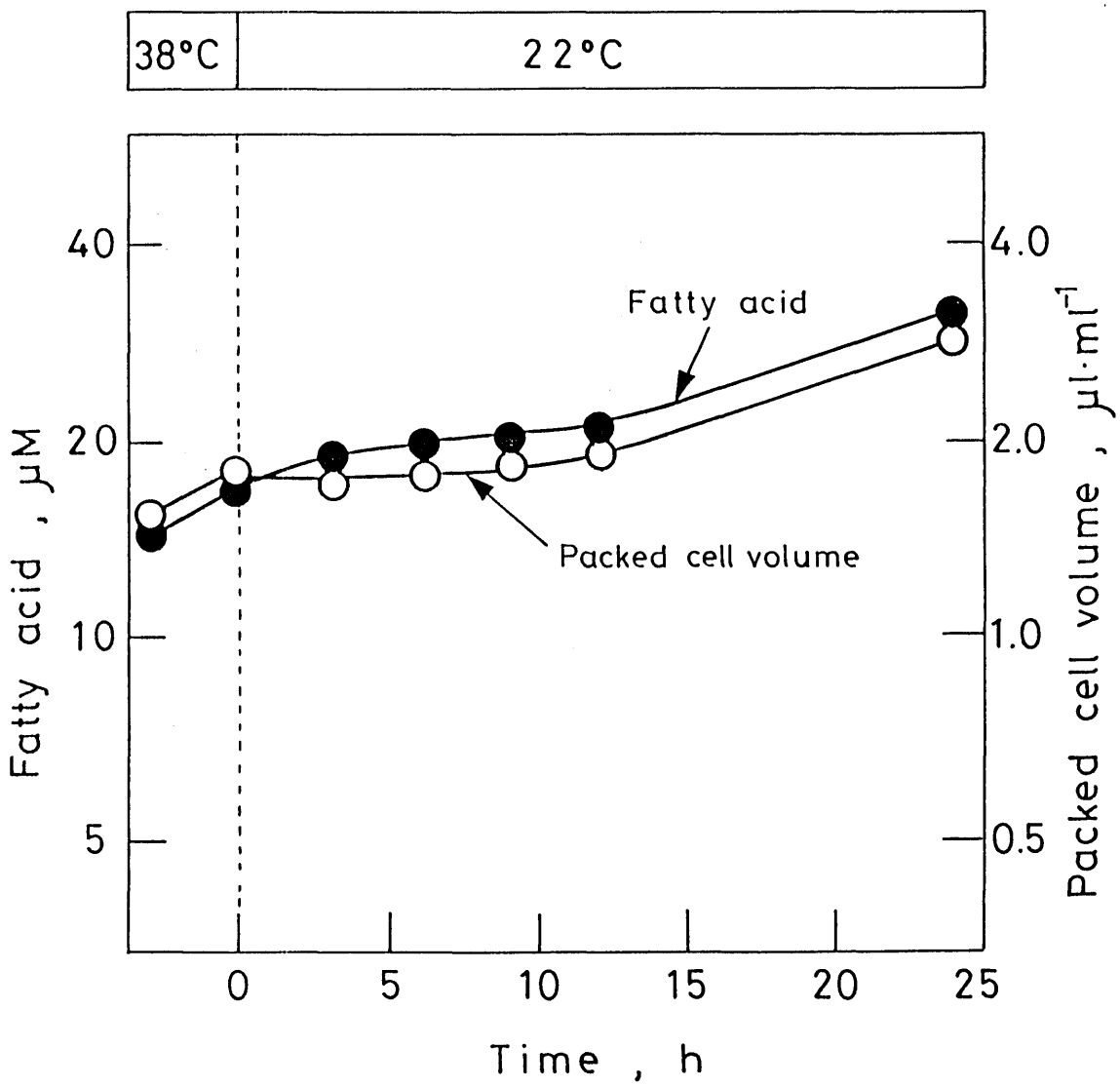


Fig. 5. Changes in the total amount of fatty acids and in the packed cell volume after a shift in temperature from 38°C to 22°C.

in temperature, significant changes in the degree of the desaturation of the C_{18} acids, but not in that of the C_{16} acids, were observed, i.e., a decrease in levels of $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$, and an increase in levels of $18:3^{\Delta 9,12,15}$, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. By contrast, the relative level of 16:0 decreased slightly, but that of $16:1^{\Delta 9}$ remained almost constant.

To characterize more precisely the desaturation of fatty acids after the downward shift in temperature, we investigated the changes in fatty acids in individual classes of lipids. Figure 7 shows that $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ of MGDG were desaturated to $18:3^{6,9,12}$ and later to $18:4^{6,9,12,15}$. However, in PG, $18:1^{9}$ and $18:2^{\Delta 9,12}$ were desaturated to $18:3^{\Delta 9,12,15}$, and $18:1^{\Delta 9}$ of SQDG was desaturated to $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$. The fatty acid composition of DGDG did not change significantly in response to the downward shift in temperature. Sato and Murata (1980) reported that, in A. variabilis, there is a rapid and transient desaturation of 16:0 to $16:1^{\Delta 9}$, which takes place at the sn-2 position in MGDG after a shift in temperature from 38°C to 22°C. This type of desaturation was not observed in Synechocystis PCC6803.

The effects of light, DCMU, cerulenin, chloramphenicol and rifampicin on the desaturation of C_{18} fatty acids in the total lipids after the shift in temperature from 38°C to 22°C are presented in Table 6. The desaturation of fatty acids and the synthesis of fatty acids were completely suppressed in the dark and partially inhibited by DCMU for 8 h after the shift in temperature. These findings suggest that the temperature-induced

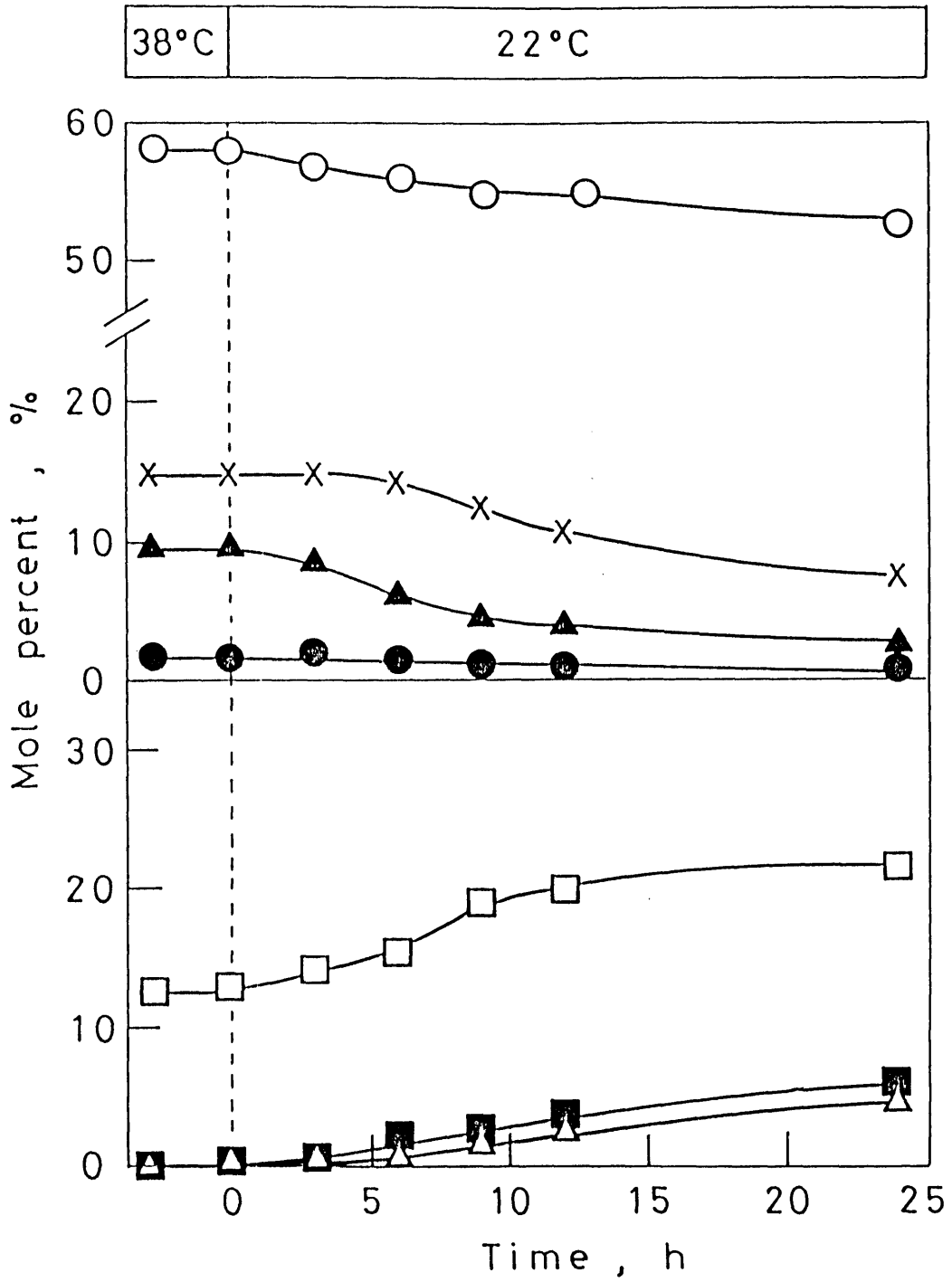


Fig. 6. Changes in the fatty acid composition of the total lipids after a shift in temperature from 38°C to 22°C. 16:0 (o), 18:0 (●), 18:1$^{\Delta 9}$ (▲), 18:2$^{\Delta 9,12}$ (×), 18:3$^{\Delta 9,12,15}$ (■), 18:3$^{\Delta 6,9,12}$ (□), 18:4$^{\Delta 6,9,12,15}$ (Δ).

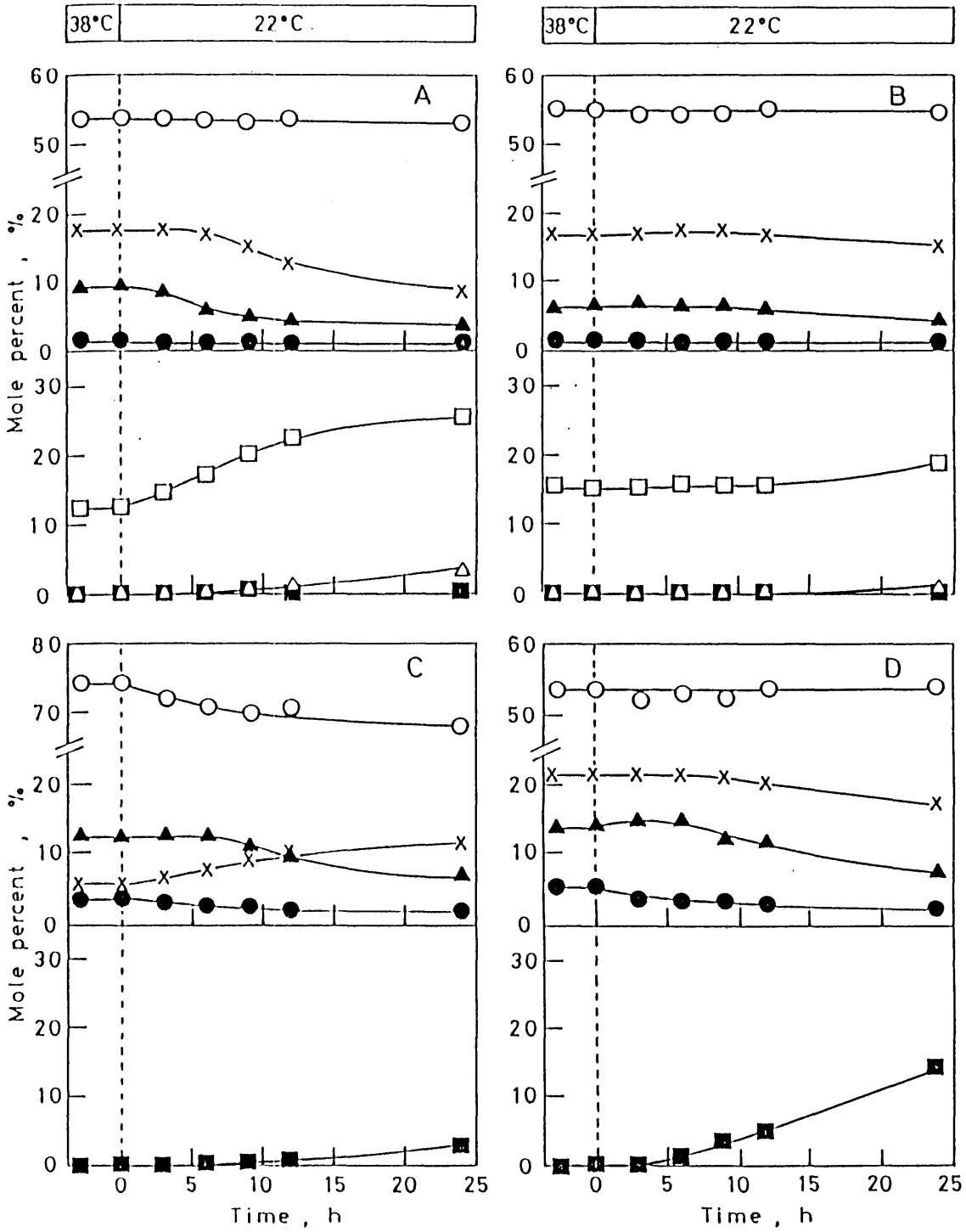


Fig. 7. Changes in the fatty acid composition of the individual classes of lipids after a shift in temperature from 38°C to 22°C. A, MGDG; B, DGDG; C, SQDG; D, PG. 16:0 (o), 18:0 (●), 18:1$^{\Delta 9}$ (▲), 18:2$^{\Delta 9,12}$ (×), 18:3$^{\Delta 9,12,15}$ (■), 18:3$^{\Delta 6,9,12}$ (□), 18:4$^{\Delta 6,9,12,15}$ (△).

Table 6 Effects of light and various inhibitors on the desaturation of C_{18} fatty acids in the total lipids from Synechocystis PCC6803 after a shift in temperature from 38°C to 22°C

Light or Dark	Inhibitor	Time after temperature shift	Total fatty acid	Fatty acid					
				18:0	18:1 Δ^9	18:2 $\Delta^{9,12}$	18:3 $\Delta^{9,12,15}$	18:3 $\Delta^{6,9,12}$	18:4 $\Delta^{6,9,12,15}$
		(h)	(μM)	(mol %)					
Initial temperature		0	29	1	10	16	0	13	t
L	No addition	8	33	1	6	14	2	16	1
D	No addition	8	28	1	9	16	0	13	t
L	DCMU	8	28	1	8	16	0	15	t
L	Cerulenin	8	29	1	5	15	1	17	1
L	Chloramphenicol	8	32	2	10	15	0	13	t
L	Rifampicin	8	32	2	10	16	0	13	t
L	Chloramphenicol + cerulenin	8	30	1	9	16	0	13	t

t, trace amount (less than 0.4%). L, Light; D, dark.

Chloramphenicol (15 μg ml^{-1}), rifampicin (15 μg ml^{-1}), cerulenin (10 μg ml^{-1}) or DCMU (20 μM) was added to the suspension of cells just before the shift in temperature. The cells were incubated at 22°C for 8 h with aeration with 1.0% CO_2 in air after the shift in temperature.

desaturation of fatty acids requires the reactions of the photosynthetic electron transport system in the thylakoid membranes.

Cerulenin (10 $\mu g\ ml^{-1}$), an inhibitor of fatty acid synthetase (Omura 1976), inhibited the increase in the total amount of fatty acids, but it did not affect the temperature-induced desaturation of fatty acids. This result demonstrates that the synthesis of fatty acids de novo is not necessary for the temperature-induced desaturation of fatty acids. Chloramphenicol, an inhibitor of protein synthesis, and rifampicin, an inhibitor of RNA synthesis, inhibited the temperature-induced desaturation of fatty acids. These findings suggest that the synthesis of RNA and protein is required for the temperature-induced desaturation of fatty acids.

Discussion

Isothermal growth conditions---Many organisms, including both prokaryotes and eukaryotes, respond to change in environmental temperature by altering the fatty acid composition of their membrane lipids. When there is a decrease in the growth temperature, the levels of more unsaturated and/or shorter-chain fatty acids increase (Russell 1984). The increase in the degree of unsaturation and the shortening of the chain lengths of fatty acids decrease the phase-transition temperature and increase the fluidity of membrane lipids (Chapman 1975). The present study indicates that Synechocystis PCC6803 responds to a change in growth temperature by altering the degree of unsaturation of the

C_{18} fatty acids at the sn-1 position of the glycerol moiety. This phenomenon is regarded as an acclimation to the environmental temperature.

Kenyon (1972) and Kenyon et al. (1972) classified the cyanobacteria into four groups with respect to the composition and metabolism of fatty acids. According to this classification, unicellular cyanobacteria belong to either the first group, containing only saturated and monounsaturated fatty acids, or to the third group, containing $18:3^{\Delta 6,9,12}$ (Kenyon 1972). In the present study we found that Synechocystis PCC6803 grown at 22°C contains a high level of $18:4^{\Delta 6,9,12,15}$, which is a specific characteristic of the fourth group in Kenyon's classification, although this alga is unicellular. This is, to our knowledge, the first report that a unicellular strain can be classified into the fourth group.

In cyanobacteria, the positional distribution of fatty acids in the sn-position of the glycerol moiety of lipids can be classified into two categories (Murata and Nishida 1987). In Anacystis nidulans, which belongs to the first category, most of the monounsaturated fatty acids are esterified to the sn-1 position and most of 16:0 is esterified to the sn-2 position in all classes of lipids. In the second category, which is represented by the filamentous cyanobacteria, such as A. variabilis, A. cylindrica, O. chalybea, N. calcicola and T. tenuis, the C_{18} acids are esterified to the sn-1 position, and the C_{16} acids are esterified to the sn-2 position in all classes of lipids. Synechocystis PCC6803 belongs to the second category

in this regard.

Temperature shift---The biosynthetic pathways of the various molecular species of glycerolipids in cyanobacteria have been studied in considerable detail in A. variabilis (Murata and Nishida 1987). The results of pulse-labeling and chase experiments with $H^{14}CO_3^-$ in vivo suggest that the primary products of lipids are 18:0/16:0-monoglucosylglycerol, 18:0/16:0-sulfoquinovosylglycerol and 18:0/16:0-glycerophosphoglycerol (Sato and Murata 1982b). MGDG is synthesized from monoglucosyl diacylglycerol by epimerization of glucose to galactose (Sato and Murata 1982a). The desaturation of fatty acids takes place in monoglucosyl diacylglycerol, MGDG, SQDG and PG, and it is accelerated at low temperature (Sato and Murata 1982b). DGDG is produced by the transfer of galactose from an unidentified galactose carrier to MGDG, and no desaturation of fatty acids has been demonstrated in DGDG (Sato and Murata 1982b). These observations suggest that only the saturated fatty acids, 18:0 and 16:0, are first esterified to the sn-1 and sn-2 positions of the glycerol moiety, respectively, and then desaturated while they are bound to the lipids. This inference was supported by experiments in vitro by Lem and Stumpf (1984a), as well as by Stapleton and Jaworski (1984), in which only saturated fatty acids were produced from [^{14}C]acetate in an extract of A. variabilis, and neither exogenously added 18:0-ACP nor 18:0-CoA was desaturated.

We have recently isolated two mutants of Synechocystis PCC6803, which are defective in Δ^6 and Δ^{12} desaturation of the

C_{18} fatty acids, respectively (Chapter 3). Based on the fatty acid composition of lipids in the mutants, a hypothetical pathway, as shown in Figure 8, is proposed for the biosynthesis of various molecular species of lipids in Synechocystis PCC6803. The desaturation reactions, which are represented by broad closed arrows in the figure, are regarded as being highly active, regardless of the temperature. The desaturation reactions, which are represented by broad open arrows, are stimulated after the shift in temperature. The data in the present study are not evident for the stimulation of desaturation pathways of MGDG from $18:1^{\Delta 9}/16:0$ to $18:2^{\Delta 6,9}/16:0$ then to $18:3^{\Delta 6,9,12}/16:0$ (represented by thin arrows) and from $18:2^{\Delta 9,12}/16:0$ to $18:3^{\Delta 9,12,15}/16:0$ then to $18:4^{\Delta 6,9,12,15}/16:0$. However, in mutants which were defective in the Δ^{12} and Δ^{6} desaturation, stimulation of these desaturation pathways was well demonstrated (Fig. 10 in Chapter 3), although these pathways are insignificant in the wild type.

The results obtained in this study demonstrate the adaptive response, in terms of fatty acid composition, of Synechocystis PCC6803 during low-temperature acclimation. This alga possesses different desaturation pathways for C_{18} acids, which correspond to the individual classes of lipids, as shown in Figure 8. The introduction of the double bond at the Δ^{6} position takes place at the sn-1 position of MGDG but is essentially absent in SQDG and PG. This difference suggests that, perhaps, the desaturase, which introduces the double bond at the Δ^{6} position, is specific with respect to the polar head-group of the lipid and the sn-glycerol

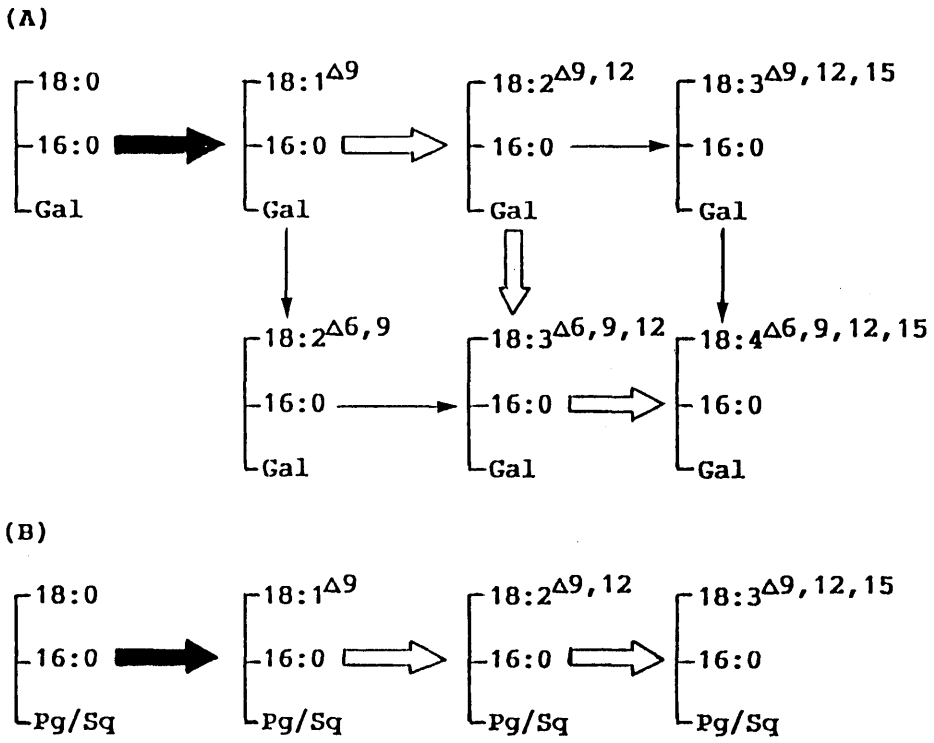


Fig. 8. The effect of a downward shift in temperature on the desaturation of fatty acids. Broad closed arrows represent desaturation reactions that are highly active regardless of the temperature; broad open arrows represent main desaturation reaction that stimulated after a shift in temperature; thin arrows represent minor desaturation reactions that are stimulated after a shift in temperature. (A) MGDG; (B), PG and SQDG. Gal, galactose; Pg, phosphoglycerol; Sq, sulfoquinovose; $18:2^{\Delta 6,9}$, $\Delta^{6,9}$-octadecadienoic acid.

position.

Sato and Murata (1981) reported that, in A. variabilis, the rapid desaturation of 16:0 to $16:1^{\Delta 9}$ in MGDG takes place in the dark as well as in the light, and that the slow desaturation of C_{18} acids is stimulated by light. However, the temperature-induced desaturation of C_{18} acids in Synechocystis PCC6803 takes place only in the light and is inhibited by DCMU, suggesting that this desaturation resembles the desaturation of C_{18} acids at the sn-1 position of lipids in A. variabilis.

Cerulenin, an inhibitor of fatty acid synthesis, did not affect the temperature-induced desaturation of fatty acids. Sato and Murata (1982b) have observed that the first step in lipid synthesis in A. variabilis is the esterification of saturated fatty acids to the glycerol moiety, and that the saturated acids are then desaturated to unsaturated fatty acids. Furthermore, they demonstrated that 16:0 is converted to $16:1^{\Delta 9}$ by a lipid-linked desaturation and not via a pathway consisting of deacylation, desaturation and reacylation (Sato et al. 1986). This type of lipid-linked desaturation is consistent with our observation that the synthesis of fatty acids de novo does not occur during the temperature-induced desaturation of fatty acids.

Fujii and Fulco (1977) reported that a desaturase in Bacillus megaterium, which introduces a double bond into the Δ^5 position of fatty acids, is transiently synthesized after a shift in the growth temperature from 35°C to 20°C (so-called "hyperinduction"). The same phenomenon was observed in A. variabilis (Sato and Murata 1981). By contrast, Skriver and

Thompson (1979) inferred that the accelerated desaturation of fatty acids in Tetrahymena pyriformis, upon a downward shift in temperature, results from the activation of membrane-bound desaturases by the change in membrane fluidity, and not by the induced synthesis of desaturases. The desaturase activity of Pseudomonas E-3 seems to be regulated in the same manner as that of T. pyriformis (Wada et al. 1987). The effect of chloramphenicol and rifampicin on the temperature-induced desaturation of fatty acids in the present study suggests that Synechocystis PCC6803 desaturates the fatty acids after the downward shift in temperature by the induction of desaturase activity, as do B. megaterium and A. variabilis.

Chapter 3

Synechocystis PCC6803 mutants defective in desaturation of fatty acids

Summary

Two mutants, which were defective in desaturation of fatty acids, were isolated from the cyanobacterium, Synechocystis PCC6803, by treating wild-type cells with ethyl methanesulfonate. They were designated as Fad6 and Fad12. Fad6 was defective in desaturation at the Δ^6 position of C_{18} fatty acids esterified to the sn-1 position of glycerol moiety in monogalactosyl diacylglycerol and digalactosyl diacylglycerol but not sulfoquinovosyl diacylglycerol and phosphatidylglycerol. This observation suggests that the desaturation affected in this mutant was specific to the sn-glycerol position, the chain length and the polar head group. Fad12 was defective in desaturation at the Δ^{12} position of C_{18} fatty acids at the sn-1 position of glycerol moiety in all the lipid classes. This observation suggests that the desaturation affected in this mutant was specific with respect to the sn-glycerol position and the chain length of fatty acids, but not to the polar head group of the lipids. At 22°C Fad6 grew as fast as the wild type, but the growth rate of Fad12 was much lower than that of wild type. At 34°C, on the other hand, the mutants and wild type grew at about the same rates.

Introduction

Cyanobacteria are prokaryotic algae, which can perform oxygenic photosynthesis with two photochemical reaction systems. The cyanobacterial cell has two membrane systems. One is an envelope, which is composed of outer and inner (plasma) membranes

separated by a peptidoglycan layer, and the other is an intracellular photosynthetic membrane system called thylakoid membranes. Its membrane structure is similar to that of the eukaryotic plant chloroplast (Stanier and Cohen-Bazire 1977). In addition, the lipid and fatty acid compositions of cyanobacterial membranes are similar to those of the chloroplast. Thus, the cyanobacterium can be regarded as a model system in the study of biosynthesis and functional roles of lipids in the eukaryotic chloroplast.

Sato and Murata (1980, 1981) have demonstrated that a cyanobacterium, A. variabilis, responds to low temperature by desaturating the fatty acids of membrane lipids. The rapid fatty acid desaturation is regarded as an emergency acclimation to compensate for the decrease in membrane fluidity due to a decrease in temperature. A number of organisms are also able to desaturate the fatty acids of membrane lipids in response to low temperature (Cossins and Sinensky 1984, Russell 1984, Thompson 1980), and this phenomenon is called "homeoviscous adaptation" (Sinensky 1974). However, the enzymes involved in the fatty acid desaturation in plants have long been in question.

In the present study we used Synechocystis PCC6803 for the isolation of mutants in fatty acid desaturation. This organism is unique because it is transformable with exogenously added DNA (Grigorieva and Shestakov 1982). This may open a molecular-biological approach to the above-mentioned problems. We isolated two mutants which were defective in the fatty acid desaturation at Δ^6 and Δ^{12} position of C_{18} fatty acids. They are designated as Fad6

and Fad12, respectively.

Materials and Methods

Organism and culture conditions--- Synechocystis PCC6803 was grown photoautotrophically at 34 or 22°C in BG-11 medium (Stanier et al. 1971) supplemented with 20 mM HEPES-NaOH (pH 7.5) under illumination of incandescent lamps (70 μE $m^{-2}s^{-1}$) with aeration of 1.0% CO_2 in air as described in Chapter 2. Cultures at the exponential growth phase were used for experiments.

Isolation of mutants---Wild type cells grown at 34°C were collected by centrifugation at 2500 x g for 10 min at the same growth temperature. The collected cells were suspended in BG-11 at a cell density of 2.5 x 10^9 cells ml^{-1}. To 1 ml of the cell suspension 1 ml of 0.4 M ethyl methanesulfonate in 30 mM K-phosphate (pH 7.0) was added. The suspension was vortexed and incubated at 37°C for 45 min in the dark. This treatment resulted in 0.1 - 1.0% cell survival. Ethyl methanesulfonate was inactivated by the addition of 5% sodium thiosulfate, and the mutagenized cells were collected by centrifugation at 2500 x g for 15 min. The cells were washed twice with 20 ml of BG-11 by centrifugation at 2500 x g for 15 min and by resuspension. Low temperature-sensitive mutants were isolated by the two following methods.

In the first method, the cells prepared as above were suspended in 15 ml of BG-11, and then divided into five test tubes. They were incubated with aeration of 1.0% CO_2 in air in the light at 22°C for 2 days. Then, the cell suspension was further incubated at 22°C for 2 days in the presence of ampicillin at 100 μg ml^{-1} for

enrichment of the low temperature-sensitive mutants. After the cells were washed with BG-11 by centrifugation at 2500 x g for 15 min and resuspension, they were suspended in 15 ml of BG-11, and then grown in five test tubes each containing 3 ml of the culture at 34°C for 3 days. The cells from each tube were plated on an agar plate of 90 mm diameter containing BG-11 and 1.2% agar and incubated in the light at 34°C until colonies were formed. The colonies were transferred to a pair of agar plates. One plate was incubated at 34°C and the other at 22°C. Four low temperature-sensitive mutants, which grew slower than the wild type at 22°C but not at 34°C, were isolated.

In the second method, the mutagenized cells mentioned above were suspended in 1 ml of BG-11 and serially diluted with BG-11 to 10^{-1}, 10^{-2} and 10^{-3}. To 0.1 ml of each of the cell suspension, 5 ml soft agar containing 0.6% agar in BG-11 supplemented with 20 mM TES-KOH (pH 8.0) and 2.5 mM sodium thiosulfate, was added and mixed. This mixture was layered onto a plate of 90 mm diameter prelayered with 15 ml of the soft agar. After solidification, another 5 ml of the soft agar was over-layered and left to solidify. The plates were incubated at 22°C for 5 days in the light. For enrichment of low temperature-sensitive mutants, 0.45 mg ampicillin in 0.1 ml water was poured onto each plate and the plates were incubated at 22°C for 2 days. Then ampicillin was hydrolyzed by adding 1.7 units of penicillinase in 0.1 ml water. Colonies which appeared after incubation at 22°C in the light for 7 - 10 days were marked. The plates were further incubated at 34°C for 6 days. Additional colonies, which were putative low temperature-sensitive mutants,

emerged. All these colonies were transferred to test tubes each containing 15 ml of BG-11 and incubated at 34°C for 14 days in the light without aeration. Then, 20 μl of each culture was inoculated to a pair of microplates each containing 0.35 ml of BG-11 in order to examine the growth rate at low temperature. One plate was incubated at 34°C and the other at 22°C both for 10 days. By comparing the growth rates of mutants at 34 and 22°C with those of wild type, 22 low temperature-sensitive mutants were isolated.

The 26 low temperature-sensitive mutants, four by the first method and 22 by the second method, were grown separately at 34°C in 15 ml of BG-11 with aeration of 1.0% CO_2 in air under illumination of 70 μE $m^{-2}s^{-1}$. By analysing fatty acid composition of their membrane lipids, two mutants defective in the desaturation of fatty acids were obtained and designated as Fad6 and Fad12 (originally obtained by the second and the first methods, respectively).

Lipid and fatty acid analyses--- Lipid and fatty acid analyses were carried out according to the methods described in Chapter 2.

Results

Lipid composition--- Lipid compositions of the wild type and desaturation mutants of Synechocystis PCC6803 are compared in Table 7. This cyanobacterium contained four major glycerolipids, MGDG, DGDG, SQDG and PG, as other cyanobacteria (Murata and Nishida 1987). In the wild type grown at 34°C the relative content of MGDG was 59% of total glycerolipids, and those of

Table 7 Lipid composition of the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803 grown at 34 and 22°C

Lipid	Growth temperature					
	34°C			22°C		
	WT	Fad6	Fad12	WT	Fad6	Fad12
	(mol %)					
MGDG	59	61	58	54	59	52
DGDG	17	18	11	18	18	15
SQDG	16	12	19	15	9	13
PG	8	9	12	13	14	20

WT, wild type.

DGDG, SQDG and PG amounted to 8 - 17%. The lipid composition of the mutants, Fad6 and Fad12, was essentially the same as that of wild type. The growth temperature had no significant effect on the lipid composition in either wild type or mutants.

Fatty acid composition---The fatty acid compositions of total lipids from wild type and desaturation mutants are shown in Table 8. The ratio of C_{18} to C_{16} acids was not affected by mutation, and 16:0 amounted to about 95% of the total C_{16} acids in the wild type and mutants. Significant changes by mutation occurred in the unsaturation of the C_{18} acids. At 34°C, the wild type contained high levels of $18:1^{\Delta 9}$, $18:3^{\Delta 6,9,12}$ and $18:2^{\Delta 9,12}$. In Fad6, as compared with the wild type, $18:2^{\Delta 9,12}$ increased and $18:3^{\Delta 6,9,12}$ decreased to a trace amount. In Fad12, $18:1^{\Delta 9}$ and $18:2^{\Delta 6,9}$ significantly increased whereas $18:3^{\Delta 6,9,12}$ and $18:2^{\Delta 9,12}$ decreased to trace amounts. It is striking that Fad6 was deficient in fatty acids having the Δ^6 double bond, and that Fad12 lacked fatty acids having the Δ^{12} double bond.

At 22°C, the wild type contained relatively high levels of $18:3^{\Delta 6,9,12}$, $18:4^{\Delta 6,9,12,15}$, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$. In Fad6, as compared with the wild type, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ decreased whereas $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ increased. In Fad12, $18:1^{\Delta 9}$ and $18:2^{\Delta 6,9}$ significantly increased at the expense of the major C_{18} acids present in the wild type. These findings indicate that Fad6 was defective in the desaturation at the Δ^6 position of C_{18} acids at 34°C, but not so strictly at 22°C, and that Fad12 at both 34 and 22°C was defective in fatty acid desaturation at the Δ^{12} position of C_{18} acids. It is likely that the desaturation at the

Table 8 Fatty acid composition of total lipids from the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803 grown at 34 and 22°C

Fatty acid	Growth temperature					
	34°C			22°C		
	WT	Fad6	Fad12	WT	Fad6	Fad12
	(mol %)					
14:0	1	1	t	t	t	1
16:0	58	57	59	51	55	53
$16:1^{\Delta 9}$	3	3	2	3	3	3
18:0	1	1	1	t	1	1
18:1*	t	2	t	t	2	t
$18:1^{\Delta 9}$	7	11	32	2	3	25
$18:2^{\Delta 6,9}$	t	t	5	t	t	16
$18:3^{\Delta 6,9,12}$	17	t	t	21	5	0
$18:4^{\Delta 6,9,12,15}$	t	0	0	8	4	0
$18:2^{\Delta 9,12}$	12	25	t	6	17	t
$18:3^{\Delta 9,12,15}$	t	t	t	8	10	t

WT, wild type; t, trace amount (less than 0.4%).

Superscripts represent the positions of cis-double bonds.

*The position of double bond differed from the Δ^9 position, but was not determined.

Δ^6 position in Fad6 is sensitive to high temperature as the Δ^{15} desaturation in a mutant of Arabidopsis thaliana, JB1 (Browse et al. 1986).

In order to determine which lipids were affected in the mutants, we examined the fatty acid compositions of individual lipid classes from the wild type and desaturation mutants. In wild type cells grown at 34°C (Table 9), 16:0, $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ were major fatty acids in all lipid classes, whereas $18:3^{\Delta 6,9,12}$ was restricted to MGDG and DGDG. The relative content of 16:0 amounted to about 50% in MGDG, DGDG and PG, but was far above 50% in SQDG. The 16:0 and $16:1^{\Delta 9}$ remained relatively constant among the wild type and mutants. In MGDG and DGDG of Fad6, as compared with the wild type, $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ contents increased whereas $18:3^{\Delta 6,9,12}$ markedly decreased. In SQDG and PG, $18:1^{\Delta 9}$ increased at the expense of $18:2^{\Delta 9,12}$, although these changes were insignificant if compared with the significant change in MGDG and DGDG. These findings suggest that Fad6 was defective in the desaturation at Δ^6 position of C_{18} acids in MGDG and DGDG, and slightly affected the desaturation of Δ^{12} position of C_{18} acids in SQDG and PG. In MGDG and DGDG of Fad12, as compared with the wild type (Table 9), $18:1^{\Delta 9}$ and $18:2^{\Delta 6,9}$ significantly increased whereas $18:3^{\Delta 6,9,12}$ and $18:2^{\Delta 9,12}$ decreased to trace amounts. In SQDG and PG, $18:1^{\Delta 9}$ increased at the expense of $18:2^{\Delta 9,12}$. These findings suggest that Fad12 is defective in desaturation at the Δ^{12} position of C_{18} acyl group in all lipid classes but capable of the desaturation at Δ^6 position in MGDG and DGDG.

The fatty acid compositions of individual lipid classes from

Table 9 Fatty acid composition of lipid classes from the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803 grown at 34°C

Fatty acid	MGDG			DGDG			SQDG			PG		
	WT	Fad6	Fad12	WT	Fad6	Fad12	WT	Fad6	Fad12	WT	Fad6	Fad12
						(mol %)						
14:0	t	t	t	2	t	1	t	1	t	3	2	1
16:0	57	52	54	58	53	55	76	72	79	56	53	55
$16:1^{\Delta 9}$	2	3	2	2	3	3	3	3	3	1	1	1
18:0	t	1	1	t	1	1	1	2	2	1	2	1
18:1*	t	1	t	t	t	t	t	3	t	t	2	t
$18:1^{\Delta 9}$	6	11	36	3	8	32	9	13	15	9	16	41
$18:2^{\Delta 6,9}$	t	t	7	t	t	8	t	0	0	t	0	0
$18:3^{\Delta 6,9,12}$	24	1	0	25	1	0	1	0	0	1	0	0
$18:4^{\Delta 6,9,12,15}$	t	t	0	t	t	0	0	0	0	0	0	0
$18:2^{\Delta 9,12}$	10	31	t	9	33	t	9	6	t	27	23	t
$18:3^{\Delta 9,12,15}$	t	t	t	t	1	t	t	0	0	2	1	t

WT, wild type; t, trace (less than 0.4%).

Superscripts represent the positions of cis-double bonds.

*The position of double bond differed from the Δ^9 position, but was not determined.

Table 10 Fatty acid composition of lipid classes from the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803 grown at 22°C

Fatty acid	MGDG			DGDG			SQDG			PG		
	WT	Fad6	Fad12	WT	Fad6	Fad12	WT	Fad6	Fad12	WT	Fad6	Fad12
						(mol %)						
14:0	t	t	t	1	t	t	1	1	t	t	1	t
16:0	50	51	52	51	52	55	63	58	66	51	52	55
$16:1^{\Delta 9}$	3	3	4	4	4	4	4	5	4	1	1	2
18:0	t	1	0	1	1	1	1	2	1	1	2	1
18:1*	t	2	t	t	t	t	t	4	t	t	2	t
$18:1^{\Delta 9}$	2	3	19	2	4	19	5	12	27	2	7	40
$18:2^{\Delta 6,9}$	t	t	24	t	t	21	0	0	1	0	0	1
$18:3^{\Delta 6,9,12}$	29	6	t	24	8	t	2	t	0	2	t	0
$18:4^{\Delta 6,9,12,15}$	10	5	0	10	6	0	t	t	0	1	0	0
$18:2^{\Delta 9,12}$	5	19	t	6	16	t	10	12	0	5	14	1
$18:3^{\Delta 9,12,15}$	0	10	t	1	8	0	13	5	0	35	20	0

WT, wild type; t, trace (less than 0.4%).

Superscripts represent the positions of cis-double bonds.

*The position of double bond differed from the Δ^9 position, but was not determined.

the wild type and desaturation mutants grown at 22°C are compared in Table 10. In the wild-type cells grown at 22°C, 16:0, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ were major fatty acids in MGDG and DGDG, whereas 16:0, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ were major fatty acids in SQDG and PG. The relative content of 16:0 amounted to about 50% in MGDG, DGDG and PG, but far above 50% in SQDG. The 16:0 and $16:1^{\Delta 9}$ remained relatively constant among the wild type and mutants. In MGDG and DGDG of Fad6, as compared with those of the wild type, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ contents increased whereas $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ contents decreased to about 5%. In SQDG and PG, $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ increased at the expense of $18:3^{\Delta 9,12,15}$, although this effect was only minor. These findings suggest that also at 22°C Fad6 was defective in the desaturation at Δ^6 position of C_{18} fatty acids in MGDG and DGDG but not so strictly as at 34°C. In MGDG and DGDG of Fad12, as compared with those of the wild type, $18:1^{\Delta 9}$ and $18:2^{\Delta 6,9}$ significantly increased whereas $18:3^{\Delta 6,9,12}$ and $18:2^{\Delta 9,12}$ decreased to trace amounts. In SQDG and PG, $18:1^{\Delta 9}$ increased at the expense of $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$. These findings suggest that also at 22°C Fad12 is defective in desaturation at the Δ^{12} position of C_{18} acyl group in all lipid classes.

Positional distribution of fatty acids---The positional distribution of fatty acids in lipids from the wild type cells was analyzed by specific hydrolysis with Rhizopus lipase. The result indicates that only 16:0 was esterified to the sn-2 position of the glycerol moiety and that the other fatty acids including all the C_{18} acids and 16:1 were esterified to the sn-1 position. A minor

contribution of 16:0 at the sn-1 position was also noticed. This type of positional distribution, in which the C_{18} and C_{16} fatty acids are selectively and exclusively bound to the sn-1 and sn-2 positions, respectively, has been observed also in A. variabilis (Murata and Nishida 1987).

Effect of temperature on growth---The growth rates at the exponential phase of the wild type and mutants are compared in Table 11. At 34°C there was no great difference among the wild type and mutants. At 22°C, the Fad6 grew at the same rate as the wild type. However, when the cells were grown in BG-11 without aeration, the growth rate of Fad6 was lower than that of the wild type. The growth rate of Fad12 was only one-third of the wild type at 22°C. This observation suggests that the Fad12 mutation has a deleterious effect on the growth rate at 22°C but not at 34°C. The effect of downward temperature shift on the growth profile of the wild type and mutants was examined (Fig. 9). When the growth temperature was changed from 34 to 22°C, the wild type and Fad6 begun to grow after a lag phase of 15 h and Fad12 did so after the lag phase of 25 h. These results suggest that the capability of adapting to the downward temperature shift is lower in Fad12 than in the wild type and Fad6.

Discussion

The present study demonstrated that only 16:0 was esterified to the sn-2 position of glycerol moiety of all the major glycerolipids, MGDG, DGDG, SQDG and PG, from Synechocystis PCC6803, and that this fatty acid was desaturated neither in the wild

Table 11 Doubling time at the exponential growth phase of the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803

Strain	Growth temperature	
	34°C	22°C
	(h)	
Wild type	14	20
Fad6	13	21
Fad12	15	59

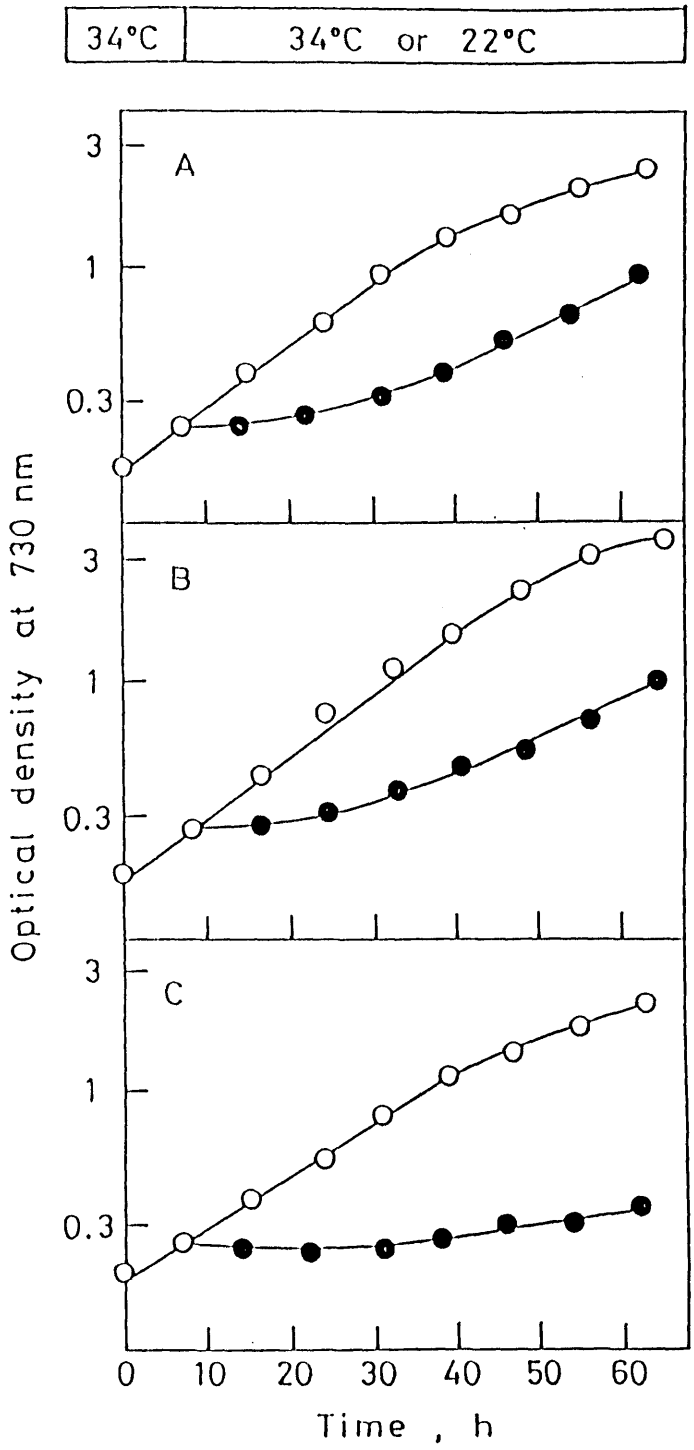


Fig. 9. The growth profile of the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803. A, wild type; B, Fad6; C, Fad12. The cells precultured at 34°C were inoculated into a pair of bottles containing 500 ml of BG-11 supplemented with 20 mM HEPES-NaOH (pH 7.5). One culture was incubated at 34°C throughout the experiment (--o--), and the other was incubated at 34°C for 8 h and then at 22°C (--•--). The incubation was done under illumination of incandescent lamps (70 μE $m^{-2}s^{-1}$) with aeration of 1.0% CO_2 in air.

type nor in the desaturation mutants (Tables 9 and 10). The C_{18} fatty acids, in turn, were esterified to the sn-1 position in all the major lipid classes, although there was a minor contribution of 16:0 and $16:1^{\Delta 9}$ at this position. These findings indicate that the major molecular species in Synechocystis PCC6803 are of a sn-1-C_{18}-sn-2-16:0 type. This type of positional distribution, sn-1-C_{18}-sn-2-C_{16}, is found in A. variabilis (Murata and Nishida 1987, Sato et al. 1979).

The biosynthetic pathways of glycerolipid molecular species in cyanobacteria have been most intensively studied in A. variabilis (Murata and Nishida 1987). The pulse-labeling and chase experiments with $H^{14}CO_3^-$ in vivo have demonstrated that the primary products of lipids are 18:0/16:0-GlcDG, 18:0/16:0-SQDG and 18:0/16:0-PG (Sato and Murata 1982b). MGDG is synthesized from GlcDG by the epimerization of glucose to galactose (Sato and Murata 1982a). The desaturation of fatty acids takes place in GlcDG, MGDG, SQDG and PG. DGDG is produced by transfer of galactose from an unidentified galactose carrier to MGDG and no desaturation of fatty acids can be demonstrated in DGDG (Sato and Murata 1982b). These observations have suggested that only saturated fatty acids, 18:0 and 16:0, are first esterified to the sn-1 and sn-2 positions of glycerol moiety, form sn-1-18:0-sn-2-16:0 type molecular species, and then desaturated while they are bound to the lipids (Sato et al. 1986). This was supported by in vitro experiments of Lem and Stumpf (1984a) as well as Stapleton and Jaworski (1984), in which only saturated fatty acids were produced from [^{14}C]acetate in the extract of A. variabilis and neither added 18:0-ACP nor 18:0-CoA

was desaturated. Based on these studies and the results in the present study, we propose hypothetical pathways of biosynthesis of glycerolipid molecular species in wild type and mutants of Synechocystis PCC6803 as well as the sites of the lesions in the mutants (See Fig. 10).

In MGDG from wild type, the major C_{18} acids were $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. This suggests that the C_{18} acids are sequentially desaturated from 18:0 to $18:4^{\Delta 6,9,12,15}$ through $18:1^{\Delta 9}$, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 6,9,12}$. Therefore, we propose the major pathway for the biosynthesis of MGDG molecular species as indicated by broad arrows in Fig. 10A. However, in Fad6 in which the Δ^6 desaturation was blocked, $18:3^{\Delta 9,12,15}$ was produced (Table 10). This demonstrates the presence of desaturation pathway from $18:2^{\Delta 9,12}$ to $18:4^{\Delta 6,9,12,15}$ through $18:3^{\Delta 9,12,15}$ as indicated by thin arrows in Fig. 10A. In Fad12 in which the Δ^{12} desaturation was blocked, $18:2^{\Delta 6,9}$ was produced. This demonstrates the occurrence of desaturation pathway from $18:1^{\Delta 9}$ to $18:3^{\Delta 6,9,12}$ through $18:2^{\Delta 6,9}$ as indicated by thin arrows in Fig. 10A. These side pathways appeared to be demonstrable by fatty acid analysis of lipids from the desaturation mutants. In PG and SQDG, no C_{18} acid contained the Δ^6 unsaturation. This suggests that the desaturation pathway of these lipids is a simple sequence as presented in Fig. 10B.

It has been suggested that one of the roles of polyunsaturated fatty acids is the maintenance of biological membranes at proper fluidity especially at low temperature (Chapman 1975). The observation that Fad6 could grow well at 22°C as the wild type in spite of the reduced level of $18:3^{\Delta 6,9,12}$, indicates that, in

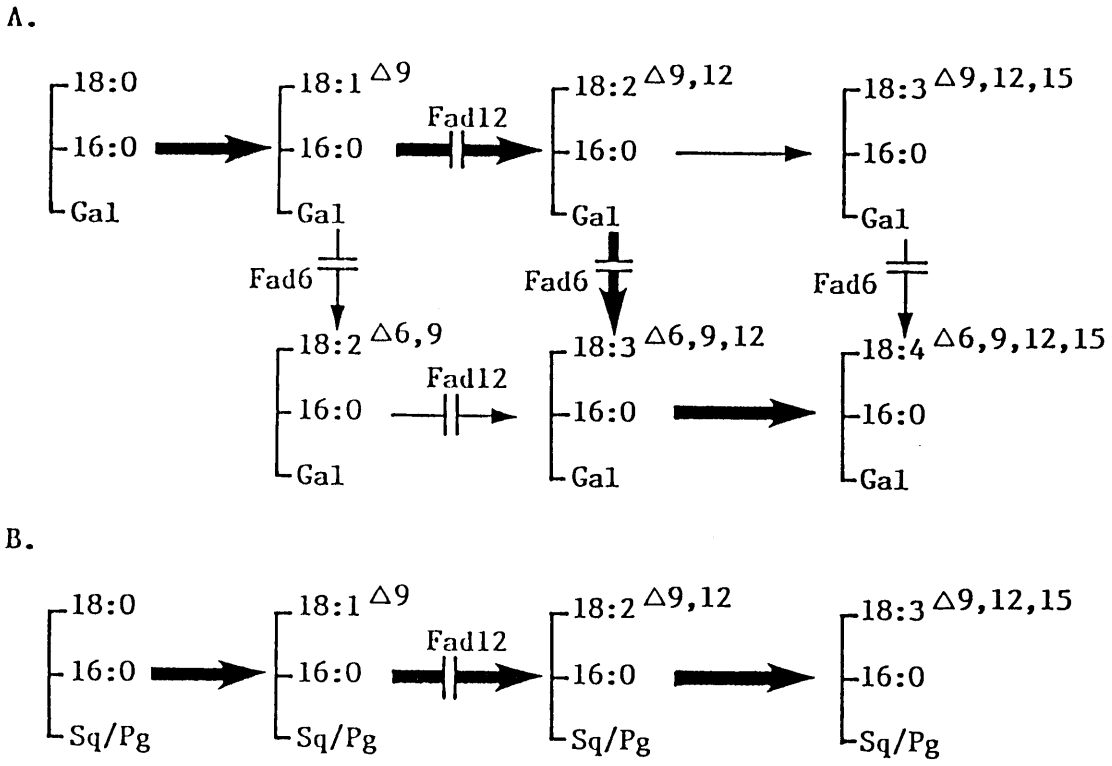


Fig. 10. Proposed scheme for the biosynthesis of glycerolipid molecular species in the wild type and desaturation mutants, Fad6 and Fad12, of Synechocystis PCC6803. Broad arrows indicate desaturations of the main pathway, and thin arrows are minor desaturations. Breaks in the arrows represent blocked desaturation reactions in the mutants. (A), MGDG; (B), SQDG and PG. Gal, galactose; Sq, sulfoquinovose; Pg, phosphoglycerol.

Synechocystis PCC6803, the desaturation from the dienoic to trienoic acid does not significantly affect the membrane fluidity with respect to the optimization for physiological activities. On the other hand, the growth rate at 22°C of Fad12 was much lower than that of wild type. This suggests that the dienoic acid (18:2) is most effective in maintaining the proper conditions of membrane fluidity at 22°C. Browse, Somerville and their colleagues isolated a mutant of A. thaliana deficient in $C_{18:3}$ and $C_{16:3}$ in leaf lipids (Browse et al. 1986, Somerville et al. 1987). They observed that this mutation had no apparent effect on growth and photosynthesis in the temperature range from 10 to 30°C (McCourt et al. 1987). The mutation also has only a slight effect on membrane fluidity as measured by fluorescence polarization. Our results in Synechocystis PCC6803 are consistent with their results in A. thaliana.

The present study clearly indicates that the mutants, Fad6 and Fad12, are defective in the desaturation of C_{18} fatty acids at the position of Δ^6 and Δ^{12}, respectively. It is reasonable to assume that the cyanobacterial cells retain several kinds of desaturases which are specific to the fatty acid, the sn-position of glycerol moiety and the polar head group. The explanation for the lack of desaturation in our mutants is that the mutation could occur at the genes of desaturases: In Fad6, a gene for one of the desaturases which introduces a cis-double bond at the Δ^6 position of C_{18} acids at the sn-1 position of MGDG was modified, whereas in Fad12, a gene for another desaturase which introduces a cis-double bond at the Δ^{12} position of C_{18} acids at the sn-1 position of MGDG, PG and SQDG was modified.

Synechocystis PCC6803 can be transformed with exogenously added DNA fragments by homologous recombination (Williams 1988). The isolation of desaturation mutants of this cyanobacterium may open an approach to desaturase genes, since these genes may be isolated from a gene library by complementation of the mutants. This hopefully leads to a study to answer how the organism synthesizes desaturases in response to the ambient temperature.

Chapter 4

Cloning and nucleotide sequence of a gene for plant-type desaturase of fatty acid (*desA*) from the cyanobacterium, *Synechocystis* PCC6803

Summary

A plasmid clone with an 8-kbp insert, which complemented the mutant of Synechocystis PCC6803 (Fad12) defective in desaturation at the Δ^{12} position of fatty acids, was isolated from the genomic library constructed in pTZ19R. Digestion of the insert with AvaI yielded a 1.5-kbp fragment which also complemented Fad12. This fragment was subcloned into a Bluescript and sequenced. It included an open-reading frame of 1053 bp which coded for 351 amino acids and corresponded to a relative molecular mass of 40494 daltons. The cloned gene was designated as desA. Anacystis nidulans R2-SPc, which is defective in the Δ^{12} desaturase activity, was transformed with the desA gene of Synechocystis using a shuttle vector pUC303. The resultant transformant of A. nidulans R2-SPc acquired a Δ^{12} desaturase activity, which was dependent on growth temperature as in Synechocystis. These findings suggest that the desA gene encodes the Δ^{12} desaturase.

Introduction

Cyanobacteria respond to low temperature by desaturating fatty acids of membrane lipids to compensate for the decrease in membrane fluidity (Murata and Nishida 1987). A number of other organisms use the same strategy to protect the cells against the low-temperature effect (Cossins and Sinensky 1984, Russell 1984, Thompson 1980). Sato and Murata (1980, 1981, 1982b) demonstrated that the low temperature-induced desaturation of fatty acids in the cyanobacterium, Anabaena variabilis, takes place in

monogalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol and phosphatidylglycerol.

Recently we studied the low temperature-stimulated desaturation of fatty acids in the cyanobacterium, Synechocystis PCC6803 (Chapter 2). This alga introduces double bonds at $\Delta^{6,9,12,15}$ positions of the C_{18} fatty acids which are esterified to the sn-1 position of glycerol moiety of glycerolipids. The unsaturation at the Δ^{6} position is restricted to monogalactosyl diacylglycerol and digalactosyl diacylglycerol but not to sulfoquinovosyl diacylglycerol and phosphatidylglycerol. When the growth temperature was shifted from 38°C to 22°C, the desaturation of C_{18} acids, but not that of C_{16} acids, was stimulated. This low-temperature-stimulated desaturation is suppressed, when de novo synthesis of proteins is inhibited in the presence of chloramphenicol, an inhibitor of protein synthesis. These results suggest that Synechocystis contains several fatty acid desaturases each of which introduces a double bond at its own specific position, and that the low temperature induces, or enhances, the syntheses of unsaturated fatty acids. In order to understand the molecular mechanism of temperature acclimation of the cyanobacterium, it is inevitable to isolate the genes for desaturases and to study the temperature-induced regulation of the expression of these genes.

The plant-type desaturases introduce a double bond into fatty acids bound to glycerolipids (Harwood 1988). In animal, in contrast, all the desaturations of fatty acids take place before they are bound to glycerolipids (Holloway 1983). None of the

desaturases has been isolated from algae or plants, because the solubilization of the enzymes and reconstitution of their activity *in vitro* are difficult (Harwood 1988).

Thus, we addressed the gene isolation by molecular-genetical method. Two mutants of *Synechocystis* PCC6803, Fad6 and Fad12, have been isolated, which are defective in the fatty acid desaturation at Δ^6 and Δ^{12} positions of C_{18} acids, respectively (Chapter 3). With use of these mutants, it is possible to isolate the corresponding desaturase genes, because this cyanobacterium can be easily transformed with exogenously added DNA (Williams 1988).

In the present study, we describe the isolation and characterization of the gene of *Synechocystis* PCC6803 designated as *desA* which encodes a fatty acid desaturase that introduces a *cis*-double bond at the Δ^{12} position of fatty acid esterified to glycerolipids.

Materials and Methods

Organisms and culture conditions---The wild type, the desaturation mutant (Fad12) and the transformant of *Synechocystis* PCC6803 (Fad12/*desA*, see text) and the wild type of *A. nidulans* R2-SPc were grown in the BG-11 medium in the light as described previously (Chapter 3). The transformants of *A. nidulans* were cultivated in the same way but in the presence of 7.5 $\mu g\ ml^{-1}$ chloramphenicol in the culture condition.

Fatty acid analysis---Lipids were extracted from the cells by the method of Bligh and Dyer (1959). The total lipids were

subjected to methanolysis with 5% HCl in methanol at 85°C for 2.5 h. The resultant methyl esters were analyzed by gas-liquid chromatography as described previously in Chapter 3.

Transformation of Fad12 with DNA---In order to enrich or clone DNAs which could complement the fatty acid desaturation of Fad12, the in situ trasformation technique (Dzelzkalns and Bogorad 1988) was employed. The Fad12 cells were mixed with agar to give a 0.6% agar in BG-11 supplemented with 20 mM TES-KOH (pH 8.0) and 2.5 mM sodium thiosulfate. This mixture was poured onto a plate prelayered with 0.6% agar in BG-11. After solidification of the mixture, the test samples of DNAs in 10 mM Tris-HCl (pH 8.0) and 1.0 mM Na_2EDTA were applied directly onto the plate. Then the plate was incubated at 22°C for 10 days. Transformants with recovered growth rates gave green spots on the agar plates (see text).

Isolation of DNA clone from Synechocystis PCC6803 DNA---The DNA of the wild-type of Synechocystis PCC6803 was prepared according to Williams (1988). Then it was digested with EcoRI. The resultant fragments were separated into 12 fractions according to their sizes by gel electrophoresis with 1.0% low melting point agarose. The individual fractions were melted at 65°C for 10 min and aliquots of the separated fractions were assayed for their ability to restore the growth of Fad12 at 22°C by the in situ transformation. A fraction containing the fragments of 8-12 kbp was obtained as an enriched DNA fraction. The DNA in this fraction was ligated to the EcoRI site of alkaline phosphatase treated pTZ19R (Pharmacia Molecular

Biochemicals) according to the standard protocols (Maniatis et al. 1982). After transformation of E. coli JM105 with the recombinant plasmids, a plasmid library of about 1500 clones was obtained. Plasmid DNAs used in cloning and transformation experiments were isolated from E. coli JM105 by boiling method for minipreparation or alkali method for middle scale preparation (Maniatis et al. 1982). The 500 clones of the enriched genomic library were tested for their ability to complement Fad12, and one positive clone, which contained an 8-kbp insert, was isolated. This insert was digested with AvaI and DraI to obtain, respectively, 1.5-kbp and 2.4-kbp fragments which were capable of complementing the fatty acid desaturation of Fad12. The 2.4-kbp DraI fragment was digested with EcoRI into a 1.1-kbp fragment by removing a 1.3-kbp fragment of the plasmid origin. The 1.1-kbp fragment was also capable of complementing the fatty acid desaturation of Fad12. The 1.5-kbp AvaI fragment and the 1.1-kbp DraI-EcoRI fragment were subcloned into a Bluescript. The resultant recombinant plasmids designated as pAV3 and pDE2 could transform Fad12.

DNA sequence analysis---The DNA insert containing the gene which complemented Fad12 were ligated to a Bluescript (Stratagene Cloning Systems), and the recombinant plasmids were amplified in E. coli JM105. The unidirectional deletions of the plasmids were performed according to the manual on Bluescript DNA sequencing system (Stratagene Cloning systems). The nucleotide sequence of deleted plasmids was determined by the dideoxy chain-termination method using double-stranded DNA templates (Tabor and Richardson

1987; also United States Biochemical Corporation manual on DNA sequencing with Sequenase T7 DNA polymerase). Some of primers for nucleotide chain elongation were prepared with a DNA synthesizer (model 381A, Applied Biosystems).

Transformation of Anacystis nidulans---In order to characterize the desA gene of Synechocystis PCC6803, this gene was ligated into a shuttle vector pUC303 (Kuhlemeier et al. 1983) by integrating the 1.5-kbp SacI fragment (SacI site originated from pTZ19R to SacI site in Bluescript) from pAV3 into the SacI site of pUC303. This vector has two markers, streptomycin and chloramphenicol resistance, and three unique restriction sites. Transformation of A. nidulans R2-SPc with a shuttle vector pUC303 was carried out according to Kuhlemeier and van Arkel (1987). A. nidulans R2-SPc and pUC303 were provided by Dr. N. Sato of University of Tokyo, who obtained them from Dr. W. E. Borrias of University of Utrecht.

Results

Isolation of the gene which complements the desaturation mutant of Synechocystis PCC6803---The desaturation mutant of Synechocystis PCC6803, Fad12, is defective in capability of introducing a double bond at the Δ^{12} position of the C_{18} fatty acids. The growth rate at 22°C of Fad12 is one-third of that of the wild type (Table 11 in Chapter 3). The transformation of Fad12 with various types of DNAs is shown in Figure 11. The wild-type DNA was able to transform the mutant to the wild type with respect to the growth rate at 22°C. The frequency of

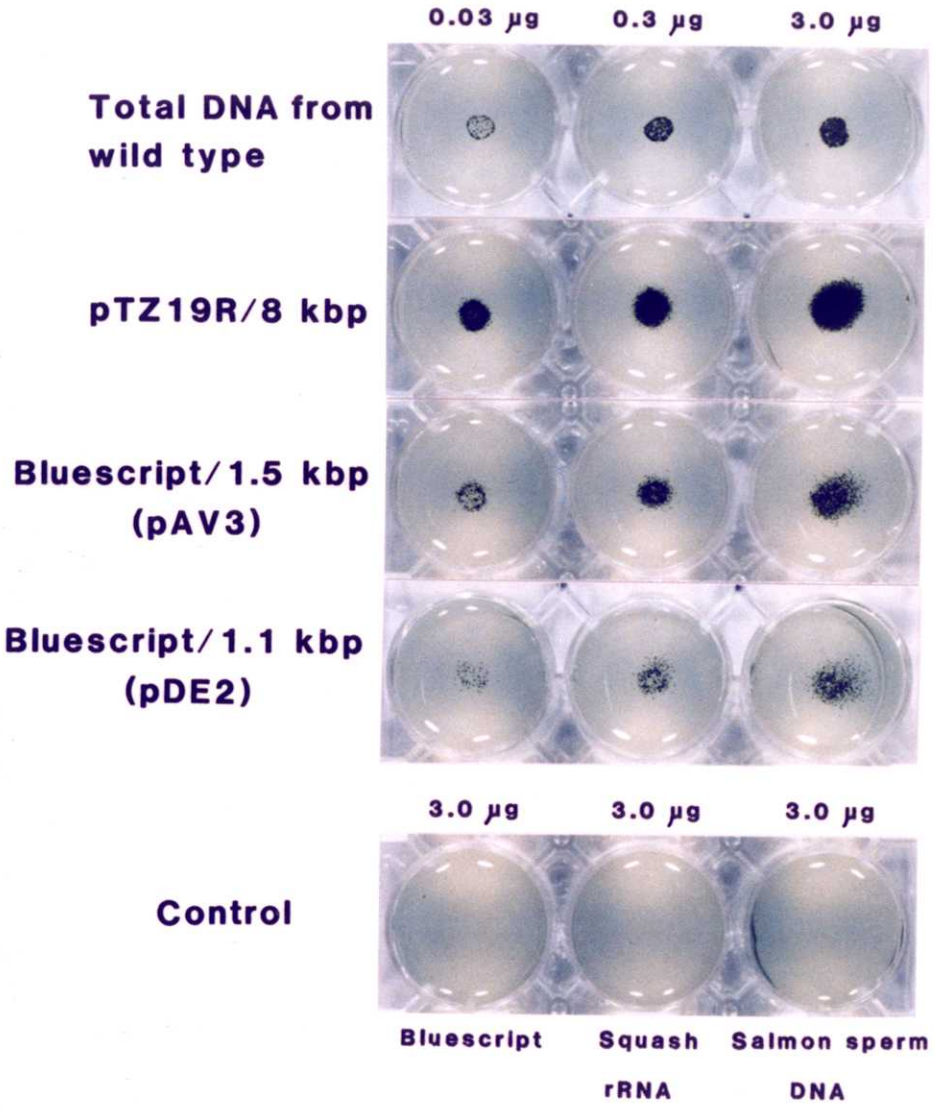


Fig. 11. Transformation with various kinds of DNA of the desaturation mutant, Fad12, of Synechocystis PCC6803. The cells of Fad12 were applied to the agar plate. The DNA from wild type of Synechocystis PCC6803, pAV3 and pDE2 dissolved in 3 µl of 10 mM Tris-HCl (pH 8.0) and 1.0 mM Na_2EDTA were spotted onto the plate. Then, the plate was incubated at 22°C.

transformation depended on DNA concentration. The pAV3 and pDE2 also could complement Fad12 as shown in Figure 11. The pAV3 contained an insert of 1505 bp including a SmaI-EcoRI 16-bp fragment which originated from pTZ19R. The pDE2 contained a 1089 bp EcoRI-DraI fragment which is identical to the EcoRI-DraI region of an insert of pAV3. Figure 12a shows the restriction map of the insert of 1489 bp in pAV3 without the 16-bp fragment from pTZ19R.

The transformants, which appeared on an agar plate as a spot (Figure 11), were isolated from the plates to investigate the fatty acid composition of total lipids. The fatty acid compositions of wild type, Fad12 and one of the transformants are shown in Table 12. The transformants of Fad12 with pAV3 and pDE2, had the same fatty acid composition as that of the wild type. This finding indicates that Fad12 was complemented to the wild type and acquired the gene involved in the desaturation of fatty acids at the Δ^{12} position.

Nucleotide sequence and deduced amino acid sequence---The nucleotide sequence of Δ^{12} desaturase gene (designated desA, see below) was determined according to the strategy shown in Figure 10b. Figure 13 shows the nucleotide sequence and the deduced amino acid sequence. A 1053-bp open-reading frame beginning at an ATG codon at position 1 and ending at a TAA ochre codon at position 1054 was found in the sequence which corresponds to 351 amino acids and a relative molecular mass of 40494 daltons. There are five ATG codons which lie in frame within the open-reading frame. A potential ribosome-binding site GGAG (Shine and Dalgarno

Table 12 Fatty acid composition of total lipids from the wild type, the desaturation mutant, Fad12, and the transformants of Fad12 with pAV3 and pDE2 (Fad12/pAV3 and Fad12/pDE2), of Synechocystis PCC6803 grown at 34°C

Fatty acid	Strain			
	Wild type	Fad12	Fad12/pAV3	Fad12/pDE2
	(mol %)			
14:0	1	t	1	1
16:0	58	59	59	57
$16:1^{\Delta 9}$	3	2	2	3
18:0	1	1	2	1
$18:1^{\Delta 9}$	8	32	9	10
$18:2^{\Delta 6,9}$	t	5	1	1
$18:2^{\Delta 9,12}$	12	t	12	12
$18:3^{\Delta 6,9,12}$	17	t	14	15

t, trace amount (less than 0.4%).

Superscripts represent the positions of cis double bonds.

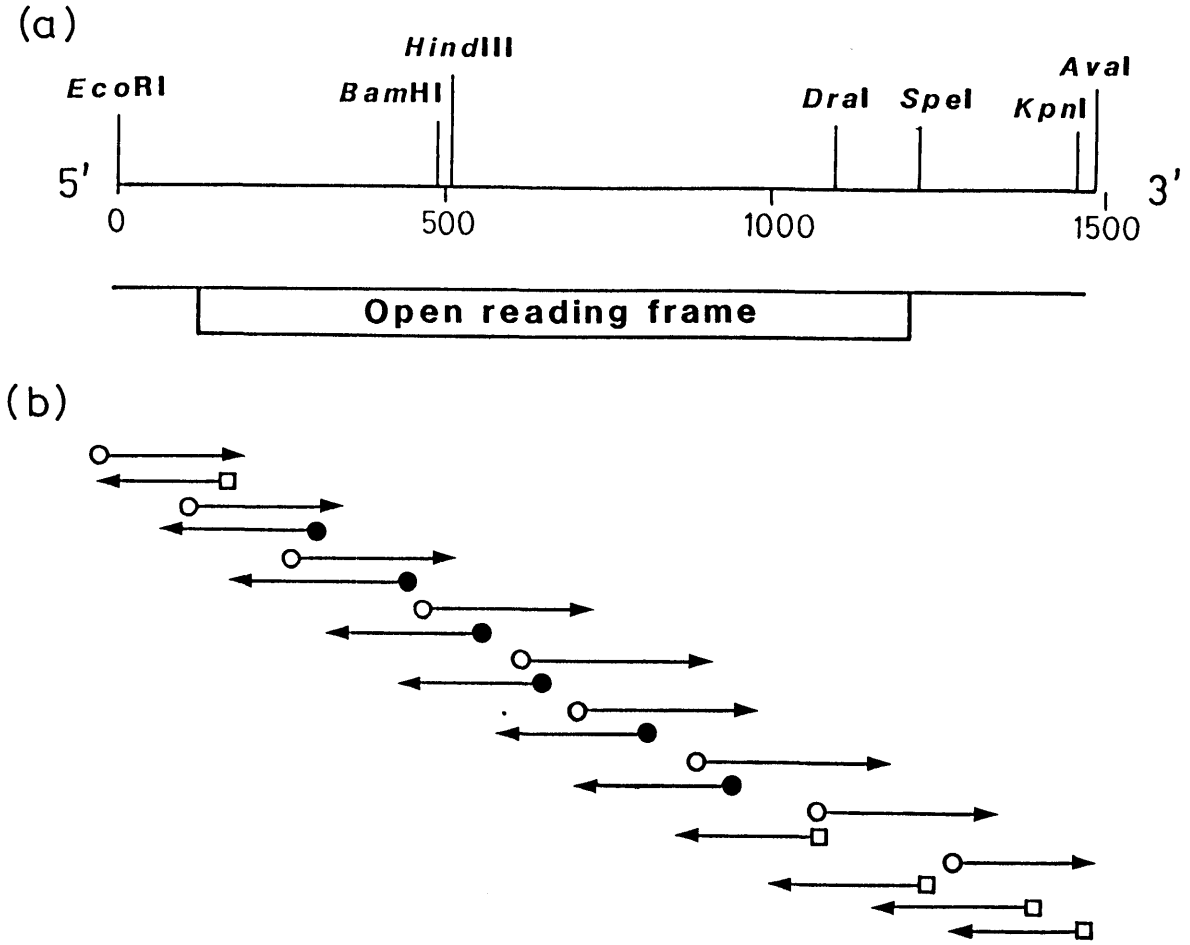


Fig. 12. (a) Restriction map of the insert of pAV3 including the gene for Δ^{12} desaturase of Synechocystis PCC6803. (b) Strategy for determination of nucleotide sequence. Arrows indicate the direction and extent of the nucleotide sequence determined for each fragment. (o) Universal primers, (•) reverse primers, (□) unique primers (17 nucleotides).

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                                    AATTCAGAAGCAATTCGGTTCCTGGTCAATGGCAACGTGTTATAAA
-120 AAGAAAAGTTTGTTTACCTGAGTATTAATTCCTAGGCACGGCAAACCTTGGCCGCTTTAT
 -60 AGCCCATGAATCCATAAACAAAATCTGTCCGACCTTCCATTTGGAGATAAACCTTTATAA

   1 ATGACTGCCACGATTCCCCCGTTGACACCAACGGTAACGCCCAGCAATCCCGATCGCCCG
   1 MetThrAlaThrIleProProLeuThrProThrValThrProSerAsnProAspArgPro

  61 ATTGCGGATCTCAAACTACAAGACATCATTAAAACCCTGCCCAAGGAATGCTTCGAGAAA
  21 IleAlaAspLeuLysLeuGlnAspIleIleLysThrLeuProLysGluCysPheGluLys

 121 AAAGCGAGCAAAGCCTGGGCTTCTGTTTTGATTACCCTAGGGGCGATCGCCGTGGGCTAT
  41 LysAlaSerLysAlaTrpAlaSerValLeuIleThrLeuGlyAlaIleAlaValGlyTyr

 181 TTGGGCATTATTTATCTGCCCTGGTACTGCTTGCCCATTACCTGGATCTGGACAGGGACA
  61 LeuGlyIleIleTyrLeuProTrpTyrCysLeuProIleThrTrpIleTrpThrGlyThr

 241 GCCTTAACGGGGGCCTTCGTTGTCGGCCATGACTGTGGCCATCGCTCCTTTGCTAAAAAA
  81 AlaLeuThrGlyAlaPheValValGlyHisAspCysGlyHisArgSerPheAlaLysLys

 301 CGCTGGGTCAATGATTTAGTGGGACATATCGCTTTTGCTCCCCTCATCTACCCTTTCCAT
 101 ArgTrpValAsnAspLeuValGlyHisIleAlaPheAlaProLeuIleTyrProPheHis

 361 AGCTGGCGCCTACTCCACGACCACCATCACCTCCACACCAACAAAATTGAGGTTGATAAC
 121 SerTrpArgLeuLeuHisAspHisHisHisLeuHisThrAsnLysIleGluValAspAsn

 421 GCCTGGGATCCCTGGAGTGTGGAAGCTTTCCAAGCCAGCCCGGCGATCGTCCGGCTTTTT
 141 AlaTrpAspProTrpSerValGluAlaPheGlnAlaSerProAlaIleValArgLeuPhe

 481 TATCGGGCCATCCGGGGTCCCTTCTGGTGGACTGGTTCCATTTTCCATTGGAGCTTAATG
 161 TyrArgAlaIleArgGlyProPheTrpTrpThrGlySerIlePheHisTrpSerLeuMet

 541 CACTTCAAACTTTCCAACTTTGCCCAAAGGGACCGCAATAAAGTCAAATTATCCATTGCC
 181 HisPheLysLeuSerAsnPheAlaGlnArgAspArgAsnLysValLysLeuSerIleAla

 601 GTTGTCTTCCTGTTTGCGGCGATCGCCTTTCCTGCCCTAATTATCACCACAGGGGTGTGG
 201 ValValPheLeuPheAlaAlaIleAlaPheProAlaLeuIleIleThrThrGlyValTrp

 661 GGTTTCGTCAAATTTTGGCTAATGCCCTGGTTGGTGTATCACTTTTGGATGAGCACTTTT
 221 GlyPheValLysPheTrpLeuMetProTrpLeuValTyrHisPheTrpMetSerThrPhe

 721 ACCATTGTGCACCACACCATTCCCGAAATTCGTTTCCGTCCCGCCGCCGATTGGAGTGCC
 241 ThrIleValHisHisThrIleProGluIleArgPheArgProAlaAlaAspTrpSerAla

 781 GCCGAAGCCCAGTTAAATGGTACTGTTCACTGCGATTATCCCCGTTGGGTGGAAGTGCTC
 261 AlaGluAlaGlnLeuAsnGlyThrValHisCysAspTyrProArgTrpValGluValLeu

 841 TGCCATGACATCAACGTCCATATTCCCCACCACCTCTCCGTTGCCATCCCTTCCTATAAC
 281 CysHisAspIleAsnValHisIleProHisHisLeuSerValAlaIleProSerTyrAsn

 901 CTACGACTAGCCCACGGAAGTTTAAAAGAAAACTGGGGACCTTTTCTTTACGAGCGCACC
 301 LeuArgLeuAlaHisGlySerLeuLysGluAsnTrpGlyProPheLeuTyrGluArgThr
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(continued)

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 961 TTTAACTGGCAATTAATGCAACAAATTAGTGGGCAATGTCATTTATATGACCCCGAACAT
 321 PheAsnTrpGlnLeuMetGlnGlnIleSerGlyGlnCysHisLeuTyrAspProGluHis

1021 GGCTACCGCACCTTCGGCTCCCTGAAAAAAGTTTAATACTGGGACAACTAGTAATTTTTG
 341 GlyTyrArgThrPheGlySerLeuLysLysVal***

1081 ACCCATGATTGGTCAGTAATTAACTTTGACTGATCCCCAGGGAGAGAAATACCAGATCAC
1141 AAATTAACTATCTTGGAATGCGGCCATCGAGCTGTATTCCTTTTTTCTTTTCTTGGTGAG
1201 GAAAAAAACTTTCTAAGTGGGCAGATGGGAGCGGTTCAGACTAGAAAGATCCACTCGGCC
1261 AAAATGAATGCTAAACGCAACCTGCATATTCTCCAAGGTTTTCACTAGCCAAGGTACCCC
1321 TTC
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Fig. 13. The nucleotide sequence and the deduced amino acid sequence of the Δ^{12} desaturase of Synechocystis PCC6803. The sequence of amino acids is numbered from the ATG codon at position 1 of nucleotide sequence. A putative ribosome binding site is underlined. Dashed underlines represent the prokaryotic-like motifs resembling the Pribnow box and '-35' consensus sequences of E. coli promoters.

1974) is located at a -15 position prior to the first, but not any other, ATG triplet. Furthermore, the prokaryotic-like motifs resembling the Pribnow box and '-35' consensus sequences of E. coli promotors (Rosenberg and Court 1979) are found at positions of -58 to -63 (TATAGC) and of -83 to -88 (TAGGCA). These findings suggest that the translation of desA starts at the first ATG codon.

Transformant of Anacystis nidulans R2-SPc with desA--- A. nidulans R2-SPc was transformed with pUC303 alone and pUC303/desA (a recombinant plasmid of pUC303 with the SacI fragment of pAV3) to yield T-pUC303 and T-pUC303/desA. The fatty acid composition of total lipids from the wild type and the transformants were shown in Table 13. The wild-type cells grown at 34°C contained 16:0, $16:1^{\Delta 9}$ as the major fatty acids. These findings indicate that this alga can introduce a double bond at Δ^9 position of 16:0 bound to lipids. In T-pUC303, the fatty acid composition of total lipids was essentially the same as that of wild type, indicating that the transformation with pUC303 alone did not affect the fatty acid composition at 34°C. In T-pUC303/desA, $16:2^{\Delta 9,12}$ and $18:2^{\Delta 9,12}$, which were lacking in the wild type and T-pUC303, emerged to a significant level at the expense of $16:1^{\Delta 9}$ and $18:1^{\Delta 9}$. This result suggests that A. nidulans R2-SPc acquired a Δ^{12} desaturase activity by the transformation, and that the 1.5-kbp insert contains the gene which encodes a Δ^{12} desaturase.

At 22°C, the wild type contained 16:0 and $16:1^{\Delta 9}$ as the major fatty acids. In T-pUC303, the fatty acid composition of the

Table 13 Fatty acid composition of total lipids from the wild type and the transformants with pUC303 and pUC303/*desA* of *Anacystis nidulans* R2-SPc grown at 34 and 22°C

Fatty acid	Growth temperature					
	34°C			22°C		
	Wild type	T-pUC303	T-pUC303/*desA*	Wild type	T-pUC303	T-pUC303/*desA*
	(mol %)					
14:0	1	1	1	1	1	2
$14:1^{\Delta 9}$	1	t	1	4	2	1
16:0	51	51	47	46	48	52
$16:1^{\Delta 9}$	36	37	29	45	42	19
$16:2^{\Delta 9,12}$	0	0	5	0	0	22
18:0	3	3	5	1	2	1
$18:1^{\Delta 9}$	6	5	2	1	2	t
$18:1^{\Delta 11}$	2	3	4	2	3	t
$18:2^{\Delta 9,12}$	0	0	5	0	0	1
18:2*	0	0	1	0	0	1

t, trace amount (less than 0.4%).

Superscripts represent the positions of *cis*-double bonds.

*The positions of double bonds differed from the $\Delta^{9,12}$ positions, but were not determined.

total lipids was essentially the same as that of the wild type, indicating that also at 22°C transformation with pUC303 alone did not affect the fatty acid composition. In T-pUC303/desA, the relative content of $16:2^{\Delta 9,12}$ emerged to a significant level at the expense of $16:1^{\Delta 9}$ and the relative content of $16:2^{\Delta 9,12}$ significantly increased as compared with 34°C-grown cells. These findings suggest that also at 22°C the Δ^{12} desaturase was expressed in T-pUC303/desA cells and its activity was significantly elevated at 22°C.

Discussion

Synechocystis PCC6803 possesses a naturally occurring transformation system that allows exogenous DNA to be incorporated into the chromosome (Williams 1988). This unique characteristic allows Fad12 to be transformed by 'in situ' transformation with wild-type DNAs, their restriction fragments and plasmids containing the restriction fragment. With use of this technique the desA gene for Δ^{12} desaturase was isolated. The transformant of A. nidulans with desA, T-pUC303/desA, acquired the Δ^{12} desaturase activity and the activity was elevated at lower temperature. These results indicate that the promoter sequence of desA may be included in the 5'-upstream region of the first ATG codon of desA (Figure 13).

The amino acid sequence of the protein deduced from the desA gene indicates that 57% of the total amino acids were hydrophobic. This suggests that the desaturase is a membrane-bound protein. The hydropathy profile was calculated from the deduced

amino acid sequence (Figure 14a). There are two clusters of hydrophobic regions consisting of 45 and 47 amino acid residues which are putative membrane-spanning domains. This result is consistent with the finding that the plant-type desaturases are regarded to be membrane-bound enzymes (Harwood 1988).

The stearoyl-CoA desaturase from endoplasmic reticulum of rat liver is membrane-bound and its cDNA has been cloned (Thiede et al. 1986). This cDNA contains an open-reading frame of 1074 bp which corresponds to 358 amino acids and a relative molecular mass of 41400 daltons. The genomic clone and cDNA for the mouse stearoyl-CoA desaturase has also been isolated from the 3T3-L1 adipocytes (Ntambi et al. 1988). The gene contains 6 exons and 5 introns, and the predicted amino acid sequence (355 residues) exhibits 92% homology to that of stearoyl-CoA desaturase of rat liver. The homology between the Δ^{12} desaturase of Synechocystis PCC6803 and the stearoyl-CoA desaturase of rat liver in their nucleotide sequences and amino acid sequences was less than 30% and 10%, respectively. However, the hydropathy profile in the region from 30 to 250 of stearoyl-CoA desaturase (Figure 14b) is similar to that in the region from 15 to 230 of the Δ^{12} desaturase, and suggests the present of two membrane spanning regions (Figure 14b).

A computer search of the EMBL data library (Release 20) with FASTA program (Pearson and Lipman 1988) failed to find any nucleotide sequence homologous to that of the desA gene from Synechocystis PCC6803.

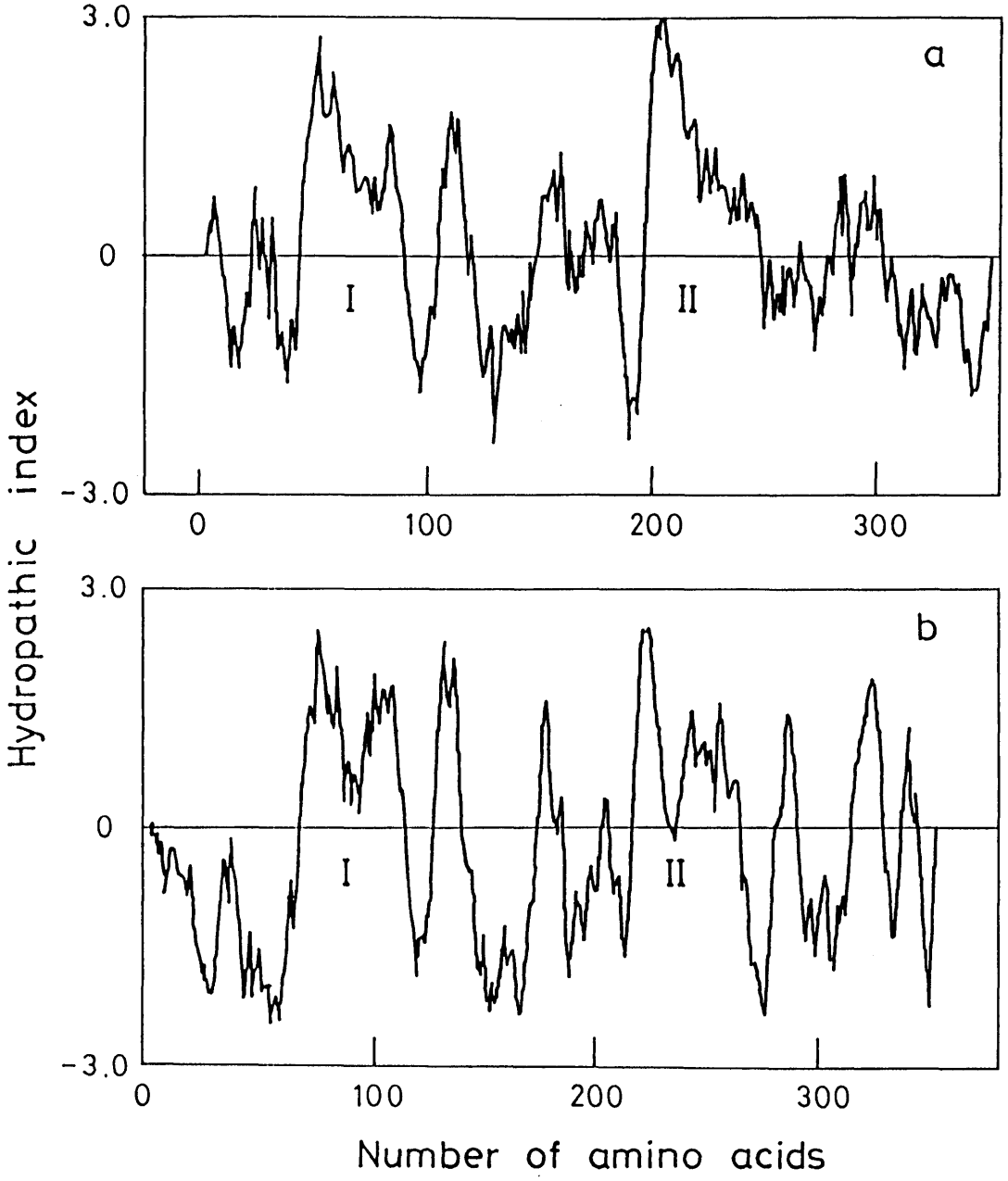

Fig. 14. Hydropathy profile of the deduced amino acid sequences. (a) The Δ^{12} desaturase of Synechocystis PCC6803; (b) stearoyl-CoA desaturase of rat liver calculated from the nucleotide sequence determined by Thiede et al. (1986). Hydrophobicity was calculated according to the algorithm of Kyte and Doolittle (1982) for a window size of 10 amino acid residues.

Chapter 5

Changes in proteins and lipids of thylakoid and cytoplasmic membranes by mutation of fatty acid desaturase of the cyanobacterium, Synechocystis PCC6803

Summary

The thylakoid membranes and the cytoplasmic membranes were isolated from the wild type, a mutant (Fad12) defective in a fatty acid desaturase at the Δ^{12} position, and a transformant (Fad12/desA) of Fad12 with a gene (desA) for the desaturase. The polypeptides of thylakoid membranes and cytoplasmic membranes were compared by gel electrophoresis. A striking change happened in a polypeptide of 35 kDa which was present in both types of membranes from wild type, was lost in those from Fad12, and emerged again in those from Fad12/desA. From this result it appears likely that this polypeptide is the Δ^{12} desaturase or a protein related to the desaturase. The lipid analysis indicates that the mutation of the Δ^{12} desaturase and the transformation with (desA) did not affect the lipid composition of both types of membranes, but changed the fatty acid composition of lipids in similar ways in the two types of membranes. These experimental results may suggest that the fatty acid desaturase may be located both in the thylakoid and cytoplasmic membranes and that the fatty acid desaturation takes place also in both types of membranes in Synechocystis PCC6803.

Introduction

Synechocystis PCC6803 can be classified into a group of cyanobacteria that contain the most unsaturated fatty acids such as $18{:}3^{\Delta 6,9,12}$ and $18{:}4^{\Delta 6,9,12,15}$ (Kenyon 1972, Table 2 in Chapter 2). It is likely that several fatty acid desaturases exist in Synechocystis PCC6803 and each desaturase introduces a

double bond at its own definite position such as Δ^6, Δ^9, Δ^{12}, Δ^{15} of C_{18} fatty acids which are esterified to the sn-1 position of glycerol moiety of glycerolipids (Table 4 in Chapter 2). Recently we isolated a mutant (Fad12) of Synechocystis PCC6803 which is defective in a desaturase of Δ^{12} position. A gene for this Δ^{12} desaturase (desA) was cloned and its sequence was determined (Fig. 11 in Chapter 4). The deduced amino acid sequence predicts the presence of two membrane-spanning regions. A transformant of Fad12 with the desA gene (Fad12/desA) is also available (Chapter 4).

Synechocystis PCC6803 is composed of an endoplasmic membrane system, i.e., the thylakoid membranes and the cell envelope (Stanier 1988). The latter consists of two distinct membrane layers, the cytoplasmic (or inner) membrane and the outer envelope membrane. The improvement of isolation technique of these membranes has enabled us to biochemically characterize the membranes (Murata and Omata 1988). In A. nidulans the thylakoid and cytoplasmic membranes are very different in their polypeptide composition, and no major polypeptides are common to both membranes (Omata and Murata 1983). On the other hand the lipid and fatty acid compositions were almost identical in the thylakoid and cytoplasmic membranes.

The present study was conducted to disclose the intracellular distribution of the Δ^{12} desaturase and Δ^{12} desaturation activity using the mutant, Fad12, and Fad12/desA. The thylakoid and cytoplasmic membranes were isolated from these strains of Synechocystis PCC6803, and their polypeptide, lipid

and fatty acid compositions were compared.

Materials and methods

Organisms and culture conditions---The wild type, Fad12, Fad12/desA of Synechocystis PCC6803 were obtained as described previously in Chapters 2, 3 and 4, and were grown photoautotrophycally at 34°C under illumination of incadescent lamps (70 $\mu E\ m^{-2}\ s^{-1}$) in the culture medium of BG-11 (Stanier et al. 1971) supplemented with 20 mM HEPES-NaOH (pH 7.5) and propagated with sterile air containing 1% CO_2 (Chapter 2).

Membrane isolation---Cytoplasmic and thylakoid membranes were isolated by the method of Omata and Murata (1983) as modified by Omata and Ogawa (1987). The cells were harvested by centrifugation at 5000 x g for 10 min, suspended in 20 mM TES-NaOH buffer (pH 7.0), 5 mM EDTA and 600 mM sucrose, and treated with lysozyme (2 mg ml^{-1}) for two hours. The cells were disrupted by passage twice through the French pressure cell (SLM, Aminco Instruments) at 160 MPa. The membranes were fractionated by flotation ultracentrifugation on a step-wise sucrose gradient described by Murata and Omata (1988). The membrane fractions were withdrawn from the sucrose gradient, diluted three fold with 20 mM TES-NaOH buffer (pH 7.0), and collected by centrifugation at 300000 x g for 1 h (Murata and Omata 1988).

Polypeptide analysis---The polypeptides of the membranes were analyzed by SDS-polyacrylamide gel electrophoresis with the buffer system of Laemmli (1970). The membrane samples were solubilized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2.0%

SDS, 10% glycerol, 5.0% mercaptoethanol and 0.001% bromphenol blue, and were incubated at room temperature for 30 min. The thickness of the gel was 0.1 cm. The stacking gel contained 5% polyacrylamide and the separation gel was composed of a linear gradient of polyacrylamide from 8% to 15%. The electrophoresis was performed at room temperature. Following the electrophoresis, the gels were stained with 0.07% Coomassie brilliant blue R-250 in $CH_3OH/CH_3COOH/H_2O$ (50:10:40, by volume) and destained with $CH_3OH/CH_3COOH/H_2O$ (30:10:60, by volume). For silver staining, the AG-stain "Daiichi" kit (Daiichi Pure Chemicals Co., Tokyo) was used. Following the silver staining procedure, the gel was washed twice with deionized water and soaked in 0.1% acetic acid in water to prevent the acceleration of coloration of the background.

Lipid and fatty acid analyses---Lipid and fatty acid analyses were carried out according to the methods described in Chapter 2.

Results

Protein composition---The protein compositions of thylakoid membranes isolated from the wild type, Fad12 and Fad12/desA were compared in Fig. 15. The polypeptide patterns of thylakoid membranes from the different strains were similar to each other. Albeit, a clear difference among the three strains of Synechocystis PCC6803 appeared in the presence and absence of two polypeptides of relative molecular masses of 27 kDa and 35 kDa. They were present in wild type, were lost in Fad12, and were

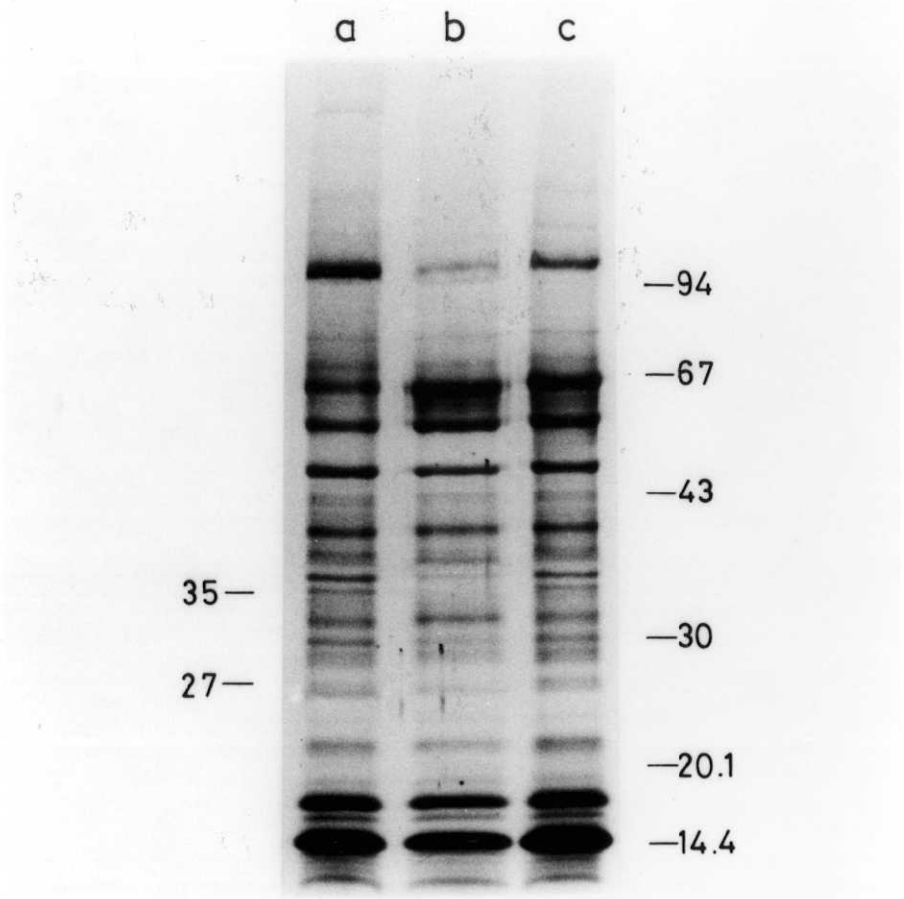

Fig. 15. SDS-polyacrylamide gel electrophoresis of thylakoid membranes isolated from wild type, Fad12 and Fad12/*desA* of *Synechocystis* PCC6803. Staining with Coomassie brilliant blue. (a) Wild type, (b) Fad12, (c) Fad12/*desA*.

recovered in Fad12/desA. ~~No other discernible changes in the polypeptide pattern were detected among the thylakoid membranes from the three strains~~. The polypeptide composition of cytoplasmic membranes (Fig. 16) was very different pattern from that of the thylakoid membranes. However, the polypeptide composition was essentially the same as the result obtained by Omata and Ogawa (1987). Two polypeptides were found at an apparent molecular mass of 35-36 kDa. The lower polypeptide band was identified as a protein closely related to the inorganic carbon transport (Omata and Ogawa), whereas the upper polypeptide was presumably the same as the 35-kDa polypeptide in the thylakoid membrane. These two polypeptides were observed in the cytoplasmic membranes from wild type (Fig. 16a) and Fad12/desA, but the upper band of 35kDa was missing in that from Fad12 (Fig. 16b). Because of many overlapping polypeptide bands, the presence or absence of the polypeptide of 27 kDa in the cytoplasmic membrane could not be answered.

Lipid composition---The lipid compositions of thylakoid and cytoplasmic membranes are presented in Table 14. MGDG, DGDG, SQDG and PG were main lipid constituents of the membranes from wild type, Fad12 and Fad12/desA in accordance with the lipid composition of other cyanobacteria (Murata and Nishida 1987). The MGDG exceeded 50% of the total lipids in both thylakoid and cytoplasmic membranes. On the other hand, the proportion of SQDG of cytoplasmic membranes was lower than that of thylakoid membranes, whereas the proportion of PG of cytoplasmic membranes was higher than that of thylakoid membranes. Albeit, in overall

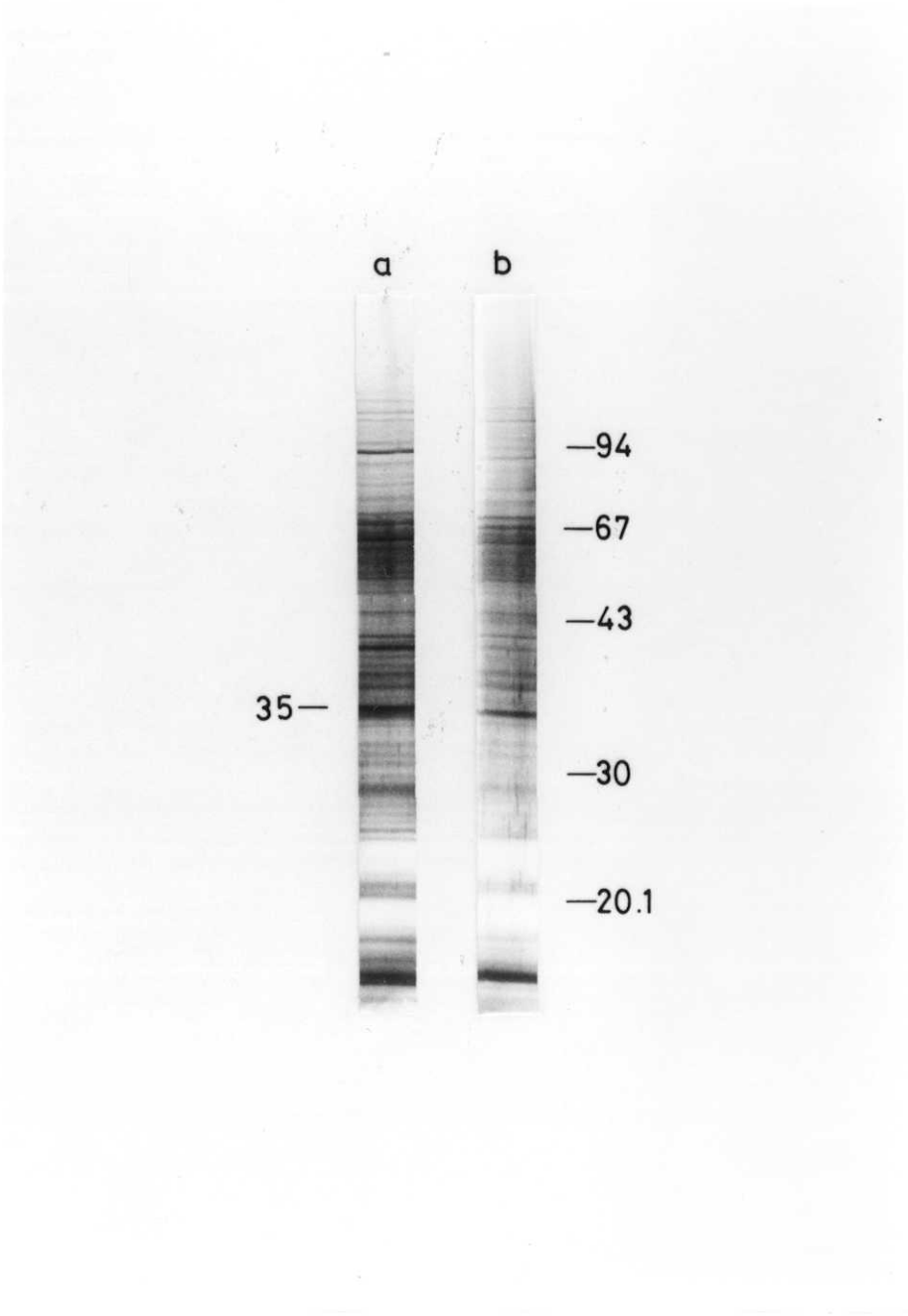

Fig. 16. SDS-polyacrylamide gel electrophoresis of cytoplasmic membranes isolated from wild type and Fad12 of Synechocystis PCC6803. Staining with silver. (a) Wild type, (b) Fad12.

Table 14 Lipid composition of thylakoid and cytoplasmic membranes isolated from wild type, Fad12 and Fad12/*desA* of *Synechocystis* PCC6803.

Lipid class	Wild type		Fad12		Fad12/*desA*	
	TM	CM	TM	CM	TM	CM
	(mol %)					
MGDG	62	59	66	67	59	62
DGDG	14	18	15	11	17	15
SQDG	18	13	13	10	16	14
PG	6	10	6	12	8	9

CM, cytoplasmic membranes; TM, thylakoid membranes.

view, both types of membranes possessed essentially the same lipid composition as was obtained from the whole cells (Table 1 in Chapter 2).

Fatty acid composition---The fatty acid composition of total lipids from the two types of membrane is shown in Table 15. In both types of membrane from Fad12, $18:2^{\Delta 9,12}$ and $18:3^{\Delta 6,9,12}$ were completely lost as seen in the total lipids from the whole cells (Table 8 in Chapter 3). Minor differences were found in the ratio of saturated to unsaturated fatty acids between the two types of membranes from both wild type and Fad12. The cytoplasmic membranes contained slightly higher levels of 16:0 and 18:0 than the thylakoid membranes. In the wild type and Fad12/desA, the cytoplasmic membrane had lower proportions of $18:2^{\Delta 9,12}$ and $18:3^{\Delta 6,9,12}$ than the thylakoid membranes. In Fad12, the cytoplasmic membrane contained lower levels of $18:1^{\Delta 9}$ and $18:2^{\Delta 6,9}$ than the thylakoid membranes. It is noteworthy that the fatty acid composition of lipids of thylakoid and cytoplasmic membranes from Fad12/desA was the same as that from wild type.

The analysis of individual lipid classes of cytoplasmic and thylakoid membranes isolated from wild type and Fad12 are presented in Tables 16 and 17. In all lipid classes the proportion of 16:0 and 18:0 of the cytoplasmic membrane was higher than that of thylakoid membranes.

Discussion

In the present study, we compared the composition of major constituents, proteins and lipids, of thylakoid and cytoplasmic

Table 15 Fatty acid composition of total lipids of thylakoid and cytoplasmic membranes isolated from wild type, Fad12 and Fad12/*desA* of *Synechocystis* PCC6803.

	Wild type		Fad12		Fad12/*desA*	
Fatty acid	TM	CM	TM	CM	TM	CM
14:0	t	t	1	t	t	t
16:0	57	62	53	58	60	63
$16:1^{\Delta 9}$	4	3	3	2	4	3
18:0	1	3	1	2	1	3
$18:1^{\Delta 9}$	9	10	37	33	8	9
$18:2^{\Delta 9,12}$	15	12	0	0	14	11
$18:2^{\Delta 6,9}$	0	0	7	4	0	0
$18:3^{\Delta 6,9,12}$	15	10	0	0	13	11
Sat/unsat	1.4	1.9	1.2	1.5	1.6	1.9
DBI	0.9	0.7	0.5	0.4	0.8	0.7

CM, cytoplasmic membranes; TM, thylakoid membranes; sat, 16:0 + 18:0; unsat, 16:1 + 18:1 + 18:2 + 18:3; DBI, double bond index; t, trace (less than 0.4%). The numbers of superscript indicate the positions of *cis*-double bonds.

Table 16 Fatty acid composition of individual lipid classes of membrane fractions isolated from wild type of Synechocystis PCC6803.

Fatty acid	MGDG		DGDG		SQDG		PG	
	TM	CM	TM	CM	TM	CM	TM	CM
14:0	t	t	1	t	t	t	1	1
16:0	55	61	57	62	74	82	62	64
$16:1^{\Delta 9}$	4	3	4	2	4	1	2	5
18:0	t	2	1	4	2	4	3	8
$18:1^{\Delta 9}$	9	11	6	9	13	10	12	12
$18:2^{\Delta 9,12}$	15	12	13	10	7	3	17	8
$18:2^{\Delta 6,9}$	0	0	0	0	0	0	0	0
$18:3^{\Delta 6,9,12}$	17	11	20	13	t	0	3	2
Sat/unsat	1.2	1.7	1.4	1.9	3.3	6.1	1.9	2.6
DBI	0.9	0.7	1.0	0.7	0.3	0.3	0.6	0.4

CM, cytoplasmic membranes; TM, thylakoid membranes; sat, 16:0 + 18:0; unsat, 16:1 + 18:1 + 18:2 + 18:3; DBI, double bond index; t, trace (less than 0.4%).

Table 17 Fatty acid composition of individual lipid classes of membrane fractions isolated from desaturation mutant, Fad12, of *Synechocystis* PCC6803.

Fatty acid	MGDG		DGDG		SQDG		PG	
	TM	CM	TM	CM	TM	CM	TM	CM
14:0	t	t	1	t	t	t	1	1
16:0	51	59	56	57	71	77	56	63
$16:1^{\Delta 9}$	3	2	3	6	3	1	2	1
18:0	t	1	2	8	4	6	3	7
$18:1^{\Delta 9}$	39	33	32	27	21	15	39	29
$18:2^{\Delta 9,12}$	0	0	0	0	0	0	0	0
$18:2^{\Delta 6,9}$	7	5	8	3	0	0	0	0
$18:3^{\Delta 6,9,12}$	0	0	0	0	0	0	0	0
Sat/unsat	1.0	1.5	1.4	1.8	3.0	4.9	1.4	2.3
DBI	0.6	0.4	0.5	0.3	0.2	0.2	0.4	0.3

CM, cytoplasmic membranes; TM, thylakoid membranes; sat, 16:0 + 18:0; unsat, 16:1 + 18:1 + 18:2; t, trace (less than 0.4%); DBI, double bond index; t, trace (less than 0.4%).

membranes from the wild type, the mutant of Δ^{12} desaturase (Fad12) and the transformant of Fad12 with the Δ^{12} desaturase gene (Fad12/desA) of Synechocystis PCC6803. In the wild type the polypeptide composition of thylakoid membranes differed from that of cytoplasmic membrane. This is comparable with the results of Omata and Murata in A. nidulans (1983) and in Synechocystis PCC6714 (1984). A similar distinct difference in the polypeptide composition was observed in the membranes from Fad12 and Fad12/desA of Synechocystis PCC6803.

The polypeptide of 35 kDa existed in the thylakoid membranes and also in the cytoplasmic membranes from wild type and Fad12/desA, but not in those of Fad12. It appears likely that this polypeptide corresponds to the Δ^{12} desaturase or a related polypeptide. The amino acid sequence of Δ^{12} desaturase deduced from the nucleotide sequence of the desA gene (Fig. 13 in Chapter 4) predicted that the relative molecular mass of the desaturase is about 40 kDa, which is compatible with the 35-kDa polypeptide detected by SDS-polyacrylamide gel electrophoresis. Assuming that the 35-kDa polypeptide is the desaturase or a protein related to the desaturase, it can be inferred that the desaturation of C_{18} fatty acids takes place at both thylakoid and cytoplasmic membranes. It can be speculated that both the thylakoid and cytoplasmic membranes are the site of glycerolipid synthesis in the cyanobacterial cells. This is supported by our previous observations, that the activity of UDP-glucose: diacylglycerol glucosyltransferase exists in both types of membranes (Omata and Murata 1986). However, Douce and Joyard (1979) proposed that in

higher plant chloroplasts only the envelope membrane is the site of esterification and desaturation of fatty acids in glycerolipid synthesis.

The lack of distinct difference in fatty acid unsaturation between thylakoid and cytoplasmic membranes can be argued from the view point of the site of fatty acid desaturation. It is clearly demonstrated that the degree of suppression of desaturation in Fad12 and the degree of recovery of desaturation in Fad12/desA are in parallel in both membranes. This result is compatible with the above-mentioned speculation that the Δ^{12} desaturase is evenly distributed in both thylakoid and cytoplasmic membranes, and that in Fad12 the desaturase disappeared in both membranes.

Chapter 6

Effects of fatty acid unsaturation on thermal properties of photosynthetic activities studied by means of mutation and transformation of a desaturase gene in Synechocystis PCC6803

Summary

Thermal properties of photosynthetic activities of a mutant and a transformant of a gene for a fatty acid desaturase were compared with those of the wild type of Synechocystis PCC6803. A mutant (Fad12) was defective in the fatty acid desaturation at the Δ^{12} position of C_{18} fatty acids of membrane glycerolipids, and therefore lacked in trienoic acids. The transformant of Fad12 with a gene (desA) for the Δ^{12} desaturase (Fad12/desA) had a fully recovered fatty acid composition. Despite of the great diversity in the fatty acid composition among wild type, Fad12 and Fad12/desA, no significant differences were observed in the temperature dependence of photosynthesis and photosystem II activities and the temperature profile of heat inactivation of photosynthesis, photosystem II and photosystem I activities. These results suggest that the desaturation at the Δ^{12} position and therefore the lack in trienoic fatty acids do not affect the thermal properties of photosynthetic activities.

Introduction

The major glycerolipid classes of thylakoid membranes in higher-plant chloroplasts and cyanobacterial cells are MGDG, DGDG, SQDG and PG (Douce et al. 1973, Murata and Nishida 1987). A major part of glycerolipids form the lipid bilayer, and the remaining part is bound to the membraneous protein complexes (Doyle and Yu 1985, Pick et al. 1985, Tremolieres et al. 1981). It is reported that SQDG is bound to CF_1/CF_0 complexes (Pick et al. 1987), and that MGDG is to PSII reaction center complex

(Murata and Miyao 1989). Therefore, it can be suggested that the characteristic of the bilayer lipids and the protein-bound lipids affect the photosynthetic activities such as photochemical and electron transport reactions which take place in the thylakoid membrane. The physical property of glycerolipids depends on the unsaturation of fatty acids esterified to the glycerolipids (Chapman 1975, Quinn 1988). The molecular motion (or fluidity) of the glycerolipids with unsaturated fatty acids is higher than that of the glycerolipids with saturated fatty acids (Chapman 1975, Silvius 1982). It is predicted therefore that the photosynthetic reactions such as photochemical and electron transport reactions can be affected by the level of fatty acid unsaturation.

Plants can acclimate to the growth temperature (Berry and Bjorkman 1980). The desaturation of fatty acids of glycerolipids stimulated at low temperature is one of the best studied phenomena in relation to the temperature acclimation (Murata 1989, Norman and Thompson 1985, Norman et al. 1985). Responses of the photosynthetic activities toward temperature changes have been studied in relation to the fatty acid unsaturation (Badger et al. 1982, Lynch and Thompson 1984, Orr and Raison 1987, Pearcy 1978, Pearcy et al. 1977, Raison and Wright 1983, Raison et al. 1982). In most of these studies, the change in fatty acid unsaturation of thylakoid lipids with growth temperature are correlated with the thermal properties of the photosynthetic activities (Pearcy 1978, Raison et al. 1982). However, it is noteworthy that the growth temperature regulates, in addition to

the enzymes involved in lipid biosynthesis, the biosynthesis of a number of enzymes (Cooper and Ort 1988, Guy et al. 1985, Mohapatra et al. 1987). Some of them can change the thermal properties of photosynthesis.

In order to elaborate the relationship between the fatty acid unsaturation of membrane lipids and thermal properties of photosynthetic activities, new techniques are necessary which can manipulate only the fatty acid unsaturation with minimum effects on other factors. The hydrogenation of fatty acids in the membrane lipids and the mutagenesis of fatty acid desaturation are regarded as powerful methods to address this problem.

The catalytic hydrogenation technique was applied to higher-plant chloroplasts (Restall et al. 1979, Vigh et al. 1985b). Although the levels of trienoic, dienoic and monoenoic fatty acids were markedly reduced by the hydrogenation, no significant alteration in thermal properties of photosynthetic activities of chloroplasts could be detected (Thomas et al. 1986, Vigh et al. 1989). Studies on a desaturation mutant of Arabidopsis having a reduced level of trienoic fatty acids lead a conclusion that the level of trienoic fatty acids does not affect the thermal stability of photosynthetic activities (McCourt et al. 1987). Nevertheless, the desaturation mutant of Arabidopsis, possessing a reduced level of dienoic fatty acids, shows higher thermal stability detected by the fluorescence yield at a high temperature region (Browse et al. 1989, Hugly et al. 1989).

However, the change in fatty acid unsaturation by the hydrogenation and mutagenesis of Arabidopsis is not discrete

because the dienoic and trienoic fatty acids are not fully removed and the remaining dienoic and trienoic fatty acids may keep the specific effect on the thermal properties of photosynthetic activities. Therefore, a clear answer regarding to the correlation between the fatty acid unsaturation and the thermal properties of photosynthetic activities can not be reached by these methods.

We recently isolated a mutant (Fad12) of fatty acid desaturation of Synechocystis PCC6803 (Chapter 3). This mutant completely lacks in desaturation at the Δ^{12} position of the C_{18} fatty acids, and contains saturated, monoenoic and dienoic, but no trienoic, fatty acids. With use of the "in situ transformation" technique, we cloned a gene (desA) for the Δ^{12} desaturase (Chapter 4), and Fad12 was transformed with the desA gene (Chapter 4). The resultant transformant (Fad12/desA) has a fully recovered level of the trienoic fatty acids. The present study was conducted to obtain with use of the mutant and the transformant a definite answer to the question whether or not the the trienoic fatty acids affect in the thermal properties of photosynthetic activities.

Materials and methods

Organisms and culture conditions---Wild type, Fad12 and Fad12/desA of Synechocystis PCC6803 were grown photoautotrophically at 34°C as described in Chapters 2, 3 and 4.

Fatty acid analysis---Fatty acid analysis was carried out

according to the methods as described in Chapter 2.

Preparation of thylakoid membrane---The mutant and wild-type cells were harvested by centrifugation at 5000 x g for 10 min. The cells were washed twice with 50 mM HEPES-NaOH (pH 7.5) containing 30 mM $CaCl_2$ (Dzelzkalns and Bogorad 1989), and were suspended in 50 mM HEPES-NaOH (pH 7.5) containing 800 mM sorbitol. The cell suspension was passed three times through a prechilled French pressure cell (SLM, Aminco Instruments) at 1600 MPa. This treatment afforded about 80% disruption of the cells. The sample was centrifuged for 5 min at 1000 x g to remove the unbroken cells, and the supernatant was recentrifuged for 15 min at 15000 x g. The pelleted membranes were suspended in 50 mM HEPES-NaOH (pH 7.5) containing 800 mM sorbitol, and were kept at $4^{o}C$ until use.

Measurement of photosynthetic activities---Three kinds of photosynthetic activities were measured by means of oxygen exchange with a Clark-type oxygen electrode: (1) Photosynthesis (oxygen evolution) in intact cells with no added electron donor nor acceptor; cells were washed twice with 50 mM Tricine-NaOH (pH 7.5), and were suspended in the same medium for measurement: (2) PSII activity measured by the electron transport from H_2O to 1,4-benzoquinone; cells were washed twice with 50 mM Tricine-NaOH (pH 7.5) and were suspended in Tricine-NaOH (pH 7.5), 0.1 mM 1,4-benzoquinone and 1.0 mM $K_3Fe(CN)_6$ for measurement (Ono and Murata 1981a): (3) PSI activity in membrane preparations measured by the electron transport from diaminodurene to methylviologen; the membranes were suspended in 50 mM HEPES-NaOH (pH 7.5), 1.0 mM

diaminodurene and 1.0 mM Na-ascorbate, 1.5 mM methylviologen, 0.01 mM DCMU and 2 mM KCN (Pakrasi et al. 1988). Light at an intensity of 3.0 Watt m^{-2} was obtained from an incandescent lamp combined with a red optical filter (Hoya, VR 620). The concentrations of intact cells and the membranes were adjusted to give about 15 and 10 μg chlorophyll/ml, respectively. Chlorophyll was determined according to Arnon et al. (1974).

Results

Fatty acid composition---In order to characterize the cyanobacterial cells used in the present study, the fatty acid composition of membraneous glycerolipids was analyzed (Table 18). The wild type and Fad12/desA contained $18:2^{\Delta 9,12}$ and $18:3^{\Delta 6,9,12}$ as the most abundant fatty acids, whereas in Fad12, these fatty acids were completely lost and the level of $18:1^{\Delta 9}$ increased with an accompany of a minor contribution of $18:2^{\Delta 6,9}$. It is noteworthy that the mutation fully eliminated the trienoic fatty acids from the membraneous glycerolipids. By comparing the wild type, Fad12 and Fad12/desA, the effect of the trienoic fatty acids on the thermal properties of photosynthetic activities can be studied without influence of other factors.

Effect of mutation and transformation on thermal properties of photosynthetic activities---The photosynthetic activities of wild type, Fad12 and Fad12/desA measured at 34°C (growth temperature) are compared in Table 19. The mutation and the transformation of fatty acid desaturase had no major effect on activities of photosynthesis, PSII and PSI.

Table 18 Fatty acid composition of total glycerolipids from wild type, Fad12 and Fad12/*desA*.

Fatty acid	Wild type	Fad12	Fad12/desA
		(mol%)	
14:0	t	t	t
16:0	57	55	60
$16:1^{\Delta 9}$	4	3	3
18:0	1	1	1
$18:1^{\Delta 9}$	9	36	8
$18:2^{\Delta 6,9}$	t	5	t
$18:2^{\Delta 9,12}$	15	0	12
$18:3^{\Delta 6,9,12}$	15	0	16
$18:3^{\Delta 9,12,15}$	t	0	t
$18:4^{\Delta 6,9,12,15}$	t	0	t

The numbers in parentheses indicate the positions of *cis*-double bonds. t, trace (less than 0.4%)

Table 19 The photosynthetic activities in wild type, Fad12 and Fad12/*desA*.

Activity	Wild type	Fad12	Fad12/*desA*
	μmol O_2 mg Chl^{-1} h^{-1}		
Photosynthesis	360 $\pm$ 20	330 $\pm$ 10	340 $\pm$ 10
PSII (H2O-BQ)	490 $\pm$ 20	520 $\pm$ 10	480 $\pm$ 10
PSI* (DAD-MV)	640 $\pm$ 30	590 $\pm$ 20	620 $\pm$ 30

*PS I was measured in isolated thylakoid membranes. The photosynthetic activities were measured at 34°C.

Fig. 17 shows the temperature dependence of photosynthesis and PSII activities in intact cells of wild type and Fad12. The temperature for 50% of the maximum activity appeared at 29-30^{o}C in both activities of wild type and Fad12. These observations suggest that the alteration in fatty acid unsaturation by mutation does not affect the temperature dependence of the photosynthetic activities.

Fig. 18 shows the time course of heat inactivation of photosynthesis in the intact cells of wild type and Fad12. The rates of heat inactivation of wild type and Fad12 were essentially the same. Treatment for 20 min was chosen for incubation for further experiments of heat inactivation. The temperature profiles of heat inactivation of the photosynthesis and PSII activities in wild type, Fad12 and Fad12/desA were compared in Fig. 19. The oxygen evolution is one of the most heat-sensitive processes in photosynthesis (Quinn and Willims 1985, Yardanov et al. 1986). One direct result of heat inactivation is release of functional Mn from PSII (Nash et al. 1985). The temperatures for 50% of heat inactivation appeared at 49-51^{o}C in both activities in the these strains. It can be concluded that no significant difference occurs in the heat stability of photosynthesis and PSII activity among wild type, Fad12 and Fad12/desA.

The temperature profiles of heat inactivation of the PSI activity in the wild type and Fad12 were compared in Fig. 20. As demonstrated previously (Katoh and San Pietro 1967, Berry and Bjorkman 1980), the PSI activity was more tolerant to heat than

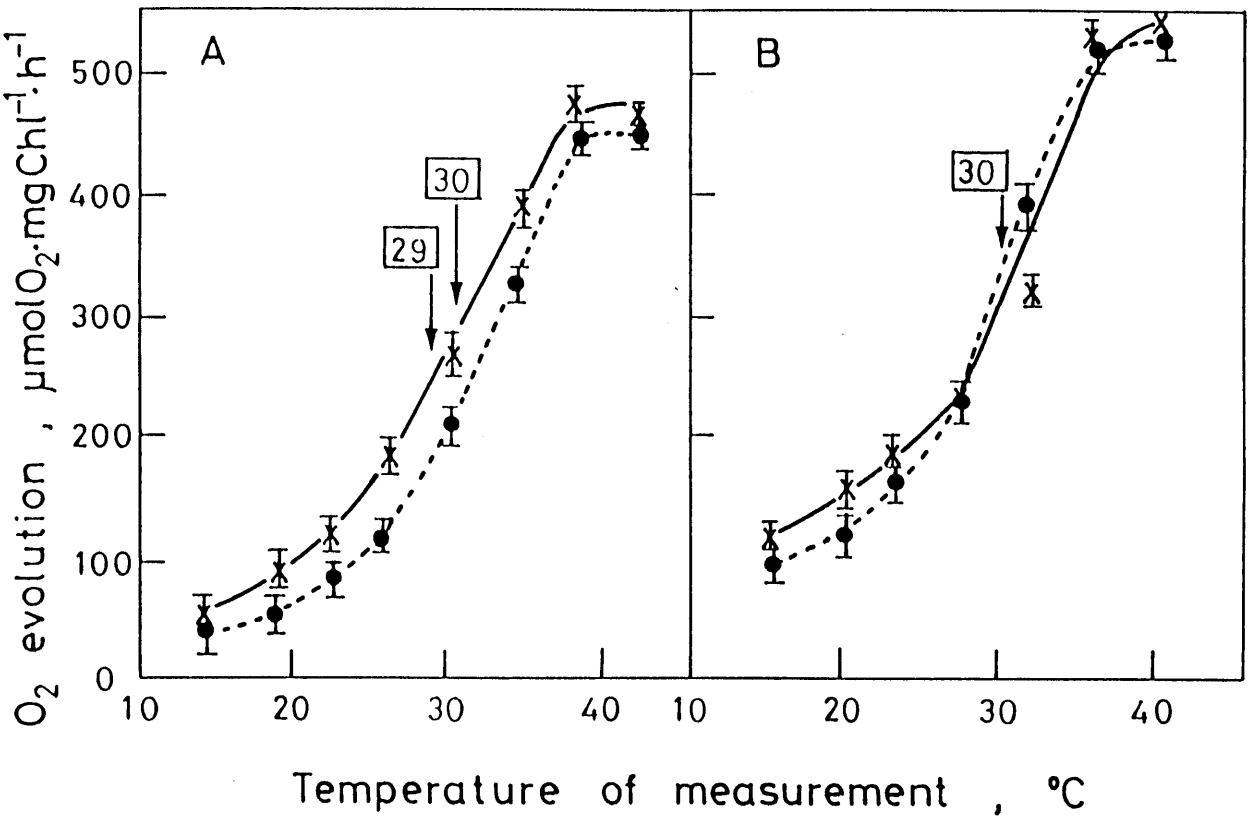

Fig. 17. The temperature dependence of photosynthesis and PSII activities in intact cells of the wild type and Fad12. (A) Photosynthetic oxygen evolution with no added electron acceptor. (B) PSII activity measured by light-induced oxygen evolution with 1,4-benzoquinone added as the electron acceptor. Wild type, (x); Fad12, (●).

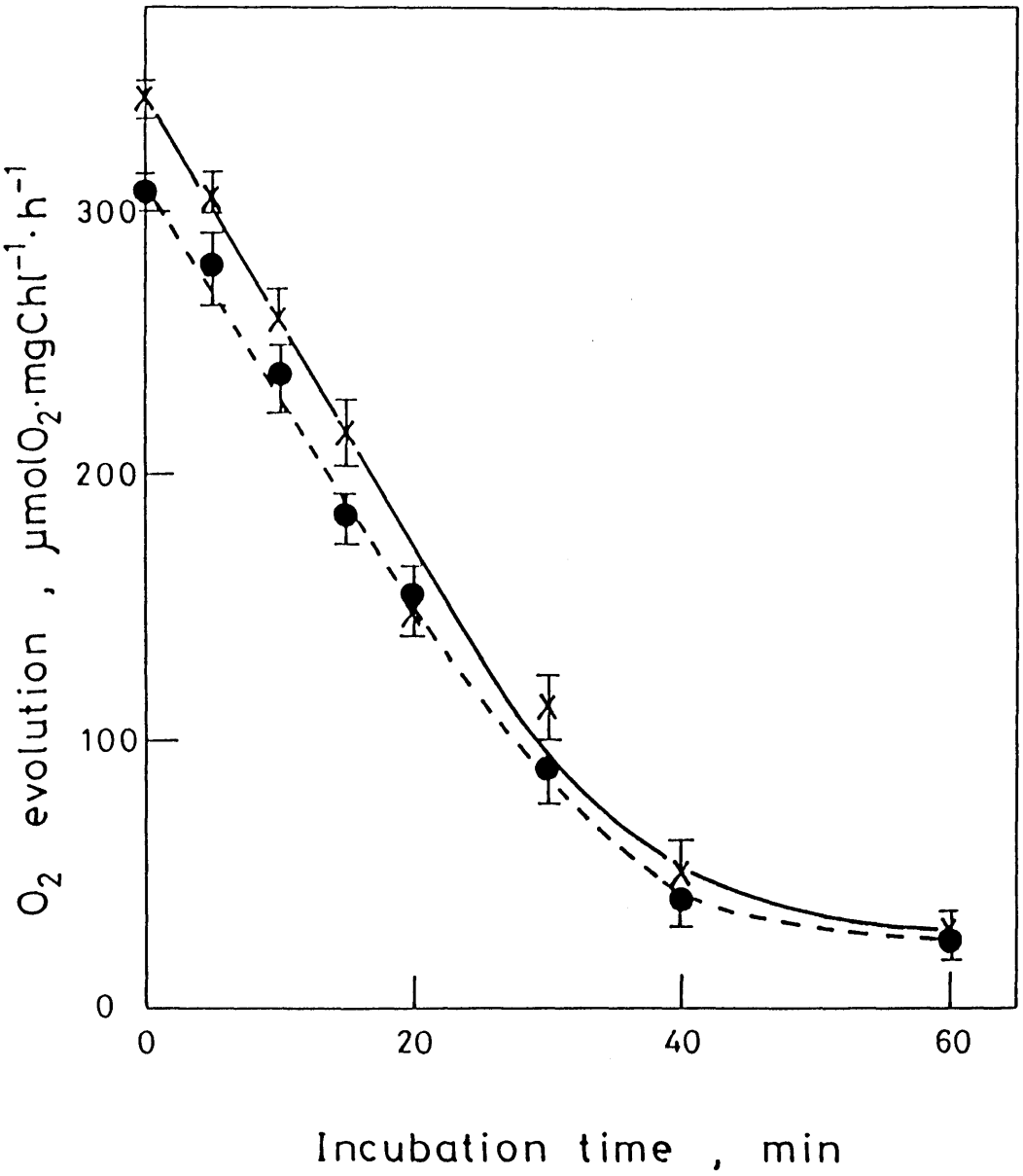

Fig. 18. The time course of heat inactivation of photosynthesis in intact cells of wild type and Fad12. The cells were incubated at 48°C in darkness and the oxygen evolution activity was measured at 34°C. Wild type, (x); Fad12, (●).

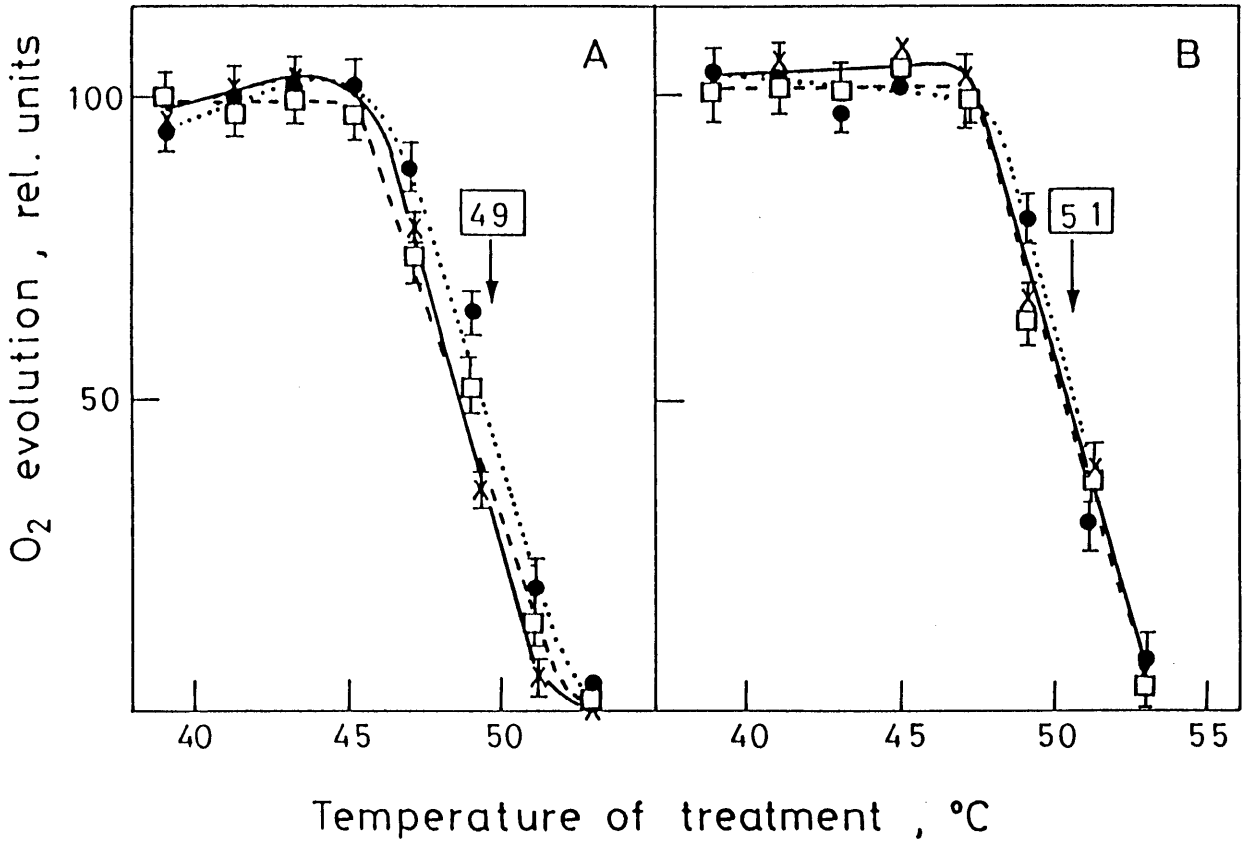

Fig. 19. The temperature profile of heat inactivation of photosynthesis and PSII activities in intact cells of wild type, Fad12 and Fad12/desA. The cells were treated for 20 min at various temperatures in darkness and the oxygen evolution activities were measured at 34°C. (A) Photosynthetic oxygen evolution with no added electron acceptor. (B) PS II activity measured by light-induced oxygen evolution with 1,4-benzoquinone added as the electron acceptor. Wild type, (x); Fad12, (●); Fad12/desA, (□).

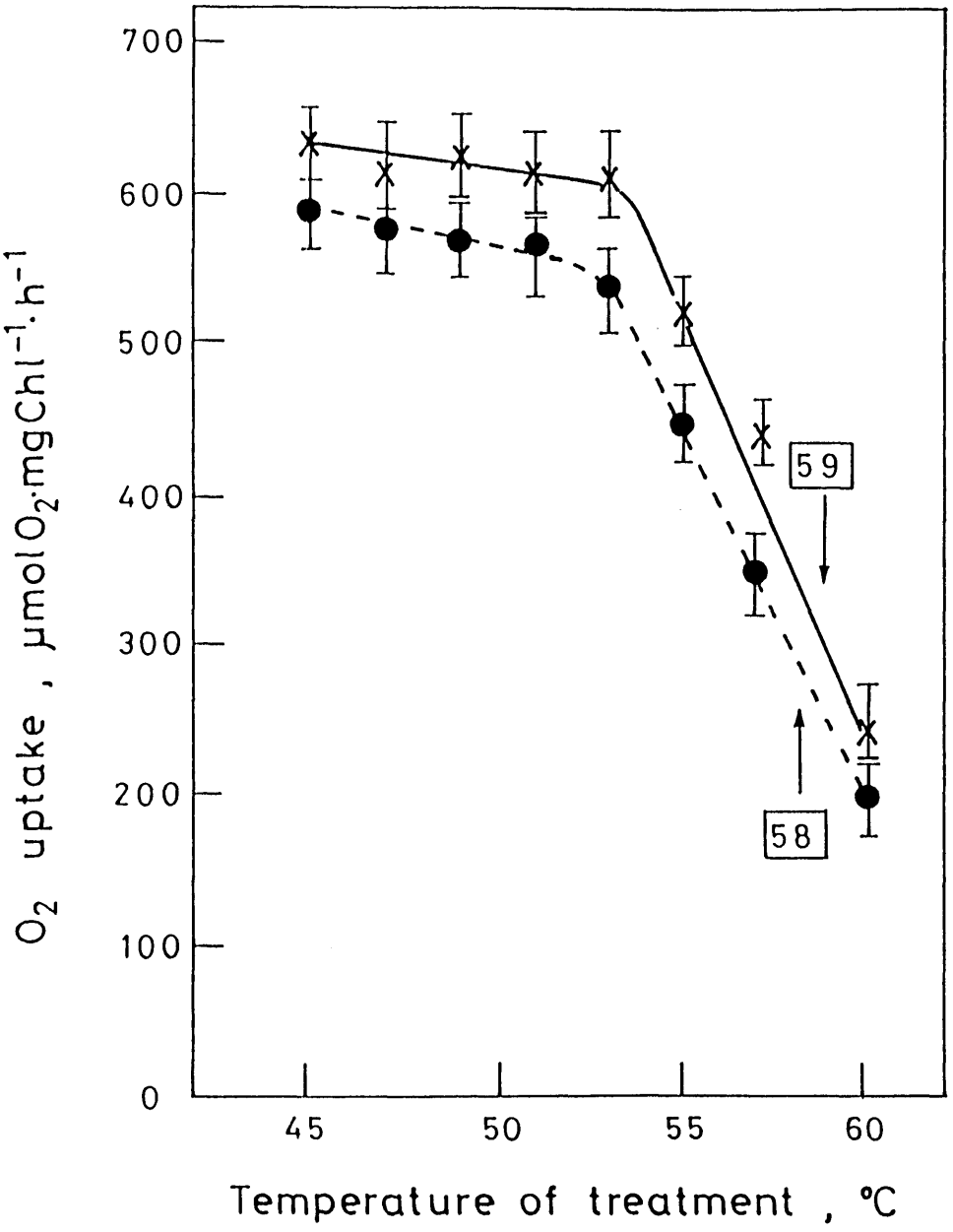

Fig. 20. The temperature profile of heat inactivation of PS I activity in thylakoid membranes from wild type and Fad12. The thylakoid membranes were treated for 20 min at various temperatures in darkness and the PSI activity (the electron transport from diaminodurene to methyl viologen was measured at 34°C. Wild type, (x); Fad12, (●).

photosynthesis and PSII activities. The temperature for 50% inactivation of the PSI activity occurred at 58-59^{o}C in the wild type and Fad12, suggesting that the heat stability was not affect by the mutation of fatty acid desaturation.

Discussion

In the present study, the mutant of fatty acid desaturation of Synechocystis PCC6803 (Fad12) was used to study the effect of trienoic fatty acids on the thermal properties of the photosynthetic activities. As a control system Fad12/desA was used, in addition to wild type. This is necessary to avoid the possibility that another unknown mutation, which is related to the thermal properties of photosynthetic activities but not to fatty acid desaturation, happened in Fad12 to effect the thermal properties. Fad12 is unable to produce fatty acids containing Δ^{12} cis-double bonds and therefore does not possess any kind of trienoic fatty acids (Chapter 3). Despite of the lack of the trienoic fatty acids, there were no significant changes in the temperature dependence and the temperature profile of heat inactivation of the photosynthetic activities. These results suggest that the trienoic acids do not play a determinative role in thermal properties of the photosynthetic activities.

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