MOLECULAR MECHANISM OF HEAT TOLERANCE OF PHOTOSYNTHESIS

光合成の高温耐性の分子機構

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MOLECULAR MECHANISM OF HEAT TOLERANCE OF PHOTOSYNTHESIS

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Abstract

Molecular mechanism of the heat tolerance of photosynthesis upon adaptation to high temperature is studied in the cyanobacterium, *Synechococcus* PCC 7002.

The environmental aspects and the primary process of photosynthesis are briefly summarized in Chapter 1. Previous studies on the response of photosynthesis to high temperature are then reviewed, in which the inactivation of photosynthesis by heat and the adaptation of photosynthesis to high temperature are mainly described. The aim of the present study and the characteristics of the experimental material, the cyanobacterium *Synechococcus* PCC 7002, are also described.

The photosynthetic adaptation to high temperature in *Synechococcus* PCC 7002 is characterized in Chapter 2. The experimental results suggest that the factor responsible for the heat tolerance of photosynthesis is associated with the thylakoid membranes.

The heat tolerance of photosynthesis acquired in the thylakoid membranes of *Synechococcus* PCC 7002 is characterized by biochemical analyses in Chapter 3. The experimental results suggest that some protein components associated with thylakoid membranes are related to the heat tolerance of photosynthetic oxygen evolution.

Cytochrome *c*-550 is identified as the factor required for the heat stability of photosynthetic oxygen evolution in *Synechococcus* PCC 7002 in Chapter 4. The gene encoding cytochrome *c*-550 is cloned from *Synechococcus* PCC 7002 and its sequence is determined.

Chapter 5 describes the conclusions deduced from the results in the present study, and the perspectives for the future study.

Abbreviations

BQ	1,4-benzoquinone
Chl	chlorophyll
$DADH_2$	1,4-diamino-2,3,5,6-tetramethylbenzene
DCIP	2,6-dichlorophenolindophenol
DPC	diphenylcarbazide
DQH_2	duroquinol
HSP	heat shock protein
MV	methylviologen
PBQ	phenyl-1,4-benzoquinone.
PS	photosystem

Chapter 1

General introduction

1.1. Environmental aspects of photosynthesis

Photosynthesis is the biochemical process that converts light energy into the chemical energy to produce organic compounds from carbon dioxide. Photosynthesis is the most fundamental activity for photosynthetic organisms including plants and photosynthetic bacteria to sustain their life. Moreover, all animals and most microorganisms rely on the products of photosynthesis. The organic compounds provide them the carbon skeletons for biosynthesis and the metabolic energy that drives all the cellular process. Oxygen molecules liberated into the atmosphere by photosynthesis are indispensable to the respiration of all the aerobic organisms.

Photosynthesis is composed of the sequential reactions; photochemical reactions upon absorption of light energy, electron-transport reactions, formation of reducing force, ATP synthesis, carbon assimilation, and the synthesis of sugar and starch, followed by the synthesis of amino acids, proteins, nucleic acids, and lipids. Extensive studies using biophysical, biochemical, and molecular biological techniques have revealed the mechanisms of the individual steps of photosynthesis in a molecular level. However, the knowledges on the structure and function of photosynthesis are mostly derived from the studies that are made under the optimum conditions with plant materials grown in the optimum conditions.

Plants are always subjected to environmental variations in their native habitat. It is well known that photosynthesis is particularly sensitive to environmental stresses among various physiological activities in plant cells. For example,

photosynthesis is primarily inhibited by strong light or high temperature, and is also strongly affected by low temperature or high concentration of salt. Nevertheless, plants maintain an high efficiency of photosynthesis in any given habitats. Individual plant species possesses not only a genetically determined tolerance of photosynthesis against the surrounding environment but also an ability to adapt their photosynthetic apparatus in response to environmental changes. The phenomena of photosynthetic responses to environment have long been observed and analyzed from physiological viewpoints, whereas the molecular mechanisms responsible for these phenomena have been little understood.

The productivity of a plant is directly related to the rate of photosynthetic carbon assimilation, which in turn is determined by an interaction between the photosynthetic apparatus and the environment. It is of importance to learn the mechanism of tolerance of photosynthesis against environmental stress, especially to reveal the key factor responsible for the tolerance, since it will allow us not only to understand the mechanism of plants to respond to the environment but also to alter the resistance of plants to environmental stress for the applicative purpose.

High-temperature stress is one of the major factors that limit the productivity of crops and the maintenance of ecology in the hot environments, particularly, in the tropical and desert area. Among several environmental factors (light, temperature, humidity, nutrient, etc), high-temperature stress was chosen for the subject in the present study. This study aims toward a goal of elucidating of the molecular mechanism for

photosynthetic response to high temperature and altering the resistance of plants to high-temperature stress.

1.2. Primary process of photosynthesis

1.2.1. Higher plants

Photochemical and biochemical process in photosynthesis are carried out in a intracellular organelle, the chloroplast, in higher plants (Govindjee 1982). The chloroplast contains three distinct membranes, outer envelope, inner envelope, and thylakoid membrane that define three separate internal compartments, the intermembrane space, the stroma, and the lumen. All the components necessary for photochemical process are localized in the thylakoid membranes. Thylakoid membranes form an extensive system of interconnected flattened vesicles and stack to form grana. The stroma contains the water-soluble enzymes responsible for the fixation of carbon dioxide. The products of photosynthesis are exported from the stroma to the cytoplasm via translocator proteins embedded in the envelopes.

The photochemical reactions are carried out by the supramolecular complexes consisting of proteins and pigments that are integrated in the lipid bilayer of thylakoid membranes. These complexes are classified as follows; the photosystem (PS) I and II, both of which involve the light-harvesting complex and the reaction center complex, the cytochrome b_6/f complex, and the proton-pomping ATP synthase (Figure 1-1).

In the PS II complex, the light energy absorbed by chlorophyll (Chl) and carotenoid molecules in the light-harvesting complex is transferred to the special pair of Chl molecules (P680) located in the reaction center complex. The transferred energy excites the reaction center Chls and causes a

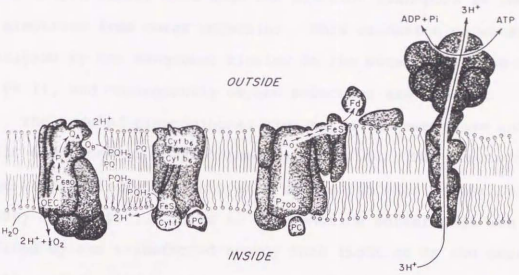

Figure 1-1. A current conceptual model of the supramolecular complexes on thylakoid membranes, related to the primary process of photosynthesis. Photosystem II complex [OEC, oxygen-evolving complex; P680, reaction center chlorophyll; Ph, pheophytin; Q_A and Q_B, primary and secondary quinone acceptors, respectively; PQ (PQH_2), mobile plastoquinone (plastoquinol)]; Cytochrome b_6/f complex (Cytb_6, cytochrome b_6; Cyt$f$, cytochrome f; FeS, Rieske Fe-S center; PC, plastocyanin); Photosystem I complex (P700, reaction center chlorophyll; A_0, primary electron acceptor; FeS, iron sulfur centers; Fd, ferredoxin); and the ATP synthase. Adapted from Govindjee and Wasielewski (1989).

charge separation. An electron is transported to the primary electron acceptor, pheophytin, and then transported to plastoquinones, designated as Q_A and Q_B. The oxidized form of the reaction center Chls upon the electron transport is reduced by electrons from water molecules. This oxidation of water is catalyzed by the manganese cluster in the water-splitting enzyme of PS II, and consequently oxygen molecules are evolved.

The reduced plastquinones migrate in the membranes and pass their electrons to a cytochrome b_6/f complex. The electrons are then transported via plastcyanin to the reaction center Chls (P700) of the PS I. In PS I, the reaction center Chls are excited by the transferred energy form light as in the case of PS II, and an electron is transported to the iron-sulfur center located in the stromal side of PS I. Consequently, the electrons are passed via ferredoxin to FNR that drives the reduction of $NADP^+$ to NADPH. The high reducing force of NADPH is supplied to the carbon-fixation metabolism (Calvin-Benson cycle) that occurs in the stroma.

This sequence of electron-transport reaction is coupled with the uptake of a proton from the stroma into the lumen across the thylakoid membranes, and the resulting generation of electrochemical potential drives the synthesis of ATP by the ATP synthase.

1.2.2. Cyanobacteria

The cyanobacteria are autotrophic prokaryotes that perform a higher-plant type of oxygenic photosynthesis (Pfennig 1978). The intracellular space is compartmented by three type of

membranes; outer and inner cytoplasmic membranes of the cell envelopes and thylakoid membranes located in the cytoplasm. The photochemical process occurs in the thylakoid membranes, and the carbon-fixation metabolism occurs in the cytoplasm. Their photosynthetic machinery contains the two photosystems and the oxygen-evolving system, and the primary process in thylakoid membranes takes place in a similar way as illustrated in Figure 1-1. There are many striking similarities between cyanobacteria and chloroplasts of higher plants. Biochemical and genetical evidences suggest that chloroplasts are descendants of cyanobacteria that were endocytosed and lived in symbiosis with primitive eukaryotic cells (Giovannoni et al. 1988).

1.3. Heat inactivation of photosynthesis

1.3.1. Heat sensitivity of photosynthesis

Temperature is one of the major factors that determine the survival of plants. In the native habitats, plants are subjected to a wide range of seasonal variation of temperature and a diurnal fluctuation of temperature. Although photosynthesis is strongly affected by temperature, plants possess the ability to maintain a high efficiency of photosynthesis in their habitats (Berry and Björkman 1980). This ability depends on partly the genotypic property resulting from the natural selection of plants that can survive in a given environment, and partly the phenotypic property involving the adjustment of their photosynthetic characteristics in response to changes in temperature.

The changes of photosynthetic activity caused by the exposure of plants at high temperatures are divided into reversible and irreversible changes. Within a normal physiological range of temperature, in general, up to 35°C, changes in photosynthetic activity are reversible. However, the exposure of plants to temperatures above this physiological range can cause an irreversible injury to photosynthesis.

The reversible changes are mostly related to the limitation of stomatal gas-exchange (Raschke 1970) and photorespiration effect (Jackson and Volk 1970, Lorimer et al. 1978). Rate-limiting steps in the Calvin cycle are also thought to be of importance (Farquhar et al. 1980, Weis 1981, 1982, Monson et al. 1982). The irreversible changes are attributed to the

susceptibility of the photosynthetic apparatus to heat damage in chloroplasts (Berry and Björkman 1980). These latter changes will be concentrated on in the present study.

The irreversible changes are characterized by a sharp decline in the rate of oxygen evolution, carbon-dioxide fixation, and photophosphorylation when the leaves or isolated chloroplasts are incubated at temperatures normally above 35°C for periods as short as a few minutes (Berry and Björkman 1980). Closure of the stomata at high temperatures is not the major reason for this inactivation, since photosynthesis under the CO_2-saturated condition is inactivated to the same degree as under the CO_2-limited condition at any given temperature (Björkman et al. 1978, Mooney et al. 1978). The heat-induced changes in the semipermeability of the partitioning membranes of the cell, such as the plasma membrane, the tonoplast, and the chloroplast envelope, occur at temperatures much higher than the inactivation of photosynthesis occurs (Krause and Santarius 1975, Thebud and Santarius 1982). The key enzymes responsible for the photosynthetic carbon metabolism, which are located in the stromal region of the chloroplast, become denatured at higher temperatures above the threshold temperatures for the irreversible changes of photosynthesis (Santarius 1975, Björkman et al. 1978). It is thus likely that the irreversible change in photosynthetic activity is associated with the primary reactions including the photochemical reaction upon absorption of light energy, the electron-transport reaction, and the phosphorylation, which occur in the thylakoid membranes.

1.3.2. Heat sensitivity of photosystem II

A number of studies have examined the sensitivity of partial reactions of photosynthetic electron-transport (Mukohata et al. 1973, Santarius 1975, Armond et al. 1978, Berry and Björkman 1980, Al-Khatib and Palsen 1989, Sabat et al. 1991, Mamedov et al. 1993). These studies demonstrate that among various electron-transport reactions, the reaction within the PS II complex is the most sensitive to heat, whereas that of PS I is rather resistant. The current conceptual model of the PS II complex is illustrated in Figure 1-2.

Mamedov et al. (1993) systematically compared the heat-sensitivity of various electron-transport and phosphorylation reactions in thylakoid membranes of the cyanobacterium *Synechocystis* PCC 6803. Temperatures for 50% inactivation of specific reactions by heat are shown in Table 1-1. The PS II-driven electron transport reaction from H_2O to PBQ, including oxygen evolution, is the most sensitive to heat. Its sensitivity exactly coincides to that of the whole electron-transport reaction from H_2O to MV including reactions in PS I and PS II. The partial electron-transport reaction of PS II from DPC to DCIP, in which DPC donates electrons to the tyrosine Z and thereby the electrons bypass the oxygen-evolving site, is more resistant than the reaction including oxygen evolution. In contrast, the electron-transport reaction from $DADH_2$ to MV, which involves PS I, and the reaction from DQH_2 to MV, which involves both PS I and cytochrome b_6/f complexes, are more resistant to heat. The phosphorylation, coupled to the cyclic

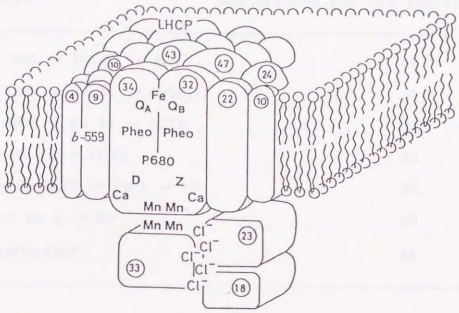

Figure 1-2. A model for the PSII complex of spinach. P680, reaction center; pheophytin (Pheo), the first electron acceptor; Q_A and Q_B, the primary and secondary quinone acceptors, respectively; Z and D, tyrosine residues (Z acts as the electron donor to P680); LHCP, the light-harvesting Chl a/b protein. Number in circles refer to the estimated relative molecular masses of proteins. Adapted from Murata and Miyao (1989).

Table 1-1. Temperature for 50% inactivation by heat of specific electron-transport reactions in thylakoid membranes of *Synechocystis* PCC 6803[a]

Reactions	Temperature for 50% inactivation, °C
$H_2O \rightarrow$ OES $\rightarrow$ PS II $\rightarrow$ PBQ	36
$H_2O \rightarrow$ OES $\rightarrow$ PS II $\rightarrow$ $Cytb_6/f \rightarrow$ MV	36
$H_2O \rightarrow$ OES $\rightarrow$ PS II $\rightarrow$ DCIP	37
DPC $\rightarrow$ PS II $\rightarrow$ DCIP	47
$DQH_2 \rightarrow$ $Cytb_6/f \rightarrow$ PS I $\rightarrow$ MV	56
$DADH_2 \rightarrow$ PS I $\rightarrow$ MV	59
ATP synthesis[b]	48

[a] Data from Mamedov et al. (1993)

[b] Coupled to cyclic electron transport in the presence of *N*-methylphenazonium methylsulfate (PMS).

OES, oxygen-evolving system; $Cytb_6/f$, cytochrome b_6/f; PBQ, phenyl-1,4-benzoquinone; MV, methylviologen; DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; DQH_2, duroquinol; $DADH_2$, 1,4-diamino-2,3,5,6-tetramethylbenzene.

transport of electrons that is mediated by PMS, is also more resistant to heat than the oxygen evolution. From all these data together, the rank order of the sensitivity to heat of the photosynthetic reactions is as follows: oxygen evolution > PS II reaction, ATP synthesis > cytochrome b_6/f reaction > PS I reaction.

1.3.3. Primary event of heat inactivation

The specific sensitivity of the oxygen-evolving system in the PS II complex to heat has long been pointed out (Berry and Björkman 1980). Katoh and San-Pietro (1967) suggested for the first time that the heat-induced inactivation of the PS II activity is due to the damage of the oxygen-evolving system. One line of the evidence to support the conclusion is that the PS II activity, which has been inactivated by heat, can be partially restored by addition of the artificial electron donors to PS II such as DPC (Yamashita and Butler 1968, Santarius 1975, Pearcy et al. 1977). Other evidences demonstrate that the inactivation of the PS II activity by heat correlates with the dissociation of manganese ions, which form the catalytic site of the oxidation of water in the PS II complex (Homann 1968, Cheniae and Martin 1970, Wydrzynski and Sauer 1980). Nash et al. (1985) demonstrated that the dissociation of two of the four manganese atoms from the PS II complex by heat results in full inactivation of oxygen evolution without a significant loss of proteins. Babcock and Sauer (1975) showed that the decline in the rate of oxygen evolution that occurs upon heat treatment of spinach chloroplasts parallels an increase in rapid ESR signal

linked to the inactivation of PS II. It is thus likely that the primary site of the heat-induced inactivation of photosynthetic reactions is the oxygen-evolving process in the PS II complex.

1.4. Photosynthetic adaptation to high temperature

1.4.1. Adaptive response in the heat stability of photosynthesis

Plants possess the ability to enhance the heat stability of their photosynthesis by adaptation to high temperatures (Berry and Björkman 1980). This acquisition of the heat tolerance of photosynthesis has been observed in a number of organisms including higher plants (Pearcy 1978, Armond et al. 1978, Raison et al. 1982, Seemann et al. 1984) and cyanobacteria (Fork et al. 1987, Lehel et al. 1993, Nishiyama et al. 1992, 1993), whereas the molecular mechanism has been little understand.

Numerous surveys of plant species from a wide range of their habitats have revealed the general correlation between the heat stability of photosynthesis and the temperature of environment surrounding the plant (Berry and Björkman 1980). Since photosynthesis is one of the most heat-sensitive activity in plant cell in spite of its importance for the viability of plants, it can be postulated that the heat stability of photosynthesis is a key factor that determines the tolerance of plants to high temperatures.

Figure 1-3 shows a typical example for the acquisition of the heat tolerance of photosynthesis upon adaptation to high temperature. *Larrea divaricata*, a desert evergreen shrub, was grown at 20°C day/15°C night and at 45°C day/33°C night, and the heat stability of photosynthesis (net CO_2 uptake) in the leaves was examined in both samples (Mooney et al. 1978). It is clear that the heat stability is markedly enhanced upon an increase in growth temperature. By measuring the fluorescence yield of

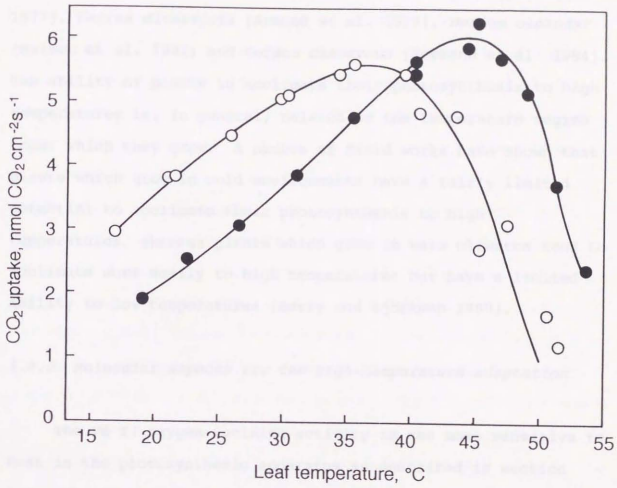

Figure 1-3. Temperature dependence of the net photosynthesis in intact leaves of *Larrea divaricata*, grown at 20°C/15°C (○) or 45°C/33°C (●). The rate of CO_2 uptake is determined after treatment for 10 min at designated temperatures. Adapted from Mooney et al. (1978).

chlorophyll a associated with PS II, a similar enhancement in the heat stability of photosynthesis has been found in several species of desert plants, *Atriplex lentiformis* (Pearcy et al. 1977), *Larrea divaricata* (Armond et al. 1978), *Nerium oleander* (Raison et al. 1982) and *Geraea canescens* (Seemann et al. 1984). The ability of plants to acclimate their photosynthesis to high temperatures is, in general, related to the temperature regime under which they grow. A number of field works have shown that plants which grow in cold environments have a fairly limited potential to acclimate their photosynthesis to high temperatures, whereas plants which grow in warm climates tend to acclimate more easily to high temperatures but have a limited ability to low temperatures (Berry and Björkman 1980).

1.4.2. Molecular aspects for the high-temperature adaptation

The PS II oxygen-evolving activity is the most sensitive to heat in the photosynthetic apparatus as described in section 1.3. Thus the acquisition of the heat tolerance of the PS II activity appears to be the mechanism of the photosynthetic adaptation to high temperature. The possible factors related to the heat tolerance of photosynthesis can be classified into membrane lipids, proteins, and the other biochemical components.

(A) Membrane lipids

Since the PS II complex is integrated in thylakoid membranes, several investigators hypothesized that physical properties of the membranes contribute to the heat tolerance of photosynthesis (Quinn and Williams 1985, Quinn 1988, Webb and

Green 1991). Pearcy (1978) have shown that an increase in the growth temperature leads to the increased level of saturation of fatty acids in the leaf lipids of *Atriplex lentiformis*. A detailed analysis of the changes in lipid composition of chloroplasts isolated from *Nerium oleander* grown at different temperatures has been also reported by Raison et al. (1982). They reported no significant changes in the composition of the neutral lipid, galactolipid, and phospholipid classes during adaptation but obvious changes in the compositions of fatty acids of each lipid class. The most significant change was a decrease in the proportion of trienoic C_{18} fatty acids in the total lipid extract. This change was accompanied by an increase in the proportions of saturated C_{16} fatty acids, monoenoic and dienoic C_{18} fatty acids. The increase in the saturation of lipids was, furthermore, accompanied by an decrease in the membrane fluidity which was determined by spin labelling. Based on these observations, they concluded that the saturation of lipids plays a major role in the heat tolerance of photosynthesis upon adaptation to high temperatures. However, it should be noted that changes in growth temperature affect not only the fatty-acid saturation but also various other metabolic factors.

Catalytic hydrogenation of membrane lipids with palladium (II) has been used to investigate the role of unsaturated fatty acids (Vigh et al. 1985). Thomas et al. (1986) observed that the heat stability of PS II activity in the thylakoid membranes of pea was enhanced after hydrogenation up to 90% of the total double bonds of membrane lipids. This observation led them to conclude the contribution of lipid saturation to the enhancement

in the heat stability of photosynthesis during adaptation to high temperature. However, it should be noted that the hydrogenation up to 40% of the total double bonds, which may correspond to changes within the physiological conditions, the heat stability of PS II was not altered at all. It is thus difficult to conclude that the saturation of lipids determine the heat stability of photosynthesis under physiological conditions.

On the other hand, recent studies using mutants and transformants of cyanobacteria and higher plants which are defective in desaturations tend to deny the contribution of lipid saturation to the heat tolerance of photosynthesis.

Gombos and his colleagues (1991, 1992, 1994) have directly evaluated the effect of the unsaturation of the glycerolipids of thylakoid membranes on the heat stability of photosynthetic oxygen evolution, using the mutants and transformants of the cyanobacterium *Synechocystis* PCC 6803. Fatty-acid composition of the total lipids from thylakoid membranes of the wild-type, Fad12, and Fad12/*desA* strain of *Synechocystis* PCC 6803 is summarized in Table 1-2. Fad12 mutant is defective in desaturation of at Δ^{12} position of C_{18} fatty acids, and consequently its thylakoid membranes lack trienoic acids (18:3) and contain a very reduced level of dienoic acids (18:2) (Wada and Murata 1989). A transformant, Fad12/*desA*, was obtained by transforming Fad12 mutant with a gene responsible for desaturation at Δ^{12} position (*desA*). This transformant fully recovers the ability to desaturate fatty acids, and consequently the contents of dienoic and trienoic fatty acids are almost the same as those of the wild type (Table 1-2). As shown in Figure

Table 1-2. Fatty acid composition of total lipids from the wild-type, Fad12 and Fad12/*desA* strains of *Synechocystis* PCC 6803[a]

Fatty acid	Strain		
	WT	Fad12 (mol%)	Fad12/*desA*
14:0	t	t	t
16:0	61	57	62
16:1	5	4	3
18:0	1	1	1
18:1(9)	8	33	8
18:2(6,9)	t	5	t
18:2(9,12)	11	0	12
18:3(6,9,12)	14	0	13
18:3(9,12,15)	t	0	t
18:4(6,9,12,15)	t	0	t

[a] Data from Gombos et al. (1991)

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

Fatty acids are represented by number 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, myrisic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1(9), oleic acid; 18:2(6,9), $\Delta^{6,9}$-octadecadienoic acid; 18:2(9,12), linoleic acid; 18:3(9,12,15), α-linolenic acid; 18:3(6,9,12), γ-linolenic acid; 18:4(6,9,12,15), $\Delta^{6,9,12,15}$-octadecatetraenoic acid.

1-4, no significant difference is found in the heat stability of oxygen evolution among these three strains (Gombos et al. 1991).

Gombos et al. (1994) also have lately demonstrated that the saturation of lipids does not increase the heat stability of photosynthetic oxygen evolution. Fad6 mutant of *Synechocystis* PCC 6803, which is defective in desaturation at the Δ^6 position of C_{18} fatty acids (Wada et al. 1992), did not show any difference in the heat stability of oxygen evolution as compared to the wild-type strain. Fad6/*desA*::Km^r strain is a transformant of Fad6 strain, in which *desA* gene responsible for the desaturation at the Δ^{12} position of C_{18} fatty acids is interrupted by the kanamycin-resistance gene cartridge (Wada et al. 1992). This strain lacks both dienoic and trienoic fatty acids and contains saturated and monoenoic species. Strikingly, this elimination of dienoic and dienoic lipid molecules even reduced, to a small but distinct extent, the heat stability of oxygen evolution.

In higher plants, the effect of lipid saturation on the heat stability of photosynthesis has been studied using mutants of *Arabidopsis thaliana* which are defective in desaturation. McCourt et al. (1987) observed that a decrease in the level of *sn*-1-18:3/*sn*-2-16:3-monogalactosylacylglycerol (MGDG) and a corresponding increase in the level of *sn*-1-18:2/*sn*-2-16:2-MGDG in a mutant strain of *Arabidopsis* did not affect the heat stability of photosynthesis. Hugly et al. (1989) observed only insignificant differences in the heat stability of photosynthetic electron transports from H_2O to MV and H_2O to DCIP in the thylakoid membranes from the *fad*C mutant of *Arabidopsis* which contained a reduced level of *sn*-1-18:3/*sn*-2-16:3-MGDG and

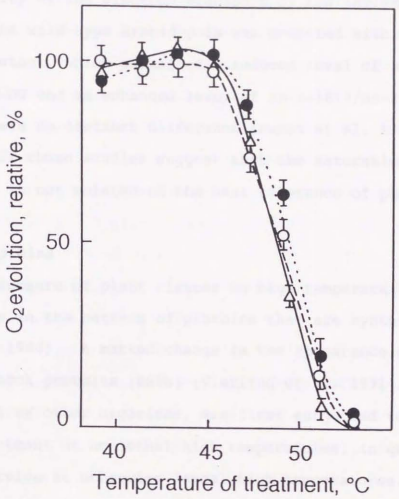

Figure 1-4. The profiles of heat inactivation of photosynthetic oxygen evolution in cells of the wild-type, Fad12, and Fad12/*desA* strains of *Synechocystis* PCC 6803, grown at 34°C. Oxygen evolution is measured at 34°C after treatment for 20 min at designated temperatures. Wild-type (Δ), Fad12 (●), Fad12/*desA* (○). Adapted from Gombos et al. (1991).

an enhanced level of *sn*-1-18:1/*sn*-2-16:1-MGDG (Browse et al. 1989) as compared to those from wild-type leaves. When the heat stability of the electron transport of the thylakoid membranes from the wild-type *Arabidopsis* was compared with that from the *fad*B mutant, which contained a reduced level of *sn*-1-18:3/*sn*-2-16:3-MGDG and an enhanced level of *sn*-1-18:3/*sn*-2-16:0-MGDG, there was no distinct difference (Kunst et al. 1989a, b).

All these studies suggest that the saturation of membranes lipids is not related to the heat tolerance of photosynthesis.

(B) Proteins

Exposure of plant tissues to high temperatures results in changes in the pattern of proteins that are synthesized (Barnett et al. 1980). A marked change is the appearance of a set of heat shock proteins (HSPs) (Vierling et al. 1991). When plants, as well as other organisms, are first subjected to a pretreatment at nonlethal high temperatures, in general, they can survive at otherwise lethal high temperatures. This acquisition of thermotolerance is normally observed under which HSPs are synthesized (Kimpel and Key 1985, Lindquist 1986), whereas the function of HSPs in thermotolerance has not been established yet.

The arguments concerning the role of HSPs in thermotolerance are well illustrated in the advanced studies with yeast. Although HSP expression correlates with the development of thermotolerance in this organism, it is also observed that mutants which lack HSP90, HSP26, HSP70, and HSP104 all possess the ability to express acquired thermotolerance (Lindquist and Craig 1988, De Virgilio et al. 1991).

In plants, HSP70, HSP60, and low-molecular weight HSPs are found to be localized in chloroplasts, although their *in vivo* function is poorly understood (Vierling 1991). Schuster et al. (1988) have shown that a 22 kDa-HSP of *Chlamydomonas* is located in granal thylakoids and protects PS II from photoinhibition during subsequent heat treatments. A GroEL-related chaperonin of *Synechocystis* PCC 6803 has been isolated (Lehel et al. 1992), and its accumulation on thylakoid membranes has been observed during exposure of the cyanobacterium to high temperatures (Kovács et al. 1994). These observation suggest the correlation of the synthesis of HSPs with thermotolerance. However, there is no direct evidence at present for the role of HSPs in the heat tolerance of photosynthesis.

It is also possible that some unknown protein factor other than HSPs, which is also regulated by temperature, protects the PS II complex against heat inactivation. The modification of the constituents of the PS II complex can also happen during the adaptation of the cell to high temperatures.

(C) Other factors

Informations concerning the factors other than membranes lipids and HSPs in relation to the photosynthetic adaptation to high temperature are quite limited. Kaiser (1984) has observed that high cellular osmotic potential correlates with the heat stability of photosynthesis. Havaux (1992) has observed that the heat stability of PS II increases in the leaves when the plant has been exposed to the water-stress conditions, which are often associated with heat stress in the natural field. These studies suggest that accumulation of some osmoprotective

compounds, such as sugar and its derivatives, may play a role in protection of photosynthetic apparatus from inactivation by heat. Glycinebetaine, an osmoregulant of halophilic plants, is localized in their chloroplasts (Hanson et al. 1985). Mamedov et al. (1991, 1993) have shown that glycinebetaine can protect the oxygen-evolving activity of PS II from heat-induced inactivation by *in vitro* experiments. Trehalose, a disaccharide, is known as an osmoregulant in *E.coli*. Studies using mutants of *E.coli* which are defective in the synthesis of trehalose have revealed that trehalose is involved in thermotolerance in stationary-phase cells of *E.coli*, but that it is not required for the development of adaptive thermotolerance in exponentially growing cells (Hengge-Aronis et al. 1991).

1.5. Aim of the present study

The photosynthetic response and adaptation to high temperature have been introduced in this chapter. Plants can acquire the heat tolerance of photosynthesis by adapting to high temperatures (Berry and Björkman 1980). It was long postulated that the key factor for the heat tolerance of photosynthesis would be the changes in the saturation level of membrane lipids according to physiological observations (Pearcy 1978, Raison et al. 1982). Recent studies, however, are giving a negative answer to this hypothesis (Gombos et al. 1991, 1994). Some unknown biochemical factors should contribute to the heat tolerance. Thus, the molecular mechanism of the heat tolerance of photosynthesis is far from understanding at present.

Photosynthesis is primarily inactivated when plants are exposed to high temperatures (Berry and Björkman 1980). The observed photosynthetic adaptation, therefore, suggests that the acquisition of the heat tolerance of photosynthesis plays an important role in the whole cellular tolerance to heat. Identification of the key factor which is responsible for the heat tolerance will allow us not only to understand the molecular mechanism of response of plants to high temperature but also to alter the heat tolerance of plants by genetic manipulation.

The cyanobacteria are prokaryotes that perform a higher-plant type of oxygenic photosynthesis (Pfennig 1978), as described in section 1.2.2. The cyanobacteria are suitable for the physiological analyses since their growth condition is easily controlled. They normally grow within periods as short

as 3 to 5 days. Some of the unicellular cyanobacteria which are classified as *Synechococcus* or *Synechocystis* are known to be transformable species that can incorporate exogenous DNA and transform their chromosomal DNA by homologous recombination (Porter 1988).

The aim of the present study is to understand the molecular mechanism of the heat tolerance of photosynthesis using *Synechococcus* PCC 7002 which belongs to the transformable cyanobacteria. The transformable feature will be useful in the future study of the molecular-biological analyses of transformants which are altered in the factor for the heat tolerance of photosynthesis. The cell wall of *Synechococcus* PCC 7002 can be mechanically disrupted more easily than other transformable cyanobacteria such as *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942. This property is useful to the biochemical analyses in more intact thylakoid membranes. The followings are major research subjects of the present study:

(1) Characterization of the photosynthetic adaptation to high temperature in cells and thylakoid membranes of *Synechococcus* PCC 7002.

(2) Biochemical analyses of the heat tolerance of photosynthesis in thylakoid membranes of *Synechococcus* PCC 7002.

(3) Identification of the factor for the heat tolerance of photosynthesis in *Synechococcus* PCC 7002.

(4) Molecular cloning of the gene encoding the factor for the heat tolerance of photosynthesis in *Synechococcus* PCC 7002.

Chapter 2

Characterization of photosynthetic adaptation to high temperature in the cyanobacterium *Synechococcus* PCC 7002

2.1. Summary

Photosynthetic adaptation to high temperature was investigated in intact cells and isolated thylakoid membranes of the cyanobacterium, *Synechococcus* PCC 7002. In intact cells, the thermal stability of photosynthesis and photosystem 2-mediated electron transport from H_2O to 1,4-benzoquinone changed in concert with growth temperature. The photosystem 2-mediated electron transport from H_2O to phenyl-1,4-benzoquinone showed greater thermal stability in thylakoid membranes isolated from cells which had adapted to high temperature than in those from non-adapted cells. Enhanced thermal stability was also observed in the thylakoid membranes in the transport of electrons from H_2O to 2,6-dichlorophenolindophenol but not in the transport of electrons from diphenylcarbazide to 2,6-dichlorophenolindophenol. These observations suggest that oxygen-evolving sites acquire enhanced thermal stability, and that factors which are responsible for thermal stability remain in isolated thylakoid membranes.

2.2. Introduction

When plants are exposed to heat, their photosynthesis can be irreversibly inactivated. However, some plants have the ability to adapt their photosynthetic apparatus to high-temperatures and to enhance the thermal stability of photosynthesis (Berry and Björkman 1980). While these phenomena have been observed in a number of organisms, including higher plants (Pearcy 1978, Armond et al. 1978, Raison et al. 1982), and cyanobacteria (Fork et al. 1987, Vigh et al. 1990), the mechanism for this adaptation has been little understood.

It has been recognized that PS II activity is the most sensitive to heat among a number of photosynthetic activities (Berry and Björkman 1980). Among the partial reactions of PS II, the oxygen-evolving process is particularly sensitive to heat (Katoh and San Pietro 1967, Yamashita and Butler 1968, Santarius 1975). The dissociation of two of the four Mn atoms from the PS II complex by heat results in full inactivation of oxygen evolution without a significant loss of proteins (Nash et al. 1985). It was predicted, therefore, that the mechanism of photosynthetic adaptation to high temperature is related to the ability of the PS II oxygen-evolving complex to protect against heat-induced inactivation.

Since the photosynthetic PS II complex is integrated with thylakoid membranes, several authors hypothesized that physical properties of the membranes may contribute to the thermal stability of photosynthesis (Quinn and Williams 1985, Quinn 1988, Webb and Green 1991). Pearcy (1978) and Raison et al. (1982) have observed that the high temperature of growth

increases the level of saturation of fatty acids of membrane lipids and enhances the thermal stability of photosynthesis. They concluded that the saturation of lipids plays an important role in the adaptation to high temperature. However, this notion should be reevaluated since it is possible that other factors, which are also regulated by growth temperature, may contribute to the observed adaptation.

Gombos et al. (1991, 1992) examined the effect of the fatty-acid saturation on the thermal stability of PS II activity, using isothermally grown cells of the mutant (Wada and Murata 1989, Wada et al. 1990) and transformant (Wada et al. 1992) of *Synechocystis* PCC 6803 which are defective in desaturation at the Δ^{12} position of C_{18} fatty acids. These studies have clearly demonstrated that thermal stability is not affected by changes in the saturation level of membrane lipids. These findings suggest that factors other than lipids are responsible for the adaptation of photosynthesis to high temperature.

In the present study, the adaptation of photosynthesis to high temperature was studied with the use of the cyanobacterium, *Synechococcus* PCC 7002. The effect of growth temperature on the thermal stability of PS II activity was examined in both intact cells and isolated thylakoid membranes. The enhanced thermal stability of PS II activity was found to be retained in the thylakoid membranes isolated from cells which had adapted to high temperature. This enhanced thermal stability was acquired in the oxygen-evolving site in the PS II complex.

2.3. Materials and Methods

Organism and culture conditions

Synechococcus PCC 7002 was obtained from the Pasteur Culture Collection. The cells were grown photoautotrophically at designated temperatures in medium A, as described by Stevens et al. (1973). The cells were aerated with 1% CO_2 in air and were illuminated with incandescent lamps at a light intensity of 15 W m^{-2}.

Preparation of thylakoid membranes

A 1.5-liter culture with a cell density at 5 μg Chl mL^{-1} was centrifuged at 8,000 x *g* for 10 min. The sedimented cells were washed twice with 100 ml of 50 mM Hepes-NaOH (pH 7.5) containing 30 mM $CaCl_2$. The above procedures were performed at 25°C.

The following procedures were performed at 0 to 4°C. Sedimented cells were suspended in 12.5 mL of 50 mM Hepes-NaOH (pH 7.5) containing 1.0 M glycinebetaine, 800 mM sorbitol, 30 mM $CaCl_2$ and 1 mM 6-amino-n-caproic acid (medium B). The suspension was mixed with an equal volume of glass beads with a diameter of 0.1 mm and the mixture was placed in a homogenizer (BEAD-BEATER; Biospec products, Bartlesville, OK, USA). Cell breakage was performed twice for 20 sec, resulting in breakage of more than 80% of the cells. The homogenate was centrifuged at 1,000 x *g* for 1 min and the supernatant was collected. The pelleted glass beads were washed with 15 mL of medium B and the suspension was centrifuged under the same conditions as above.

The supernatant was collected, and the two kinds of supernatant were combined. This combined supernatant was centrifuged at 10,000 x *g* for 10 min to remove unbroken cells. The resulting supernatant was then centrifuged at 60,000 x *g* for 60 min. The sedimented thylakoid membranes were suspended in 20 mL of medium B and the suspension was centrifuged again at 60,000 x *g* for 60 min. The pelleted thylakoid membranes were suspended in 2 mL of medium B, and were kept at 4°C for no longer than 1 day before being used. Thylakoid membranes were also prepared in the absence of glycinebetaine in medium B, while the other conditions were the same as above. The concentration of Chl was determined by the method of Arnon et al. (1974).

Heat treatment

The cells in medium A at a concentration corresponding to 5 μg Chl mL^{-1} were incubated in darkness for 60 min at designated temperatures. Thylakoid membranes were incubated in darkness for 20 min at designated temperatures in 50 mM Tricine-NaOH (pH 7.5), 600 mM sucrose, and 30 mM $CaCl_2$ (medium C) in the presence or absence of 1.0 M glycinebetaine, and at a thylakoid membrane concentration corresponding to 10 μg Chl mL^{-1}. After incubation, the suspension was promptly cooled to 25°C, and a suitable electron acceptor was then added to measure the evolution of oxygen.

Measurement of photosynthetic activities

The photosynthetic evolution of oxygen was measured by monitoring the concentration of oxygen in the suspension medium with a Clark-type oxygen electrode. Photosynthesis in intact cells was measured at 30°C in medium A in the absence of any exogenously added electron acceptor. The PS II-mediated electron transport from H_2O to BQ in intact cells was measured at 30°C in medium A supplemented with 1 mM BQ and 1 mM $K_3Fe(CN)_6$. The PS II-mediated electron transport from H_2O to PBQ in thylakoid membranes was measured at 25°C in medium C supplemented with 100 μM PBQ. The reaction mixture was equilibrated at 25°C for 3 min before measurement. Red actinic light at an intensity of 640 W m^{-2} was provided from an incandescent lamp through the combination of a heat-absorbing optical filter (HA50; Hoya, Tokyo, Japan) and a red optical filter (R-60; Toshiba, Tokyo, Japan).

Reduction of DCIP in the light was determined by monitoring changes in absorbance at 580 nm, with a reference beam of 500 nm, using a dual-wavelength spectrophotometer (UV-300; Shimadzu, Kyoto, Japan). The PS II-mediated electron transport from H_2O to DCIP was measured in medium C supplemented with 50 μM DCIP. For assay of the PS II-mediated electron transport from DPC to DCIP, the thylakoid membranes were incubated in darkness in 800 mM Tris-HCl (pH 8.4) for 30 min at 4°C and the mixture was centrifuged at 60,000 x *g* for 60 min. The pelleted membranes were suspended in medium C and the suspension was subjected to the heat treatment described above. The electron transport from DPC to DCIP was measured in medium C supplemented with 50 μM

DCIP and 1 mM DPC. Red actinic light at an intensity of 1200 W m^{-1} was obtained from an incandescent lamp using two optical filters as described above.

2.4. Results

Thermal stability of photosynthetic activities in cells

Figure 2-1 shows the profiles of heat inactivation of photosynthesis measured by the evolution of oxygen in intact cells grown at 25°C and 40°C. Growth temperature had a distinct effect on the thermal stability of photosynthesis. The temperature for 50% inactivation appeared at 46°C and 50°C in cells grown at 25°C and 40°C, respectively. This observation indicates that the cells grown at 40°C acquired more enhanced thermal stability of photosynthesis than cells grown at 25°C.

A similar change in the profile of heat inactivation was observed in the PS II-mediated electron transport from H_2O to BQ as measured by the evolution of oxygen in the presence of BQ as the electron acceptor. As shown in Figure 2-2, 50% inactivation appeared at 44°C and 48°C in cells grown at 25°C and 40°C, respectively. These results suggest that PS II activity, which is the most sensitive to heat, can adapt to high temperature.

In order to elucidate the mode of thermal stability acquisition in *Synechococcus* PCC 7002, the profile of heat inactivation was examined in cells grown at various temperatures. Figure 2-3 shows cells grown at various temperatures compared according to the temperature for the 50% inactivation of photosynthetic activities. The thermal stabilities of these reactions gradually increased in almost linear relationship to growth temperature.

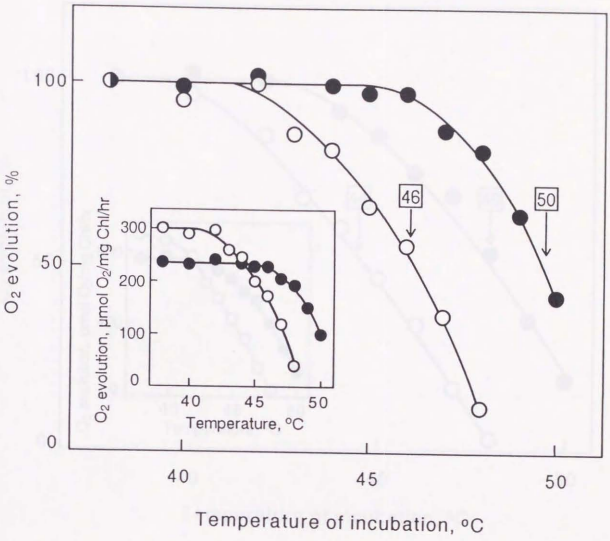

Figure 2-1. Profiles of heat inactivation of photosynthesis in cells of *Synechococcus* PCC 7002 grown at 25°C and 40°C. The cells were treated for 60 min at designated temperatures in darkness, and the photosynthetic activity was measured by oxygen evolution at 30°C without exogenous electron acceptor. Cells grown at 25°C, (○) cells grown at 40°C, (●). The numbers in rectangles are temperatures for 50% inactivation.

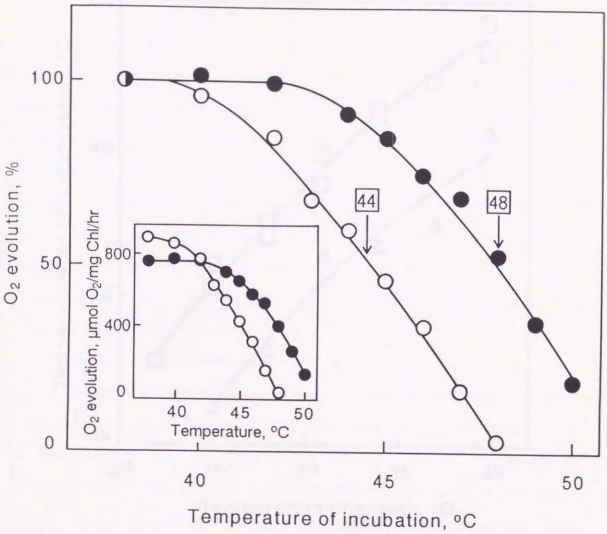

Figure 2-2. Profiles of heat inactivation of PS II-mediated electron transport in cells of *Synechococcus* PCC 7002 grown at 25°C and 40°C. The cells were treated for 60 min at designated temperatures in darkness, and the electron transport from H_2O to BQ was measured by oxygen evolution at 30°C in the presence of 1 mM BQ and 1 mM $K_3Fe(CN)_6$. Cells grown at 25°C, (○); cells grown at 40°C, (●). The numbers in rectangles are temperatures for 50% inactivation.

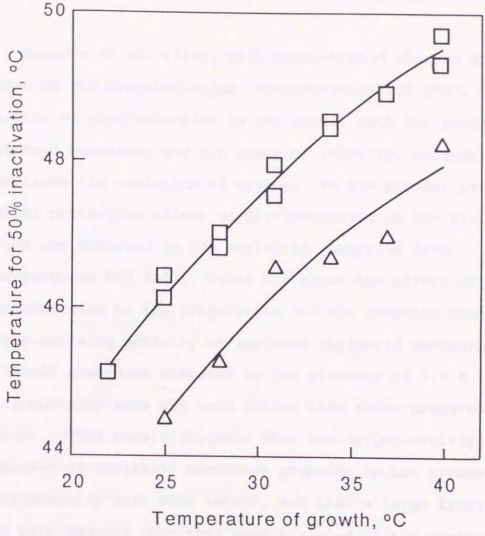

Figure 2-3. The relationship between growth temperature and thermal stability of photosynthesis and the PS II-mediated electron transport in cells of *Synechococcus* PCC 7002. The temperatures for 50% inactivation of photosynthesis and the PS2 activity were plotted against growth temperature. Conditions for measurements of photosynthesis and PS II-mediated electron transport were the same as in Figures 2-1 and 2-2. Photosynthesis, (□); PS2 activity, (Δ).

The effect of glycinebetaine on the photosynthetic activities of isolated thylakoid membranes

Mamedov et al. (1991) have demonstrated that in another strain of the cyanobacterium, *Synechocystis* PCC 6803, the presence of glycinebetaine in the media, both for preparation of thylakoid membranes and for assay of activity, enhances and stabilizes the evolution of oxygen. In the present study, a similar protective effect of glycinebetaine on the evolution of oxygen was observed in the thylakoid membranes from *Synechococcus* PCC 7002. Table 2-1 shows the effect of adding glycinebetaine to the preparation and the reaction media on the oxygen-evolving activity of isolated thylakoid membranes. The thylakoid membranes prepared in the presence of 1.0 M glycinebetaine were 40% more active than those prepared in its absence. This result suggests that the oxygen-evolving complexes of thylakoid membranes prepared in the presence of glycinebetaine were more intact, and that a large fraction of them were damaged when they were prepared in the absence of glycinebetaine.

Table 2-1 also shows that activities of both types of thylakoid membranes were enhanced by approximately 20% using exogenously added glycinebetaine. In addition, the incubation of thylakoid membranes for 10 h in medium C in the presence of glycinebetaine at 25°C did not diminish the evolution of oxygen with PBQ as the electron acceptor, while incubation for 10 h in

Table 2-1. Effects of glycinebetaine in the preparation and the reaction media on the oxygen-evolving activity of thylakoid membranes of cells of *Synechococcus* PCC 7002 grown at 34°C. The results are averages ± S.D. of determinations from three independent experiments.

Type of preparation	Glycinebetaine during measurement of activity	
	None	1.0 M
	[μmoles O_2 (mg Chl)$^{-1}$ h^{-1}]	
Thylakoid membranes prepared with 1.0 M glycinebetaine	327 ± 35	400 ± 37
Thylakoid membranes prepared without glycinebetaine	254 ± 23	302 ± 20

the same medium without glycinebetaine resulted in a 50% decrease in activity. Therefore, both the medium used for isolation of the thylakoid membranes and the medium used for the assay of photosynthetic activities were supplemented with 1.0 M glycinebetaine.

Thermal stability of PS II activities in isolated thylakoid membranes

Figure 2-4 shows the profiles of heat inactivation of the PS II-mediated electron transport from H_2O to PBQ in thylakoid membranes isolated from cells grown at 25°C and 40°C. It is clear that growth temperature had a marked effect on the thermal stability of PS II activity. The temperature for 50% inactivation was 38°C and 42°C in the thylakoid membranes from cells grown at 25°C and 40°C, respectively. The difference of this midpoint temperature by 4°C was the same as that observed in intact cells. This indicates that the thylakoid membranes from cells grown at 40°C retained greater thermal stability of PS II activity than those from cells grown at 25°C. However, the temperatures for 50% inactivation were lower than those for intact cells.

Since glycinebetaine protects the evolution of oxygen against inactivation by heat (Mamedov et al. 1993), we also examined the thermal stability of thylakoid membranes which were isolated and incubated in the absence of glycinebetaine. The temperature profile was essentially the same as Figure 2-4,

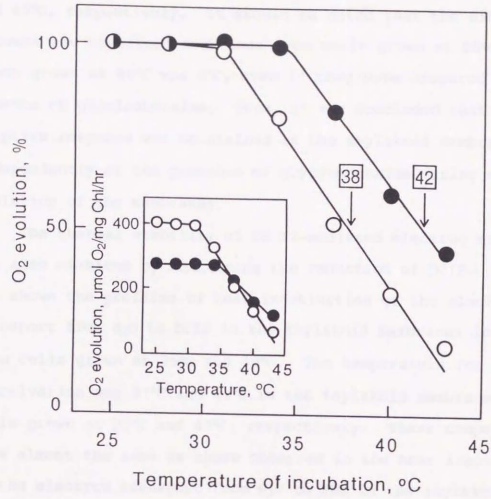

Figure 2-4. Profiles of heat inactivation of PS II-mediated electron transport from H_2O to PBQ in thylakoid membranes isolated from cells of *Synechococcus* PCC 7002 grown at 25°C and 40°C. The membranes were treated for 20 min at designated temperatures in darkness, and the electron transport from H_2O to PBQ was measured by oxygen evolution at 25°C. Thylakoid membranes from cells grown at 25°C, (○); thylakoid membranes from cells grown at 40°C, (●). The numbers in rectangles are temperatures for 50% inactivation.

although the temperature for 50% inactivation in PS II activity of these membranes was 35°C and 39°C from cells grown at 25°C and 40°C, respectively. It should be noted that the difference between the thylakoid membranes from cells grown at 25°C and those grown at 40°C was 4°C, even if they were prepared in the absence of glycinebetaine. Thus, it was concluded that the adaptive response was maintained in the thylakoid membranes independently of the presence of glycinebetaine during the isolation of the membranes.

The thermal stability of PS II-mediated electron transport was also examined by monitoring the reduction of DCIP. Figure 2-5 shows the profiles of heat inactivation of the electron transport from H_2O to DCIP in the thylakoid membranes isolated from cells grown at 25°C and 40°C. The temperature for 50% inactivation was 37°C and 42°C in the thylakoid membranes of cells grown at 25°C and 40°C, respectively. These temperatures were almost the same as those observed in the heat inactivation of the electron transport from H_2O to PBQ in the thylakoid membranes.

The electron transport from DPC to DCIP was assayed in the thylakoid membranes which had been treated with 800 mM Tris-HCl (pH 8.4) to allow the electrons to by-pass the oxygen-evolving site (Yamashita and Butler, 1969). These Tris-treated membranes ceased all electron transport activities from H_2O to DCIP. Figure 2-6 shows the profiles of heat inactivation of the electron transport from DPC to DCIP in the Tris-treated thylakoid membranes from cells grown at 25°C and 40°C. The temperature for 50% inactivation was 55°C, in the thylakoid membranes from cells grown at both 25°C and 40°C, indicating

that growth temperature had no effect on the thermal stability of the electron transport from DPC to DCIP. The thermal stability of this electron transport was much higher than that which included the oxygen-evolution process. These results suggest that enhanced thermal stability is acquired in the oxygen-evolving site, but not in the other electron transport reactions of PS II.

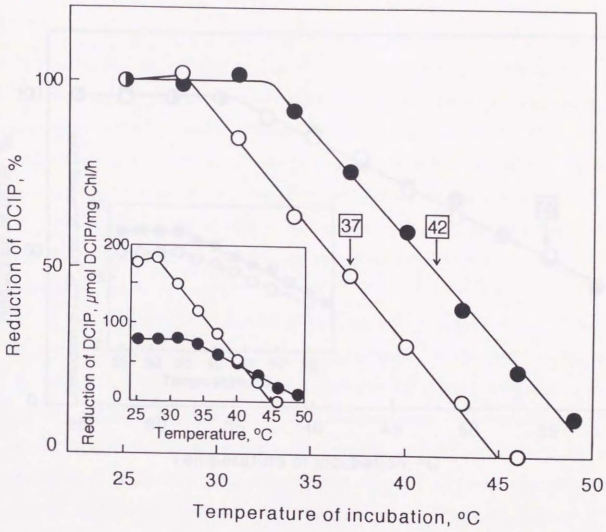

Figure 2-5. Profiles of heat inactivation of PS II-mediated electron transport from H_2O to DCIP in thylakoid membranes isolated from cells of *Synechococcus* PCC 7002 grown at 25°C and 40°C. The membranes were treated for 20 min at designated temperatures in darkness, and the electron transport from H_2O to DCIP was measured by reduction of DCIP at 25°C. Thylakoid membranes from cells grown at 25°C, (○); thylakoid membranes from cells grown at 40°C, (●). The numbers in rectangles are temperatures for 50% inactivation.

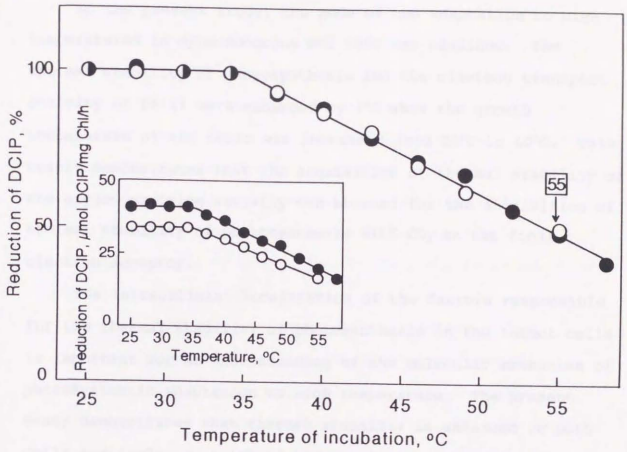

Figure 2-6. Profiles of heat inactivation of PS II-mediated electron transport from DPC to DCIP in thylakoid membranes isolated from cells of *Synechococcus* PCC 7002 grown at 25°C and 40°C. Following the treatment with 800 mM Tris-HCl (pH 8.4), the membranes were treated for 20 min at designated temperatures in darkness, and the electron transport from DPC to DCIP was measured by reduction of DCIP at 25°C. Thylakoid membranes from cells grown at 25°C, (○); thylakoid membranes from cells grown at 40°C, (●). The numbers in rectangles are temperatures for 50% inactivation.

2.5. Discussion

In the present study, the mode of the adaptation to high temperatures in *Synechococcus* PCC 7002 was examined. The thermal stability of photosynthesis and the electron transport activity of PS II were enhanced by 4°C when the growth temperature of the cells was increased from 25°C to 40°C. This result demonstrates that the acquisition of thermal stability of the oxygen-evolving activity can account for the acquisition of thermal stability of photosynthesis with CO_2 as the final electron acceptor.

The intracellular localization of the factors responsible for the thermal stability of photosynthesis in the intact cells is important for an understanding of the molecular mechanism of photosynthetic adaptation to high temperature. The present study demonstrates that thermal stability is enhanced in both cells and isolated thylakoid membranes, i.e, the temperature for 50% inactivation increases by 4°C upon an increase in growth temperature from 25°C to 40°C. This result indicates that factors responsible for thermal stability are associated with the thylakoid membranes, and that even after isolation, the thylakoid membranes retain these factors. This association between some thermo-stabilizing factor and the thylakoid membranes has not been previously reported.

The present study also demonstrates that only the oxygen-evolving site, which is particularly sensitive to heat, can acquire enhanced thermal stability in the PS II complexes. Therefore, the protection of the oxygen-evolving site against inactivation by heat may be the mechanism by which

photosynthetic adaptation to high temperature is achieved, and further, the thermo-stabilizing factor can protect the oxygen-evolving site.

As shown in Figure 2-4, isolated thylakoid membranes are more susceptible to heat when they have been isolated than they are in the cells. This shift may be attributed to the loss of the interaction between the thylakoid membranes and the cytosol. Nevertheless, the extent of the observed shift in thermal stability due to growth temperature is retained after the isolation. It is likely that once the factors for thermal stability have been induced, their effects are independent of any interaction with the cytosol.

It is interesting to note that the enhanced thermal stability is acquired in linear relationship to growth temperature (Figure 2-3). This finding indicates that thermal protection does not occur abruptly above a certain temperature. In this respect, it is worth while to discuss whether HSPs play a role in enhancing the thermal stability of photosynthesis. It has been reported that a 22 kDa-HSP of *Chlamydomonas* associates with granal thylakoids and protects against photoinhibition during heat treatment (Schuster et al. 1988). A GroEL-related chaperonin of *Synechocystis* PCC 6803 has been isolated, and its accumulation on thylakoid membranes has been observed during exposure of the cyanobacterium to high temperatures (Vigh et al. 1990, Lehel et al. 1992). Thus far, however, there is no direct evidence for the role of HSPs in the thermal stability of photosynthesis. These HSPs are synthesized in great amounts only above certain threshold temperatures. The linear relationship between thermal stability and growth temperature

suggests that HSPs are not involved in the adaptation of photosynthesis to high temperatures. Further biochemical and molecular biological studies are necessary to identify the factors responsible for the thermal stability of photosynthesis.

Chapter 3

Biochemical analyses of the heat tolerance of photosynthesis in thylakoid membranes from the cyanobacterium *Synechococcus* PCC 7002

3.1. Summary

Thylakoid membranes isolated from cells of the cyanobacterium *Synechococcus* PCC 7002, which had adapted to high temperature, possessed a greater heat stability of oxygen evolution than those from non-adapted cells (Chapter 2). The heat tolerance acquired in the thylakoid membranes was characterized by biochemical analyses. Treatment of the thylakoid membranes with 1.0 M NaCl did not affect the heat stability of oxygen evolution. In contrast, treatment of the thylakoid membranes with 0.1% Triton X-100 resulted in a remarkable decrease in the heat stability. The heat stability, however, was restored when the membranes were reconstituted with the components dissociated from the membranes with Triton X-100. When the dissociated components were boiled, they failed to restore the heat stability. Furthermore, addition of these components to the heat-sensitive thylakoid membranes, which had been isolated from non-adapted cells, resulted in an enhancement in the heat stability of oxygen evolution. These findings suggest that some protein factors, which are dissociated from thylakoid membranes with Triton X-100, are responsible for the heat tolerance of photosynthetic oxygen evolution.

3.2. Introduction

When plants are exposed to heat, their photosynthesis can be irreversibly inactivated. However, plants possess the ability to adapt to high-temperature environment and to enhance the heat stability of photosynthesis (Berry and Björkman 1980). This acquisition of the heat tolerance of photosynthesis has been observed in a number of plants (Berry and Björkman 1980) and some cyanobacteria (Fork et al. 1987, Lehel et al. 1992, Nishiyama et al. 1992, 1993), whereas the molecular mechanism has been little understood.

It was long hypothesized that the saturation of fatty acids of membrane lipids upon adaptation of plants to high temperature was a major factor for the heat tolerance of photosynthesis (Pearcy 1978, Raison et al. 1982). However, using mutants and transformants of the cyanobacterium *Synechocystis* PCC 6803 (Wada and Murata 1989, Wada et al. 1990, 1992), Gombos et al. (1991, 1994) have clearly denied the contribution of fatty-acid saturation to the heat tolerance of photosynthesis. It is therefore likely that some unknown biochemical factor is responsible for the heat tolerance of photosynthesis.

The oxygen-evolving activity is more sensitive to heat than other photosynthetic activities (Berry and Björkman 1980, Mamedov et al. 1993). Nash et al. (1985) demonstrated that a direct result of heat inactivation is the release of functional manganese ions from the PSII complex. Thus, it is likely that the thermal protection of the oxygen-evolving activity plays a key role in the heat tolerance of photosynthesis.

In a previous study, the author has shown that in the cyanobacterium *Synechococcus* PCC 7002, thyakoid membranes isolated from the cells, which have adapted to high temperature, possess a greater heat stability of oxygen evolution than those from non-adapted cells (Chapter 2, Nishiyama et al. 1993). This finding suggests that factors responsible for the heat tolerance of oxygen evolution are associated with thylakoid membranes even after the isolation of the membranes.

In the present study, the effect of NaCl and Triton X-100 on the heat tolerance of oxygen evolution in the thylakoid membranes of *Synechococcus* PCC 7002 was examined in order to characterize the factor for the heat tolerance. Disruption of the vesicular structure of thylakoid membranes with a low concentration of Triton X-100 resulted in the release of some protein factors associated with the heat tolerance of oxygen evolution.

3.3. Materials and Methods

Preparation of thylakoid membranes

Synechococcus PCC 7002 was obtained from the Pasteur Culture Collection. The cells were grown photoautotrophically at 25°C and 40°C for 3 days, and thylakoid membranes were isolated from the cells as previously described (Chapter 2, Nishiyama et al. 1993). The isolated thylakoid membranes were then suspended in medium A (50 mM HEPES-NaOH pH 7.5, 800 mM sorbitol, 30 mM $CaCl_2$, 1.0 M glycinebetaine, 1 mM 6-amino-*n*-caproic acid) and stored at 0 to 4°C for a maximum period of one day.

Treatments of thylakoid membranes

For NaCl-washing, thylakoid membranes were incubated for 10 min at 4°C in medium B (50 mM HEPES-NaOH pH 7.5, 800 mM sorbitol, 30 mM $CaCl_2$) in the presence and absence of 1.0 M NaCl in the dark, at a Chl concentration of 0.1 mg mL^{-1}. The membrane suspension was centrifuged at 60,000 x *g* for 60 min, and the sedimented membranes were suspended in medium A.

For treatment with Triton X-100, thylakoid membranes were incubated in the dark at 4°C for 5 min in medium A containing 0.1% (w/v) Triton X-100 at a Chl concentration of 0.5 mg mL^{-1}, at which the ratio of Triton X-100 to Chl was 2:1 (w/w). The concentration of Chl was determined according to the method of Arnon et al. (1974).

Reconstitution of thylakoid membranes

After the components were dissociated with Triton X-100, they were separated into soluble and membrane fractions by centrifugation at 200,000 x *g* for 2 h. The Triton X-100 was removed from the soluble fraction using column chromatography with a 1 cm x 10 cm column of Bio-beads (Bio-Rad, Richmond, VA, USA) equilibrated with medium A. The fractions eluted with medium A (non-adsorbed fractions) were collected and concentrated approximately 20-fold using a Centricon-10 centrifugal microconcentrator (Amicon, Beverly, MA, USA).

To reconstitute the thylakoid membranes, 10 μL of the thylakoid membrane suspension, previously treated with 0.1% Triton X-100, was mixed with 90 μL of the concentrated extract. The mixture was incubated in the dark at 25°C for 10 min. The suspension was then mixed with 900 μL of medium C (50 mM Tricine-NaOH pH 7.5, 600 mM sucrose, 30 mM $CaCl_2$, 1.0 M glycinebetaine) and subjected to heat treatments at designated temperatures, for 20 min in the dark. Following each heat treatment, the suspension was cooled to 25°C and the oxygen-evolving activity was measured.

Measurement of photosynthetic activity

Photosynthetic oxygen evolution was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode. Measurements were carried out at 25°C in the presence of 100 μM PBQ, to be used as an electron acceptor. Red actinic light, at an intensity of 430 W m^{-2}, was provided by an incandescent lamp

used in conjunction with a heat-absorbing optical filter (Filter HA50; Hoya, Tokyo, Japan) and red optical filter (Filter R-60; Toshiba, Tokyo, Japan).

3.3. Results

Figure 3-1 shows the effect of 1.0 M NaCl-washing on the heat stability of oxygen evolution in the thylakoid membranes from the cells which had grown at 40°C. The heat tolerance of oxygen evolution, which had been acquired in the thylakoid membranes, was not significantly affected upon this treatment. This suggests that the factors responsible for the heat tolerance may not bind to the outer surface of the thylakoid membranes with an electrostatic interaction.

Figure 3-2 shows the effect of the treatment with 0.1% (w/v) Triton X-100 on the heat stability of oxygen evolution in two types of thylakoid membranes from cells which had grown at 25°C and 40°C. Treatment with Triton X-100 did not affect the heat stability of the thylakoid membranes from cells grown at 25°C (Figure 3-2A). In contrast, this treatment remarkably reduced the heat tolerance acquired in the membranes from cells grown at 40°C (Figure 3-2B). The temperature for 50% inactivation of oxygen evolution decreased from 43°C to 38°C; nearly the level of the corresponding temperature of the membranes from cells grown at 25°C. This suggests that the treatment resulted in the dissociation of components from the thylakoid membranes that are essential for the heat tolerance of oxygen evolution.

The components dissociated with Triton X-100 from the thylakoid membranes from cells grown at 40°C were separated from Triton X-100 and concentrated 20-fold. The resulting components were then added back to the thylakoid membranes from cells grown at 40°C which had been treated with Triton X-100. The

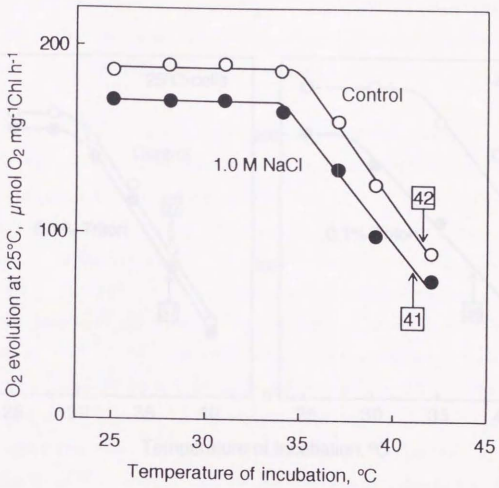

Figure 3-1. Effect of 1.0 M NaCl-washing on the heat stability of oxygen evolution in the thylakoid membranes from cells of *Synechococcus* PCC 7002 grown at 40°C. The washed membranes were treated for 20 min at designated temperatures in darkness, and the oxygen-evolving activity was measured at 25°C in the presence of PBQ as a electron acceptor. Thylakoid membranes after washing in the presence (●) and absence (○) of NaCl. The numbers in rectangles are temperatures for 50% inactivation.

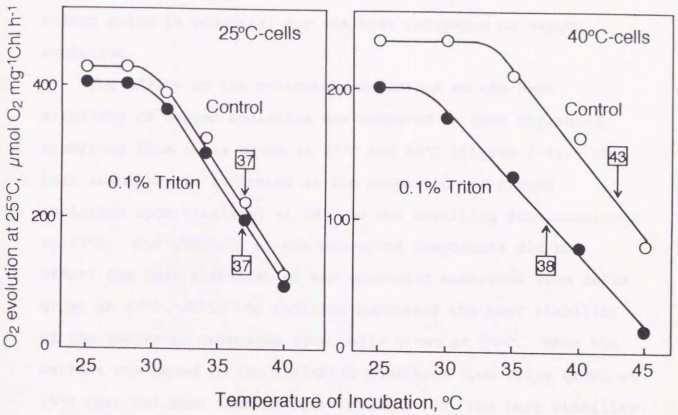

Figure 3-2. Effect of 0.1% Triton X-100 on the heat stability of oxygen evolution in the thylakoid membranes from cells of *Synechococcus* PCC 7002 grown at 25° (**A**) and 40°C (**B**). Conditions for heat treatment and measurement of oxygen evolution are the same as in Figure 3-1. Thylakoid membranes after incubation in the presence (●) and absence (○) of 0.1% Triton X-100. The numbers in rectangles are temperatures for 50% inactivation.

concentrated components were able to restore the heat stability of oxygen evolution; the temperature for 50% inactivation increased from 38°C to 42°C (Figure 3-3). These observations indicate that at least one of the components extracted with Triton X-100 is essential for the heat tolerance of oxygen evolution.

The effect of the extracted components on the heat stability of oxygen evolution was compared in both thylakoid membranes from cells grown at 25°C and 40°C (Figure 3-4). The heat stability is indicated as the proportion of oxygen evolution upon treatment at 38°C to one resulting from treatment at 25°C. The addition of the extracted components did not affect the heat stability of the untreated membranes from cells grown at 40°C, while the addition increased the heat stability of the untreated membranes from cells grown at 25°C. When the extract was added to the thylakoid membranes from cells grown at 25°C that had been treated with Triton X-100, the heat stability increased more greatly. Thus, the extracted components were able to enhance the heat stability of oxygen evolution in the heat-sensitive thylakoid membranes. Furthermore, when the extracted components were boiled at 100°C for 10 min, they lost the activity to increase the heat stability of oxygen evolution. When the extracted components were filtrated through a Centricon-10 filter, they also lost the activity (data not shown). Thus, the functional component appears to be a protein.

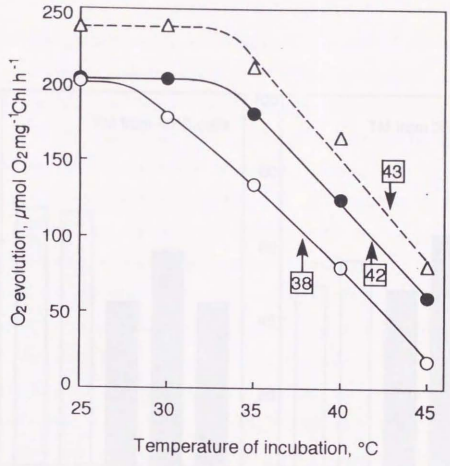

Figure 3-3. Temperature profiles of the heat inactivation of photosynthetic oxygen evolution in thylakoid membranes from *Synechococcus* PCC 7002. The thylakoid membranes were treated with 0.1% Triton X-100, and were then reconstituted with the concentrated Triton X-100 membrane extract. The membranes were incubated at designated temperatures in the dark for 20 min, and oxygen evolution was measured at 25°C in the presence of PBQ as an electron acceptor. Thylakoid membranes with no treatment (Δ); thylakoid membranes after treatment with Triton X-100 (○); thylakoid membranes reconstituted with the extract (●). The numbers within the squares signify temperatures for 50% inactivation.

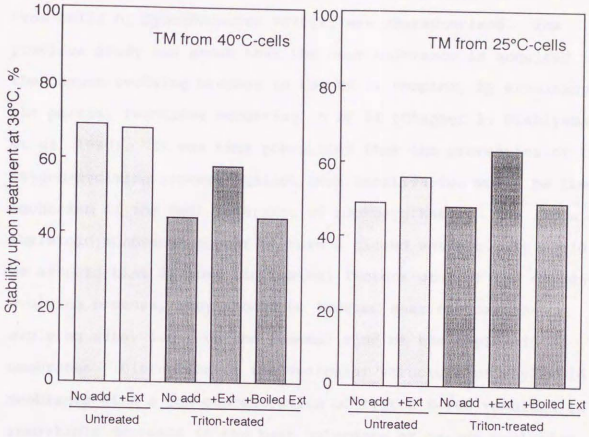

Figure 3-4. Effect of the extract on the heat stability of oxygen evolution in the thylakoid membranes from cells grown at 40°C (**A**) and 25°C (**B**). Heat stability is indicated as a ratio of oxygen-evolving activity upon treatment at 38°C to that upon treatment at 25°C. Conditions for heat treatment and measurement of oxygen evolution are the same as in Figure 3-1.

3.5. Discussion

In the present study, the heat tolerance of oxygen evolution, which had been acquired in the thylakoid membranes from cells of *Synechococcus* PC7002, was characterized. The previous study has shown that the heat tolerance is acquired in the oxygen-evolving process in the PS II complex, by examining the partial reactions occurring in PS II (Chapter 2, Nishiyama et al. 1993). It was thus postulated that the protection of the oxygen-evolving process against heat inactivation would be the mechanism of the heat tolerance of photosynthesis. The isolated thylakoid membranes appear to form a closed vesicle. It could be assumed that if some biochemical factors protect the oxygen-evolving process, they should be located near the oxygen-evolving site, i.e., on the lumenal side of the thylakoid membranes. Disruption of the vesicular structure of thylakoid membranes with a low concentration of Triton X-100 resulted in a remarkable decrease in the heat tolerance of oxygen evolution (Figure 3-2). The heat stability was restored by reconstitution of the thylakoid membranes with the component dissociated with Triton X-100 (Figure 3-3). These results can account for the existence of some factors which protect the oxygen-evolving activity against heat, located on the lumenal side of thylakoid membranes. Moreover, the components extracted with Triton X-100 enhanced the heat stability of oxygen evolution in the heat-sensitive thylakoid membrane from non-adapted cells (Figure 3-4). The extract no longer increased the heat stability when they were boiled before the reconstitution. These findings suggest that a protein factor appears to determine the heat

tolerance of oxygen evolution. The present study is the first demonstration for the contribution of a protein factor to the heat tolerance of photosynthesis. Further study is inevitable to identify the protein factor responsible for the heat tolerance of oxygen evolution.

Chapter 4

Identification of cytochrome *c*-550 as a factor required for the heat stability of oxygen evolution in the cyanobacterium *Synechococcus* PCC 7002

4.1. Summary

Factors responsible for the heat stability of photosynthetic oxygen evolution were investigated by examining thylakoid membranes from the cyanobacterium *Synechococcus* PCC 7002. Treatment of the thylakoid membranes with 0.1% Triton X-100 resulted in a remarkable decrease in the heat stability of oxygen evolution, and the heat stability could be restored by reconstituting the membranes with the components that had been extracted by Triton X-100 (Chapter 3). The protein responsible for the restoration of heat stability was purified from the Triton X-100 extract by two successive steps of chromatography. The purified protein had a molecular mass of 16 kDa and exhibited the spectrophotometric properties of a *c*-type cytochrome with a low redox potential. The dithionite-minus-ascorbate difference spectrum revealed a α-band maximum at 551 nm. The gene encoding this cytochrome was cloned from *Synechococcus* PCC 7002, based on the partial amino-terminal amino-acid sequence. The deduced amino-acid sequence revealed a gene product consisting of a 34-residue transit peptide and a mature protein of 136 residues. The mature protein is homologous to cytochrome *c*-550, a cytochrome with a low redox potential. Thus our results indicate that cytochrome *c*-550 greatly affects the heat stability of oxygen evolution.

4.2. Introduction

When plants are exposed to temperatures above the normal physiological range, photosynthesis is permanently inactivated. The inactivation of photosynthesis occurs at relatively low temperatures; additional severe damage and the thermal breakdown of cellular integrity occur at higher temperatures (Berry and Björkman 1980). Thus it is likely that the heat stability of photosynthetic activity determines the cellular heat tolerance.

The PS II complex is the most susceptible to heat among various components of the photosynthetic apparatus (Berry and Björkman 1980, Mamedov et al. 1993). Among the partial reactions taking place in PS II, the oxygen-evolving process is particularly heat sensitive (Katoh and San Pietro 1967, Yamashita and Butler 1968, Santarius 1975, Mamedov et al. 1993). Therefore, the heat stability of oxygen evolution should determine the overall heat tolerance of the photosynthetic process. Nash et al. (1985) demonstrated that the heat inactivation of oxygen evolution is caused by the release of functional manganese ions from the PS II complex. In order to understand the mechanisms contributing to the heat stability of photosynthesis, the biochemical factors responsible for the heat stability of oxygen evolution must be examined.

The glycerolipids of thylakoid membranes were initially thought to play a key role in the heat stability of photosynthesis (Quinn 1988). Several studies suggested that levels of saturated fatty acids in thylakoid glycerolipids correlated with the heat stability of PS II activity (Pearcy 1978, Raison et al. 1982, Thomas et al. 1986). Recently,

however, direct experimental evidence has been obtained, using mutants and transformants defective in desaturases (Gombos et al. 1991, Mamedov et al. 1993, Wada et al. 1994), that contradicts the previously suggested importance of saturated lipids in maintaining heat stability. Therefore, factors other than membrane lipids must be involved in determining the heat stability of photosynthetic oxygen evolution.

In the present study, thylakoid membranes from the cyanobacterium *Synechococcus* PCC 7002 were examined in order to find the molecular basis for the heat stability of oxygen evolution. Destruction of the vesicular structure in the thylakoid membranes resulted in a radical decrease in the heat stability of oxygen evolution, and the heat stability could be restored by adding the components that had been extracted with Triton X-100 (Chapter 3). Using a reconstitution assay, the protein component responsible for the heat stability of oxygen evolution was determined to be cytochrome c-550, a cytochrome with a low redox potential.

4.3. Materials and methods

Preparation of thylakoid membranes

Synechococcus PCC 7002 was obtained from the Pasteur Culture Collection. The cells were grown photoautotrophically at 40°C for 3 days, and thylakoid membranes were isolated from the cells as previously described (Chapter 2, Nishiyama et al. 1993). The isolated thylakoid membranes were then suspended in medium A (50 mM HEPES-NaOH pH 7.5, 800 mM sorbitol, 30 mM $CaCl_2$, 1.0 M glycinebetaine, 1 mM 6-amino-*n*-caproic acid) and stored at 0 to 4°C for a maximum period of one day.

Treatment of thylakoid membranes

In order to disrupt the closed vesicular structure of the thylakoid membranes, the membranes were incubated in the dark at 4°C for 5 min in medium A containing 0.1% (w/v) Triton X-100 at a Chl concentration of 0.5 mg mL^{-1}, at which the ratio of Triton X-100 to Chl was 2:1 (w/w).

Reconstitution of thylakoid membranes

To reconstitute the thylakoid membranes, 10 μL of the thylakoid membrane suspension, previously treated with 0.1% Triton X-100, was mixed with 90 μL of the column chromatography fractions, or cytochrome *c*-550. The mixture was incubated in the dark at 25°C for 10 min. The suspension was then mixed with 900 μL of medium B (50 mM Tricine-NaOH pH 7.5, 600 mM sucrose,

30 mM $CaCl_2$, 1.0 M glycinebetaine) and subjected to heat treatments at designated temperatures, for 20 min in the dark. Following each heat treatment, the suspension was cooled to 25°C and the oxygen-evolving activity was measured.

To assay the column chromatography fractions for heat-stabilization activity, the 20-min heat treatment was always performed at 38°C. The extent that each fraction prevented heat inactivation was measured, and the measurement was considered as the heat-stabilization activity.

Protein purification

Thylakoid membranes, with concentrations of 20 mg Chl, were treated with 0.1% Triton X-100 under the same conditions as described above, except that medium A was replaced with medium C (20 mM HEPES-NaOH pH 7.5, 800 mM sorbitol, 1.0 M glycinebetaine). The membrane suspension was then separated into soluble and membrane fractions by centrifugation at 200,000 x *g* for 2 h. The soluble fraction was applied to a 2.6 cm x 8 cm column of DEAE-Toyopearl 650C (TOSOH, Tokyo, Japan), equilibrated with medium D (20 mM HEPES-NaOH pH 7.5, 0.2% *n*-octyl-β-D-glucopyranoside). The column was washed with 100 mL of medium D, and was then developed with a linear gradient ranging from 0 M to 0.3 M NaCl in 150 mL of medium D. Active fractions were collected and dialyzed in medium E (25 mM N-methylpiperazine-HCl pH 5.5), and the dialysate was applied to a Mono-P HR 5/5 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with medium E. The column was developed with 20 mL of medium F (10% (v/v) Polybuffer 74 (Pharmacia LKB

Biotechnology)-HCl pH 4.0). The active fractions were recovered, and the medium was replaced with medium G (20 mM HEPES-NaOH pH7.5, 10 mM NaCl) by centrifugal concentration in a Centricon-10.

Measurement of photosynthetic activity

Photosynthetic oxygen evolution was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode. Measurements were carried out at 25°C in the presence of 100 μM PBQ, to be used as an electron acceptor. Red actinic light, at an intensity of 430 W m^{-2}, was provided by an incandescent lamp used in conjunction with a heat-absorbing optical filter (Filter HA50; Hoya, Tokyo, Japan) and a red optical filter (Filter R-60; Toshiba, Tokyo, Japan).

Analysis of the amino-acid composition and amino-terminal amino-acid sequence

Purified protein was hydrolyzed in 5 N HCl at 110°C for 24 h or 72 h, and the amino-acid composition was determined using an automatic amino-acid analyzer (Model 835; Hitachi, Tokyo, Japan). The amino-terminal sequence was determined by automatic Edman degradation using a protein sequence analyzer (Model 477A; Applied Biosystems, Foster City, CA, USA).

Nucleotide sequence analysis

The DNA fragment corresponding to 25 of the amino-acid residues in the amino-terminus of the purified protein was amplified using the polymerase chain reaction (PCR) technique. First, genomic DNA was isolated from *Synechococcus* PCC 7002 cells, according to the method described by Williams (1988), and was used as template DNA for the PCR. Mixed oligonucleotides, encoding for the amino-acids at positions 1 to 5 and 21 to 25, were synthesized using a DNA synthesizer (Model 391; Applied Biosystems Japan, Tokyo, Japan) and used as forward and reverse primers, respectively. The PCR amplification product of approximately 70 bp was then cloned into a plasmid vector using the TA Cloning System (Invitrogen, San Diego, CA, USA). The nucleotide sequence was determined, to ensure that the cloned gene coded for a portion of the purified protein.

We also created a genomic library from *Synechococcus* PCC 7002 DNA. The genomic DNA was partially digested with *Sau*3AI, and the resulting DNA fragments were ligated into the *Bam*HI site of the phage vector, λ*DASH*II (Stratagene, La Jolla, CA, USA). The genomic library was then screened by plaque hybridization as described by Ausubel et al. (1987). Approximately 4,000 plaques were transferred onto nylon membranes (GeneScreen *Plus*; NEN Research Products, Boston, MA, USA), and hybridized at 50°C for 20 h in a medium containing 10% dextran sulfate, 5 x Denhardt's solution, 6 x SSC, 0.1% SDS, and 100 μg mL^{-1} salmon sperm DNA. The library was screened using the 70-bp PCR product described above, labeled with [α-^{32}P] dCTP (Random Primer Labeling Kit, Takara, Tokyo, Japan). Following hybridization with the probe,

membranes were washed at 50°C for 2 h in a solution containing 1 x SSC and 0.5% SDS, and were then exposed to X-ray film (WIF50; Konica, Tokyo, Japan).

A total of 24 positive clones were obtained, and DNA fragments from the clones were analyzed using restriction digestion and Southern blotting. The Southern blots were probed with the previously described 70-bp probe, under the same hybridization and wash conditions used to screen the genomic library. A 5.6-kb *Eco*RI fragment, contained in the 12-kb insert of one positive clone, hybridized strongly to the probe, and was subcloned into a plasmid vector (pBluescript II KS+; Stratagene, La Jolla, CA, USA). The recombinant plasmid was designated pCC55.

We then determined the nucleotide sequence of a 1.3-kb *Bam*HI-*Pst*I fragment within the insert of pCC55, using a DNA sequencer (Model 373A; Applied Biosystems Japan, Tokyo, Japan) to carry out dideoxy sequencing (Sanger et al. 1977). The sequencing reactions were performed, using either the Dye Dedoxy Terminator Cycle Sequencing Kit or the Dye Primer Cycle Sequencing Kit (Applied Biosystems Japan, Tokyo, Japan), as described by the manufacturer.

Other analytical methods

The Chl concentration was determined using the method of Arnon et al. (1974). The absorption spectrum of the purified protein was measured with a slit-beam spectrophotometer (Model UV300; Shimadzu, Kyoto, Japan) in the presence or absence of sodium dithionite and sodium ascorbate. SDS-polyacrylamide gel

electrophoresis was performed according to the method of Laemmli (1970), using a 15% polyacrylamide gel. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO, USA). Protein concentrations were determined using a protein-assay solution (Bio-Rad) with bovine serum albumin (Sigma Chemical Co.) as a standard (Bradford 1976).

4.4. Results

Identification of cytochrome c-550 as a component involved in heat stability

The component responsible for the restoration of the heat stability of oxygen evolution was purified from thylakoid membranes by extraction with Triton X-100 followed by two successive steps of column chromatography, first with DEAE-Toyopearl 650C and then with Mono-P HR 5/5. Figure 4-1 shows the elution profile of proteins from the DEAE-Toyopearl column and the activity for the restoration of the heat stability of oxygen evolution. The fractions that were able to restore heat stability were recovered at NaCl concentrations of 0.2 to 0.3 M NaCl in medium D. No activity was recovered in effluent that contained 0.3 to 1.0 M NaCl.

Using the Mono-P column, the activity was recovered at pH 4.0 as a single peak, whereas proteins with no activity were eluted within a pH range of 4.0 to 5.5 (Fig. 4-2). This result indicates that the functional protein has an isoelectric point of 4.0. Figure 4-3 shows the profiles obtained by SDS gel electrophoresis of the active fractions from each purification step. The active fraction obtained from Mono-P chromatography contained a single protein of 16 kDa, without any sign of contamination.

The 16-kDa protein exhibited a unique absorption spectrum with a maximum at 409 nm (Fig. 4-4). Upon the addition of sodium dithionite, the absorption maxima appeared at 551, 523, and 417 nm (Fig. 4-4). An absorption change of this nature,

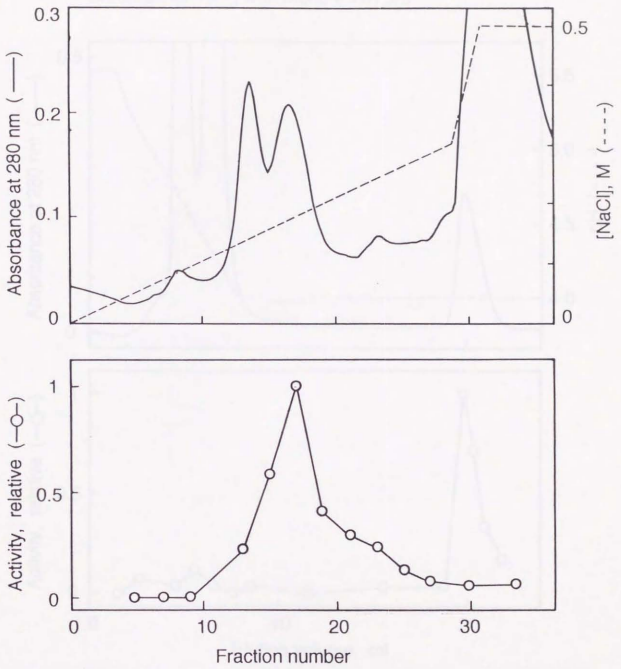

Figure 4-1. DEAE-Toyopearl 650C column chromatography of the components extracted with Triton X-100 from the thylakoid membranes of *Synechococcus* PCC 7002. Fraction activity was assayed as described in Materials and Methods.

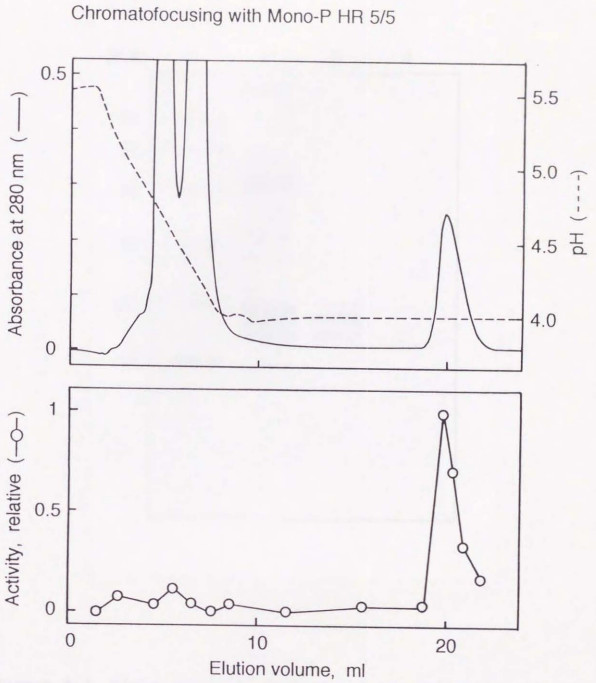

Figure 4-2. Chromatofocusing using Mono-P HR 5/5 column chromatography of the active fractions obtained from DEAE-Toyopearl 650C column chromatography. Fraction activity was assayed as described in Materials and Methods.

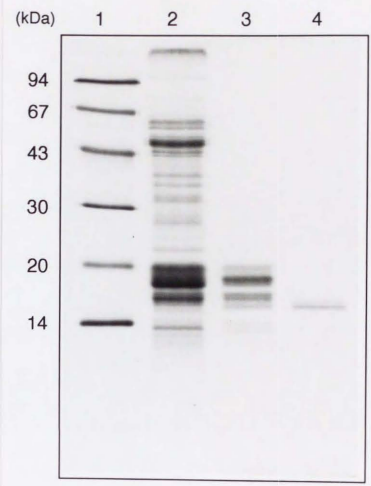

Figure 4-3. Electrophoretic analysis of the active fractions from each purification step. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel. Lane 1 shows the molecular mass markers. Lanes 2, 3, and 4 show the active fractions obtained after extraction with Triton X-100, DEAE column chromatography, and chromatofocusing, respectively.

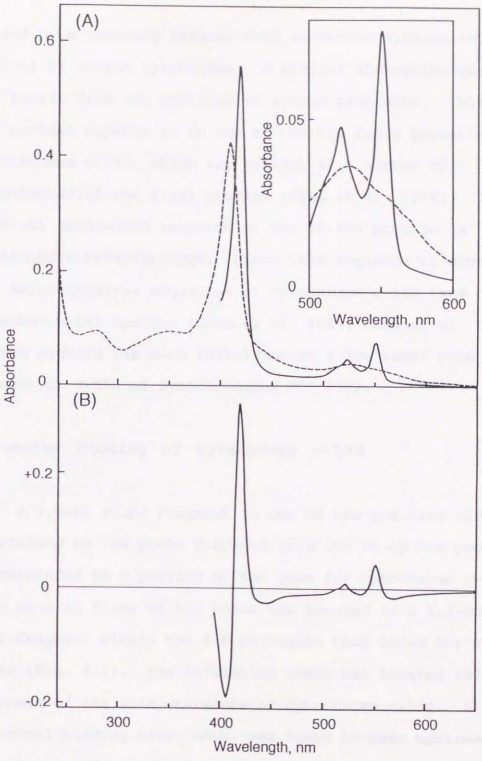

Figure 4-4. **(A)** Absorption spectra of the protein purified from thylakoid membranes of *Synechococcus* PCC 7002. The spectra were measured in the presence (solid line) and absence (dashed line) of sodium dithionite. **(B)** The dithionite-minus-ascorbate difference spectrum.

caused by a reducing reagent such as sodium dithionite, is typical of *c*-type cytochrome. A similar absorption change did not result from the addition of sodium ascorbate. Thus, the 16-kDa protein appears to be one of the low redox potential cytochromes *c*-550, which are present in a number of cyanobacterial and algal species (Alam et al. 1984). The amino-terminal amino-acid sequence of the 16-kDa protein is TALREVDRTVNLNETETVVLSDQQV. Since this sequence is similar to the amino-terminal sequences of cytochrome *c*-550 from other cyanobacterial species (Cohn et al. 1989, Shen et al. 1992), the 16-kDa protein has been identified as a low redox potential cytochrome *c*-550 of *Synechococcus* PCC 7002.

Molecular cloning of cytochrome *c*-550

A 5.6-kb *Eco*RI fragment in one of the positive clones hybridized to the probe prepared from the 70-bp PCR product that corresponded to a portion of the gene for cytochrome *c*-550. An open reading frame of 510 bases was located in a 1.3-kb *Bam*HI-*Pst*I fragment within the 5.6-kb region that coded for cytochrome *c*-550 (Fig. 4-5). The initiation codon was located 102-base upstream of the amino-terminus of cytochrome *c*-550. A typical ribosomal binding site, GAGG, was found 10-base upstream of the initiation codon. The deduced amino-acid sequence indicates that the product of this open reading frame consists of a transit peptide of 34 residues and a mature protein of 136 residues. The deduced amino-acid composition of the mature protein is identical to that of purified cytochrome *c*-550 (data not shown); thus, the identity of the gene is confirmed. The

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GGATCCTAAAAAAAAGTAAAACGCAGCACGAAATATTGCTATTTTTTACGGATTTATAAG    60

AAAAATTCACCCCAATGGCATGATATGTATGGTTAGTTGCCCTGGCTGACTGGTGCCGTT   120

GGCAACACAAATTTAAATAAACAAAGATAAGCACATTTACTTACAGCCTTGATAAATTCT   180

TGCCTGTAGGTCTGCCTTCACAGTTAAATAGGTGGAGATAGATTTATTTACGTTATTGCT   240

GTATTGAGGAAAAACATGAATAAAATTCTGGGGATCGACCCACTCAAAAAATTTATTTTC   300
      SD        M  N  K  I  L  G  I  D  P  L  K  K  F  I  F    15'

GGGATTAGCGCATTCGTTTTACTATTTTGGCAACTCAATGTCGGCGCTGCCAATGCCACC   360
G  I  S  A  F  V  L  L  F  W  Q  L  N  V  G  A  A  N  A  T     35'
                                                    ▲

GCCCTCCGGGAAGTAGACCGCACCGTCAACCTCAATGAAACCGAAACGGTCGTCCTCAGT   420
A  L  R  E  V  D  R  T  V  N  L  N  E  T  E  T  V  V  L  S     55'

GACCAGCAGGTTGCCAAAGGAGAGCGGATCTTTATCAACACCTGTTCCACCTGCCACAAC   480
D  Q  Q  V  A  K  G  E  R  I  F  I  N  T  C  S  T  C  H  N     75'
                                                HBS

AGCGGCCGGACAAAGAGCAACCCCAACGTAACCCTTTCTCTAGTCGATCTAGAAGGGGCA   540
S  G  R  T  K  S  N  P  N  V  T  L  S  L  V  D  L  E  G  A     95'

GAACCCCGCCGCGATAATATCCTGGCCATGGTGGACTATCTCAAAAATCCCACCTCCTAC   600
E  P  R  R  D  N  I  L  A  M  V  D  Y  L  K  N  P  T  S  Y    115'

GATGGTGAATTAGACCTATCTCAGTTACACCCCAATACCGTCCGTGCCGACATCTGGAGT   660
D  G  E  L  D  L  S  Q  L  H  P  N  T  V  R  A  D  I  W  S    135'

AGCATGCGTAACCTCAACGAAGAGGATCTGCAAAATGTTTCCGGTTATGTCCTCGTTCAA   720
S  M  R  N  L  N  E  E  D  L  Q  N  V  S  G  Y  V  L  V  Q    155'

GCCCAGGTGCGTGGTGTTGCTTGGGGTGGTGGTAAAACCGTTAACTAAAACACGATTGAT   780
A  Q  V  R  G  V  A  W  G  G  G  K  T  V  N  *                170'

CGACTTTATTTAAATCGTCTTTTTATCTAAAATTCGCAAAATGAAGTACCGAAGACAACC   840

ATAATGAGGTTGTCTTTTTAATTGTCTCTAAAAGCGATCGCCTCCGGTTTCGACACAAAA   900
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Figure 4-5. The nucleotide sequence of the gene for cytochrome *c*-550 from *Synechococcus* PCC 7002. The deduced amino-acid sequence is shown in one-letter code under the nucleotide sequence. The amino-terminal sequence that was determined directly from the protein is underlined. The putative ribosomal binding site (SD) and heme-binding site (HBS) are double-underlined. The arrow indicates the cleavage site for removal of the transit peptide.

presence of the transit peptide suggests that cytochrome *c*-550 is located on the lumenal side of the thylakoid membranes. In addition, a conserved heme binding site, Cys-X-X-Cys-His, was found at residues 70 to 74.

The thermoprotective role of cytochrome *c*-550

Figure 4-6A shows the effect of the addition of cytochrome *c*-550 on the temperature profile of heat inactivation of oxygen evolution in thylakoid membranes treated with Triton X-100. Cytochrome *c*-550 was added to the thylakoid membrane suspension, with a Chl concentration of 5 μg mL^{-1}, at a final concentration of 2 μg mL^{-1}. The addition of cytochrome *c*-550 clearly resulted in an increase in the heat inactivation profile of the membranes; the temperature for 50% inactivation shifted from 38°C to 42°C. Thus, the addition of cytochrome *c*-550 restored the heat stability of thylakoid membranes to initial levels (see Fig. 4-1).

The restoration of heat stability in the thylakoid membranes was examined using various concentrations of cytochrome *c*-550 (Fig. 4-6B). Heat stability increased linearly with increases in the amount of added cytochrome *c*-550, up to a concentration of 0.6 μg mL^{-1}, at which the molecular ratio of added cytochrome *c*-550 to the PS II reaction center was approximately 1.4. The addition of greater amounts of cytochrome *c*-550 resulted in a much slower increase in heat stability (Fig. 4-6B).

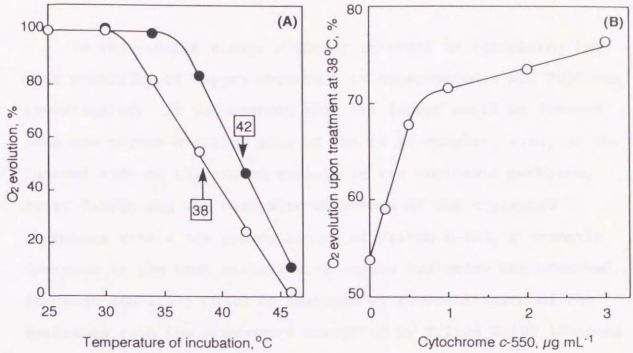

Figure 4-6. Effect of cytochrome *c*-550 on the heat stability of oxygen evolution in the thylakoid membranes of *Synechococcus* PCC 7002. **(A)** Temperature profile of the heat inactivation of oxygen evolution. Cytochrome *c*-550 at a final concentration of 2 μg mL^{-1} was added to the thylakoid membrane suspension that had been treated with Triton X-100 (●); control (no cytochrome *c*-550) (○). The oxygen-evolving activities taken as 100% were 195 and 191 μmol O_2 mg^{-1} Chl h^{-1}, respectively. The numbers within the squares signify temperatures for 50% inactivation. **(B)** The relationship of cytochrome *c*-550 concentration to the restoration of heat stability. Various amounts of cytochrome *c*-550 were added to the thylakoid membrane suspension that had been treated with Triton X-100. The heat stability is shown as the proportion of oxygen evolution upon treatment at 38°C compared to that resulting from treatment at 25°C.

4.5. Discussion

In the present study, a factor involved in sustaining the heat stability of oxygen evolution in *Synechococcus* PCC 7002 was investigated. It was assumed that the factor would be located near the oxygen-evolving site of the PS II complex, i.e., on the lumenal side of the closed vesicle in the thylakoid membrane. After disrupting the vesicular structure of the thylakoid membranes with a low concentration of Triton X-100, a dramatic decrease in the heat stability of oxygen evolution was observed. The heat stability could be restored by reconstitution of the membranes with the components extracted by Triton X-100 (Chapter 3). In this study, the author demonstrated that the component responsible for the restoration of the heat stability of oxygen evolution in *Synechococcus* PCC 7002 is a cytochrome *c*-550. In addition, this is the first report for cloning of the gene for a cyanobacterial cytochrome *c*-550. Nucleotide sequence obtained from the cloned cytochrome *c*-550 gene revealed the presence of a transit peptide, indicating that cytochrome *c*-550 is located on the lumenal side of the thylakoid membranes.

The amino-acid sequence of the mature cytochrome *c*-550 from *Synechococcus* PCC 7002 was compared with cytochrome *c*-550 sequences from other cyanobacteria (Fig. 4-7). The sequence of cytochrome *c*-550 from *Synechococcus* PCC 7002 showed 48% identity to the cytochrome sequence from *Microcystis aeruginosa* (Cohn et al. 1989) and 47% identity to a partial sequence of 119 residues from *Aphanizomenon flos-aquae* (Cohn et al. 1989).

Cytochrome *c*-550 is a *c*-type, monoheme cytochrome, with a molecular mass of 15 kDa and an unusually low redox potential

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S.PCC7002       TALREVDRTVNLNET-ETVVLSDQQVAKGERIFINT CSTCH NSGRTKSNPN 50
M.aeruginosa    LE*D*KTL*IT**DAG*S*T*TSE*ATE*QKL*VAN *TK** LQ*K**T*N* 51
A.flos-aquae    LE*D*TI***P**DKGG*****LE**-*EGKL*NYA *AQ** AG*V**T*Q* 50

S.PCC7002       VTLSLVDLEGAEPRRDNILAMVDYLKNPTSYDGELDLSQLHPNTVRADIWS  101
M.aeruginosa    *S*G*G**AK***P***L**LI***EH*******D***E****VS*P**FP  101
A.flos-aquae    *G*EPEA*A**L*N*?????????M****T****EEI*EI?*SIKS*N*F-   90

S.PCC7002       SMRNLNEEDLQNVSGYVLVQAQVRGVAWGGGKTVN                  136
M.aeruginosa    EL***T*D*VY**AA*M**APRLDER-***TIYF                   135
A.flos-aquae    --***TD***KAIAEHI*LEPL*V*TK***-*                     119
```

Figure 4-7. Aligned amino-acid sequences of cyanobacterial cytochrome *c*-550. *Amino-acids in the cytochrome *c*-550 of *Synechococcus* PCC 7002 identical to those in *Microcystis aeruginosa* or *Aphanizomenon flos-aquae* cytochrome *c*-550. Gaps introduced to optimize alignment are indicated by "-". The heme binding sites are boxed.

with an E_0 of -260 mV (Holton and Myers 1967a, b). Holton and Myers (1963) first discovered cytochrome *c*-550 in *Anacystis nidulans*, and it has since been found in a variety of cyanobacteria (Ho et al. 1979, Alam et al. 1984) and in some eukaryotic algae (Yamanaka et al. 1967, Kamimura et al. 1977, Evans and Krogmann 1983). The physiological function and cellular localization of cytochrome *c*-550 have not yet been established, although there are several hypotheses. The cellular content of cytochrome *c*-550 varies greatly among different species of cyanobacteria and may depend on growth conditions (Ho et al. 1979). Krogmann and Smith (1990) observed that, in some cyanobacterial species, the abundance of cytochrome *c*-550 increases with cell density when the availability of light and oxygen becomes limited. They suggested that cytochrome *c*-550 may eliminate excess electrons in a fermentative pathway in order to maintain cell viability in a dark, anaerobic environment.

In addition to the water-soluble cytochrome *c*-550 described above, a membrane-bound cytochrome *c*-550 has been observed in some cyanobacteria. The membrane-bound cytochrome *c*-550 resembles the water-soluble cytochrome *c*-550 in size, spectrum, and redox properties. The bound cytochrome can be separated from the thylakoid membranes using Tween 20 and acetone (Ho et al. 1979), Triton X-100 (Krinner et al. 1982), or 1.0 M NaCl (Hoganson et al. 1990). It is unclear whether the membrane-bound cytochrome *c*-550 is the same as the water-soluble one. Hoganson et al. (1990) observed that the two cytochromes differ slightly in EPR spectra. Additional data, such as amino-acid sequences, are needed to address the question (Krogmann 1991).

It should be noted here that the cytochrome that is located on the lumenal side of the thylakoid membranes (as in the present study) can also be released from the membranes during cell breakage by treatment with a French pressure cell, shaking with glass beads, or freeze-thawing and be recovered in the aqueous phase.

Recently, Shen et al. (1992) found that a PS II core complex, prepared from the thermophilic cyanobacterium *Synechococcus vulcanus* using lauryldimethylamine *N*-oxide and dodecyl β-D-maltoside, contained a stoichiometric amount of cytochrome *c*-550 as an extrinsic component of the PS II complex. They demonstrated that cytochrome *c*-550 enhanced oxygen evolution and facilitated the binding of another extrinsic protein, of 12 kDa, to the PS II complex (Shen and Inoue 1993a). In addition, they noted that the cytochrome was exclusively located on the lumenal side of PS II complex (Shen and Inoue 1993b). It is unclear whether the cytochrome from *Synechococcus vulcanus* is the same as the cytochrome in the present study. The partial amino-terminal sequence of the cytochrome *c*-550 from *Synechococcus vulcanus* (Shen et al. 1992) is similar to that from *Synechococcus* PCC 7002 with 12 identical residues out of 35 (data not shown). However, the two cytochromes differ in their association with the thylakoid membranes. The cytochrome *c*-550 from *Synechococcus vulcanus* required the presence of 1.0 M $CaCl_2$ or 1.0 M Tris (pH 8.5) during sonic oscillation to release it from the thylakoid membranes (Shen et al. 1992, Shen and Inoue 1993b). A cytochrome *c*-550 identical to that from *Synechococcus vulcanus* was also found in PS II particles prepared from the thermophilic cyanobacterium *Phormidium laminosum* (Bowes et al.

1983). In contrast, the cytochrome *c*-550 from *Synechococcus* PCC 7002 could be dissociated from the thylakoid membranes at neutral pH and under low-salt conditions. This suggests that the cytochrome in *Synechococcus* PCC 7002 is loosely bound to the PS II complex. Omata and Murata (1984) observed that cytochrome *c*-550 from *Anacystis nidulans* was also released from the thylakoid membranes by sonic oscillation under low-salt conditions. Thus it appears that there may be two distinct type of cytochrome *c*-550.

The redox function of cytochrome *c*-550 is unlikely to occur in the high-redox-potential environment surrounding the donor side of the PS II complex (Shen and Inoue 1993b). It seems that the apoprotein portion of the cytochrome, rather than heme, is responsible for the structural stabilization of the oxygen-evolving complex against heat denaturation.

With respect to the photosynthetic adaptation to high temperature, it appears that only the cytochrome *c*-550 is not responsible for the heat tolerance of oxygen evolution upon the adaptation, since the addition of cytochrome *c*-550 was not able to enhance the heat stability of the heat-sensitive thylakoid membranes from the cells that had been grown at 25°C (data not shown). For a better understanding of the *in vivo* function of cytochrome *c*-550 in the heat stability, it will be necessary to study mutants of *Synechococcus* PCC 7002, depleted of the cytochrome *c*-550 gene.

Chapter 5

Conclusions

Photosynthesis is one of the central activities indispensable for the viability of plants, whereas it is particular sensitive to heat among various physiological activities in the cell. It thus appears that the acquisition of the heat tolerance of photosynthesis is a key process for plants to adapt to the high-temperature environment. Although many studies on the photosynthetic adaptation to high temperature have been descriptive from physiological viewpoints, the basic principle of the molecular mechanism of the adaptation has been far from clear (Chapter 1).

In the present study, the molecular mechanism of the heat tolerance of photosynthesis has been investigated in the cyanobacterium *Synechococcus* PCC 7002. Studies on the heat stability of photosynthesis in intact cells, grown at various temperatures, has characterized the mode of the acquisition of heat tolerance; the heat tolerance of the PS II activity, which is the most sensitive to heat among various photosynthetic activities, is acquired in concert with an increase in growth temperature (Chapter 2). Studies on the isolated thylakoid membranes have revealed that the heat tolerance of PS II activity, which has been acquired in the cells adapted to high temperature, is retained in the isolated thylakoid membranes. This finding suggests that factors responsible for the heat tolerance are associated with thylakoid membranes, even after isolation. This fact allowed the further analyses of the heat tolerance of photosynthesis on a molecular level. Analyses of the partial reactions of PS II-driven electron transport in the thylakoid membranes have clarified that the oxygen-evolving

process, a particularly heat-sensitive process in PS II, can acquire the heat tolerance upon adaptation to high temperature.

Biochemical analyses of the heat tolerance in the thylakoid membranes have revealed that the heat tolerance of oxygen evolution is associated with some protein factors that can be dissociated from the thylakoid membranes by disruption of their vesicular structure with a low concentration of Triton X-100 (Chapter 3).

The protein component required for the heat stability of oxygen evolution has been identified (Chapter 4). The result has demonstrated that cytochrome *c*-550 is essential in maintaining the heat stability of oxygen evolution in *Synechococcus* PCC 7002. The nucleotide sequence obtained from the cloned cytochrome *c*-550 gene has revealed the existence of the transit peptide, suggesting the localization of the cytochrome *c*-550 on the lumenal side of the thylakoid membranes. As far as the present data obtained, however, only the role of cytochrome *c*-550 can not account for the heat tolerance of photosynthesis upon adaptation to high temperature. It is likely that other unknown factor(s) should exist together with cytochrome *c*-550 to acquire the heat tolerance of photosynthesis. Further studies are necessary to reveal the factor(s) that directly determine the heat tolerance upon the adaptation to high temperature. According to the low-redox potential, the cytochrome *c*-550 is unlikely to act as a redox substance in the high-redox-potential environment surrounding the donor side of the PS II complex. The question should be addressed why the component required for heat stability needs to contain the heme.

For an understanding of the *in vivo* function of cytochrome *c*-550 in the heat stability, it will be necessary to study mutants of *Synechococcus* PCC 7002, depleted of the cytochrome *c*-550 gene. Although the importance of the heat tolerance of photosynthesis in the cellular heat tolerance can be assumed, there is no direct evidence to demonstrate the assumption so far. Studies on the deletion mutants will also allow us to directly evaluate the role of the heat stability of photosynthetic oxygen evolution in the overall heat tolerance of the cell.

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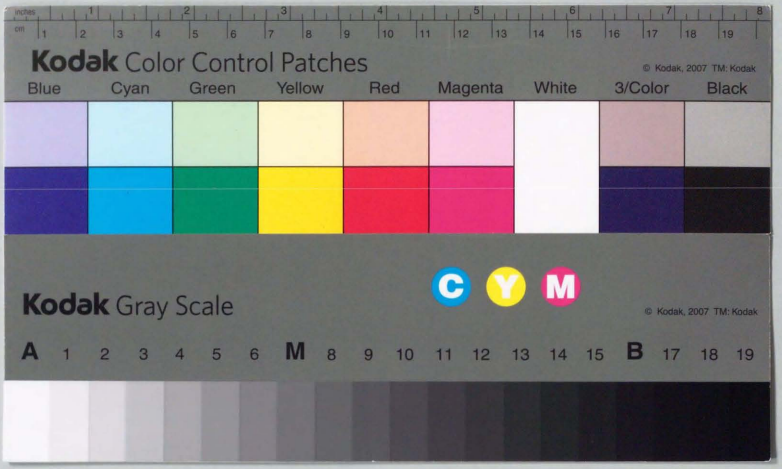
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