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Bioenergetic Responses of *Synechocystis* 6803 Fatty Acid Desaturase Mutants at Low Temperatures

R. A. Dilley,[1,2,4] Y. Nishiyama,[2] Z. Gombos,[2,3] and N. Murata[2]

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Fatty acid composition of the membrane lipids in the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 was altered in earlier work by targeted mutagenesis of genes for fatty acid desaturases. In this work, cells of several mutant strains, depleted in the unsaturated fatty acids in membrane lipids, were grown at 34°C. Spheroplasts (permeabilized cells) were prepared by lysozyme digestion of the cell wall followed by gentle osmotic shock. The bioenergetic parameters ATP formation, electron transport, and H^+ uptake were measured at various temperatures. All three bioenergetic parameters for spheroplasts from wild-type cells (which had abundant polyunsaturated fatty acids) were active down to the lowest temperatures used (1°–2°C). In two strains, which lacked the capacity to desaturate fatty acids at the Δ12 position and at the Δ12 and Δ6 positions (designated as *desA*$^-$ and *desA*$^-$/*desD*$^-$, respectively), the spheroplasts lost the capacity to form ATP (measured as phenazine methosulfate cyclic phosphorylation) at about 5°C but retained electron transport (water oxidation-dependent ferricyanide reduction) and H^+ uptake linked to phenazine methosulfate cyclic electron transport. It appears that the absence of the unsaturation of fatty acids in the Δ12 and Δ6 positions blocks the ability of the photosynthetic membranes to couple a bioenergetically competent proton-motive force to the ATP formation mechanism at temperatures below 5°C. It remains to be determined whether the loss of ATP formation in the mutant strains is the failure of available protons to properly flow into the CF_0CF_1-ATP synthase or a failure in the CF_1 part of the complex in coupling the dissipative H^+ flow to the enzyme mechanism of the synthase.

KEY WORDS: Bioenergetics; fatty acid desaturase mutants; *Synechocystis* sp. PCC 6803.

INTRODUCTION

Photosynthetic electron transport linked to the synthesis of ATP occurs in chloroplast thylakoid membranes of higher plants and cyanobacterial cells. Despite many decades of research concerning the bioenergetic reactions associated with diverse membrane systems, there are still many unanswered questions about actual mechanisms of bioenergetic membrane functions. One such area needing study is the role of lipids in membrane function. The major glycerolipids of thylakoid membranes are monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and phosphatidylglycerol (Block *et al.*, 1983; Murata and Nishida, 1987). These lipids are involved not only in the formation of membrane bilayers, but, in some cases, may also be specifically required in the function of membrane-bound proteins (Doyle and Yu, 1985; Pick *et al.*, 1985). For example, monogalactosyl diacylglycerol with highly saturated fatty acids (unusual in thylakoid lipids) is associated with and seems necessary for the structure and/or function of the reaction center complex of photosystem II (PSII) (Murata *et al.*, 1990). Sulfoquinovosyl diacylglycerol is bound to the ATP synthase and the evidence suggests that it is necessary for the complex to function (Pick *et al.*, 1985; Barber and Gounaris, 1986). This sulfolipid is also bound to the PSII core complex of *Chlamydomonas reinhardtii*

[1] Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907.
[2] Department of Regulation Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan.
[3] Present address: Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, P.O. Box 521, Hungary.
[4] To whom correspondence should be addressed at email: ddilley@bilbo.bio.purdue.edu

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and the light-harvesting complex of PSII and may influence PSII function, although a mutant lacking the sulfolipid had about 60% of the wild-type PSII activity (Sato *et al.*, 1995).

The physical properties of glycerolipids depend on the degree of unsaturation of the component fatty acids. The molecular motion (or fluidity) of a glycerolipid with unsaturated fatty acids is greater than that of a glycerolipid with saturated fatty acids (Chapman, 1975; Silvius, 1982). In response to decreases in ambient temperature, plants and cyanobacteria adjust the fatty acid biosynthesis patterns so as to increase the extent of fatty acid unsaturation in membrane lipids, which is largely controlled by acyl–lipid desaturases (Jaworski, 1987; Sato and Murata, 1981; Tasaka *et al.*, 1996; Harwood, 1996). Genetic manipulation of acyl–lipid desaturases in cyanobacteria has demonstrated that unsaturation of fatty acids of membrane lipids is important for the tolerance of the photosynthetic machinery to low temperatures (Wada *et al.*, 1990; Gombos *et al.*, 1992). Studies of the molecular basis of low-temperature tolerance have revealed that unsaturation of membrane lipid fatty acids protects the PSII complex from photoinhibition at low temperatures by enhancing the recovery from the photoinhibitory damage (Tasaka *et al.*, 1996; Gombos *et al.*, 1997; Kanervo *et al.*, 1997). However, the role of fatty acid unsaturation in other photosynthetic membrane components, such as the ATP synthase, remains to be elucidated.

In the present study, we examined the role of unsaturated fatty acids in membrane lipids on photosynthetic electron transport, proton uptake, and ATP formation activities at temperatures from near 2°–4°C up to room temperature. We compared these bioenergetic activities in the wild-type *Synechocystis* sp. PCC 6803 and the two mutant lines, *desA*$^-$, in which the *desA* gene for Δ12 desaturase activity was inactivated (Jaworski, 1987), and *desA*$^-$/*desD*$^-$, in which both the *desA* gene and the *desD* gene for Δ6 desaturase activities were inactivated (Tasaka *et al.*, 1996).

In the spheroplasts of wild-type cells, the three bioenergetic activities decreased, as expected, with decrease in temperature, but were still easily measurable down to 1°–2°C. In contrast, in both the *desA*$^-$ and the *desA*$^-$/*desD*$^-$ spheroplasts, ATP formation was completely lost below 5°C, but electron transport and H^+ uptake were both still active down to 1°–2°C.

METHODS AND MATERIALS

Organisms and Culture Conditions

Wild-type and mutant cells of *Synechocystis* sp. PCC 6803 were grown at 34°C in the light in BG-11 medium, as described previously (Wada and Murata, 1989). Two transformant strains of *Synechocystis* PCC 6803 were primarily used: *desA*$^-$ and the double mutant *desA*$^-$/*desD*$^-$; their development was described by Tasaka *et al.* (1996). A third strain (*desD*$^-$), which allows synthesis of the 18:2 (9, 12) fatty acid but not the synthesis of 18:2 (6, 9) was used for comparison. Samples of cultures used in the these bioenergetics studies were assayed for fatty acid content as previously described (Wada and Murata, 1989). The growth of cells was measured in terms of turbidity at 730 nm with a spectrophotometer.

Preparation of Spheroplasts and Thylakoid Membranes

Thylakoid membranes (or spheroplasts) were prepared from cells (when they reached a 730-nm turbidity near 1.2) by the lysozyme cell wall digestion method of Scholts *et al.* (1996). Chlorophyll was assayed by the method of Arnon (1949). The spheroplasts were stored in liquid nitrogen after supplementing the buffer medium of Scholts *et al.* (1996) with 1.0 M glycine betaine (hereafter referred to as betaine). Protease inhibitors were added as indicated in (Scholts *et al.*, 1996). The betaine storage medium provided excellent protection against loss of electron and H^+ ion transport and ATP formation activities during the freeze-thaw cycles (Mamedov *et al.*, 1991).

ATP Formation, Electron Transport, and H^+ Uptake Assays

The bioenergetic parameters were assayed in a medium having a low buffer capacity using the pH electrode method (Dilley, 1972). All the parameters could be assayed sequentially (as shown in Fig. 1) in the same reaction mixture by first adding PMS to catalyze ATP formation or H^+ pumping (in that case ADP was not added) during a set of dark–light–dark cycles, followed by addition of the uncoupler pair nigericin (1 μM) and valinomycin (1 μM) to inhibit H^+ gradients and ATP formation. K_3 $(CN)_6$ (FeCN) (0.5 mM) was added and the light was then turned on to measure electron transport (because H^+ ions were released during PSII water oxidation in the FeCN Hill reaction). The pH of the reaction mixture contained in a thermostated glass reaction cuvette was adjusted with standard base or acid and calibration of the pH changes was made with aliquots of standard HCl. Figure 1 shows a typical reaction using that technique, where the PMS-catalyzed ATP formation was detected first, followed by addition of the uncoupler and FeCN; oxidation of water (proton release) was then measured.

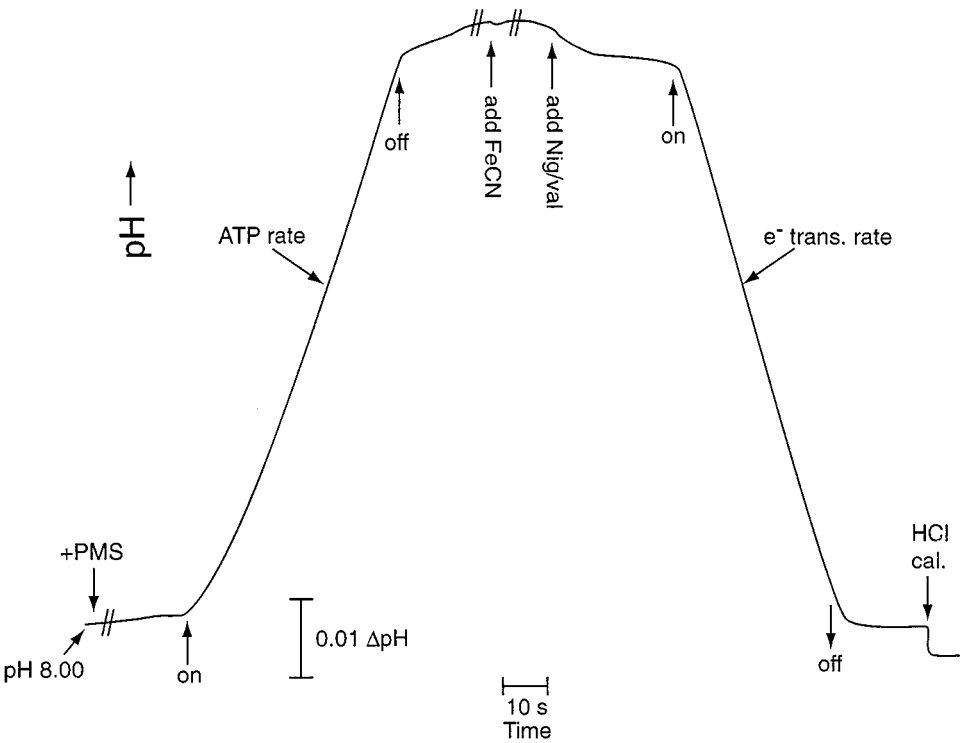

Fig. 1. A tracing from a typical strip chart recorder record of the data obtained for ATP formation and electron transport rates in a spheroplast preparation of wild-type cells of *Synechocystis* sp. PCC 6803 (See Section Materials and Methods for components in the assay mixture and other conditions). Before turning on the actinic illumination, 10 μM phenazine methosulfate (PMS) was added as indicated and illumination was provided to energize cyclic ATP formation. After turning off the light, 0.5 mM potassium ferricyanide (FeCN) and the uncoupling pair, nigericin (1 μM) and valinomycin (1 μM), were added to inhibit ATP formation but allow noncyclic electron transport to proceed after once again turning on the actinic illumination. The ensuing acidification represents H^+ release in PSII water oxidation. Both the alkalization (photophosphorylation rate) and the acidification (electron transport rate) were calibrated using an addition of standard HCl as indicated.

The assay medium was: 800 mM mannitol, 2 mM tricine, 5 mM Mg Cl_2, 50 mM KCl, 50 mM NaCl, 3 mM K_2H PO_4, 1 mM caproic acid (protease inhibitor), and spheroplasts to give 20 μg Chl $(ml)^{-1}$. The pH was adjusted to 8.0. When added, ADP was 0.2 mM, nigericin and valinomycin (in MeOH) were each at 1 μM, and FeCN was at 0.5 mM. The glass reaction cuvette was temperature controlled to $\pm 0.1^\circ$C from a Lauda Model RM6 circulating temperature bath and the cuvette was illuminated by red light using a 500-W tungsten light source with a Corning 2304 red glass filter and a Corning heat filter.

RESULTS AND DISCUSSION

Fatty acid analysis of representative wild-type, *desA*$^-$/*desD*$^-$, *desD*$^-$, and *desA*$^-$ cells used in the experiments reported here is presented in Table I. The replacement of 18:2 (9, 12) and 18:3 (6, 9, 12) fatty acids by 18:1 (9) fatty acid in *desA*$^-$/*desD*$^-$ cells was evident, as well as the *desA*$^-$ having no 18:2 (9, 12) or 18:3 (6, 9, 12), but having abnormally high levels of 18:2 (6, 9).

Wild-type spheroplasts preparations retained ATP synthesis and electron transport activity down to 1°–3°C (Fig. 2A). Spheroplasts prepared from the *desD*$^-$ cells were similar to wild-type preparations in having active ATP formation and electron transport activities down to 1°–2°C (data not shown). The spheroplasts from the *desA*$^-$ and the *desA*$^-$/*desD*$^-$ cells both lost ATP formation activity near and below 5°C (Figs. 2B and 2C), but the electron transport (PSII-dependent Hill reaction) activity was active at 2°–3°C. The loss of photophosphorylation activity below about 5°C in the *desA*$^-$/*desD*$^-$ cells was reproducible in several preparations from different cell cultures widely separated in time, as was the continuation of good ATP formation activity in the wild-type spheroplasts at temperatures in the 1°–5°C range.

Table I. Fatty Acid Composition of the Total Glycerolipids from Wild-Type and Mutant Cells of *Synechocystis* sp. PCC 6803[a]

	Fatty acid (mole%)									
Stain	16:0	16:1 (9)	18:0	18:1 (9)	18:1 (11)	18:2 (6, 9)	18:2 (9, 12)	18:3 (9, 12, 15)	18:3 (6, 9, 12)	18:4 (6, 9, 12, 15)
Wild type	55	5	1	7	tr		22	tr	10	tr
desA$^-$	55	2	1	36		5	0	0	0	0
desD$^-$	55	3	1	12	tr		28	1	0	0
desA$^-$/*desD*$^-$	55	3	1	42	tr	0	0	0	0	0

[a] Cells were grown at 34°C. tr, Trace amount (less than 0.5%). Experiments were repeated twice; deviations were within ±3% of individual values. Numbers in parentheses indicate the positions of double bonds in the *cis* configuration.

It is noted that the electron transport rates for the *desA*$^-$ and *desA*$^-$/*desD*$^-$ strains were significantly less at 2°–3°C compared to the wild-type cells. The lower electron transport rates for those two strains persisted (in comparison to the wild-type strain) over the entire range of temperatures tested (Fig. 2). Perhaps the absence of the Δ(9, 12) diunsaturated fatty acid in the *desA*$^-$ and *desA*$^-$/*desD*$^-$ strains causes PSII-dependent redox function to be more labile in isolated spheroplasts, although for intact cells of the *desA*$^-$/*desD*$^-$ strain, Tasaka *et al.* (1996) reported both photosynthetic oxygen evolution and respiration rates to be very nearly the same as for wild-type cells over a temperature range of about 15° to 35°C

Because the FeCN Hill reaction and PSI–PMS cyclic electron/H^+ flow can be affected quite differently by the events of cell breakage and/or storage (PSII activities are generally more labile than PSI cyclic reactions), an additional and probably a better gauge of the thylakoid energization status than PSII electron transport is the PMS-supported H^+ uptake. Figure 3 shows that a spheroplast preparation (different cultures than used for the Fig. 2 data) of wild-type and the *desA*$^-$/*desD*$^-$ double mutant cells showed that the *desA*$^-$/*desD*$^-$ spheroplasts had more PMS-supported H^+ gradient formation at 3.8°C than the wild-type preparation [146 and 111 nmol H^+ (mg chl)$^{-1}$, respectively], but the wild-type spheroplasts had an active ATP formation and the *desA*$^-$/*desD*$^-$ preparation had very little, if any, photophosphorylation activity, in agreement with the results shown in the spheroplast samples used for the Fig. 2 experiments. The greater H^+ uptake in the *desA*$^-$/*desD*$^-$ spheroplast preparation below 5°C is what one would predict if there is normal H^+ pump activity and H^+ permeability properties but no ATP formation activity to dissipate the gradient.

The passive H^+ efflux rate constant—which indicates the H^+ ion leakiness of thylakoid membranes—is an important parameter for evaluating possible reasons why a particular thylakoid preparation may have a low ATP formation rate in comparison to an actively phosphorylating membrane preparation. For example, an excessive H^+ leak could be the cause of the *desA*$^-$/*desD*$^-$ vesicles losing ATP formation capacity below 5°C, as shown in Figs. 2 and 3. However, both in the spheroplast preparations used for the Fig. 2 experiment and for the preparation from which the Fig. 3 data were obtained, H^+ pump assays using the PMS cyclic system (no ADP added in the first several light–dark cycles) gave H^+ efflux kinetics in the *desA*$^-$/*desD*$^-$ strain either similar to the wild-type preparation or slower. Figure 3 shows that at 3.8°C the H^+ efflux $t_{1/2}$, (which relates linearly to the first-order rate constant as $k = 0.69/t_{1/2}$) in the *desA*$^-$/*desD*$^-$ spheroplasts was greater than the half-time for the wild-type spheroplasts (16 versus 6 s for the wild type), indicating a slower rate constant (k) for the H^+ leak in the *desA*$^-$/*desD*$^-$ spheroplasts. With the spheroplast preparations used for the Fig. 2 experiment, the $t_{1/2}$ values at 5.9°C for H^+ efflux were identical, within experimental error, for both wild-type and the *desA*$^-$/*desD*$^-$ spheroplast preparations (data not shown); although the ATP formation rates at 5.9°C were zero in the *desA*$^-$/*desD*$^-$ spheroplasts and near 120 μmol ATP (mg chl • hr)$^{-1}$ for the wild-type spheroplsts. We can conclude that the loss of ATP formation activity at low temperatures, in the *desA*$^-$/*desD*$^-$ spheroplast preparation was not caused by an excessive H^+ leak in the thylakoid membranes.

Wild-type cells of *Synechocystis* sp. PCC 6803 are chilling-resistant and spheroplasts retain photophosphorylation activity below 5°C, but the spheroplasts from *desA*$^-$ and *desA*$^-$/*desD*$^-$ cells lacking the 18:2 (9, 12) fatty acids were inactive for ATP formation near and below 5°C (Figs. 2 and 3). The biochemical step(s) responsible for the low-temperature loss of ATP formation in those strains has not been identified, but clearly the problem is not in the capacity to energize the H^+ gradient, because, as Fig. 3 shows, the *desA*$^-$/*desD*$^-$ spheroplasts compared well to the wild-type spheroplasts and had a more than

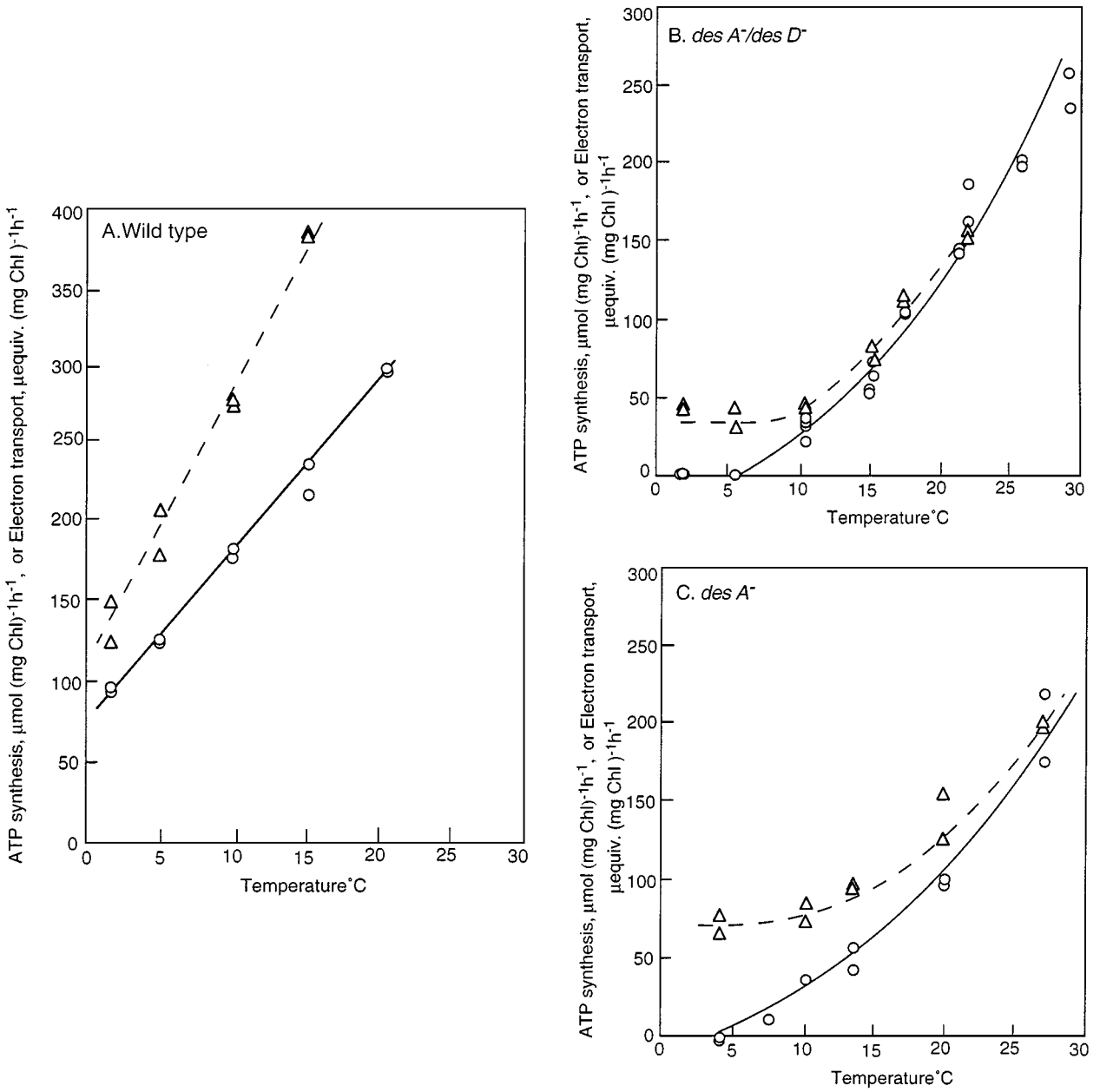

Fig. 2. (A–C) Photophosphorylation and electron transport rates versus temperature in spheroplast preparations of wild-type (A), *desA*$^-$/*desD*$^-$ (B), and *desA*$^-$ (C) cells of *Synechocystis* sp. PCC 6803 (See Section Materials and Methods for assay details). For all three spheroplast preparations, the PMS cyclic photophosphorylation rate is given by the open circles (o); FeCN electron transport rate is illustrated by the open triangles (Δ).

adequate light-dependent H^+ uptake activity, more than 50% of which was dissipated by the uncoupler treatment indicating that an electrochemical potential H^+ gradient was present before addition of uncoupler. The uncoupler-resistent H^+ uptake and release is not understood, but it has been observed before with *Synechocystis* spheroplasts (Van Walraven, (1996) personal communication) and it may represent electron transport-driven ion exchange unrelated to the proton-motive force responsible for ATP formation. The block in energy transfer may be at the level of the terminal steps of the CF_1 enzyme turnover or it may be a problem in the earlier steps, which link the energetically down hill flow of H^+ ions through the CF_0 H^+ channel to the final energy-requiring steps which, in the current thinking, may be conformational changes in the CF_0–CF_1 complex that result in driving ATP formation

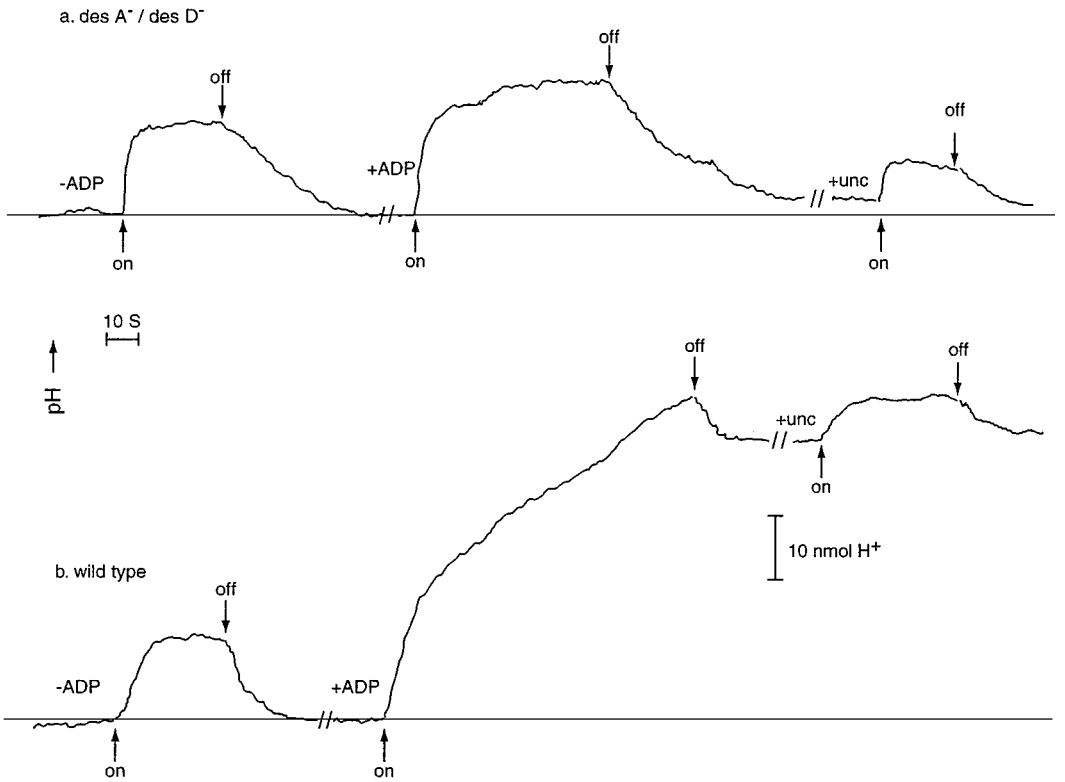

Fig. 3. Recorder tracings of data at 3.8°C for the proton gradient formation (extent of H^+ uptake) driven by PMS cyclic electron flow (no ADP was present) followed by the addition of ADP to test for the capacity for ATP formation in wild-type (b) and *desA*$^-$/*desD*$^-$ (a) spheroplast preparations of *Synechocystis* sp. PCC 6803. The spheroplast preparations were from different cultures of the two strains than those used for the Fig. 2 data. The extent of H^+ uptake (−ADP) was 146 and 111 nmol H^+ (mg chl)$^{-1}$, for the *desA*$^-$/*desD*$^-$ (top tracings, a) and wild-type (bottom tracings, b), respectively, which is rather typical for such preparations. After addition of ADP and adjusting the pH back to 8.0, the photophosphorylation rate for the *desA*$^-$/*desD*$^-$ sample (top center tracing) was essentially zero. Three other assays of this sample verified that at 3.8°–3.9°C the rate of photophosphorylation was so close to zero as to be zero or less than 2% of the wild-type rate. The wild-type photophosphorylation rate under these conditions (bottom center trace) was 10 μmol ATP (mg chl • hr)$^{-1}$.

(Boyer, 1993). The "conformation change coupling" hypothesis also referred to as the "adenine nucleotide-binding energy change" hypothesis of Boyer (1993), is not yet fully elucidated—especially in the functioning of the H^+ channel (CF_0) part of the energy transduction process—but it seems clear that either the proper arrangement of the CF_0 subunits for H^+ ion channel function and/or the proper H^+ ion conduction steps through the CF_0 complex, requires the presence of the correct membrane lipid mileau. For proper function, cell and organelle membranes must be in the liquid crystalline state, i.e., above the temperature where transition to the gel state occurs (Linden *et al.*, 1973). The absence of the Δ(9, 12) fatty acids in the *desA*$^-$ and *desA*$^-$/*desD*$^-$ strains may lead to formation of the nonfunctional gel phase in the region near the CF_0CF_1 complex.

Pick *et al.* (1987) using the isolated CF_0CF_1 from spinach showed that the chloroplast "sulfolipid" (sulfoquinovosyl diacyclglycerol, SL; cf. (Barber and Gounaris, 1986) for a discussion of the possible role the sulfolipid might have in photosynthetic tissue) is specifically required for ATP–P_i exchange activity of the reconstituted CF_0CF_1 complex (ATP–P_i exchange has similar enzyme requirements as ATP synthase activity, including activation of the complex by a protonmotive force). Van Walraven *et al.* (1984) found that the negatively charged phosphatidylglycerol (PG) or phosphatidylcholine (PC) in combination with MGDG may play the same role in the cyanobacterial systems. In any event, it is possible that the SL or the PG may have to be present with unsaturated bonds in the Δ(9,12) positions to confer chilling resistance of the photosynthetic bioenergetic reactions of cyanobacteria. Hagio *et al.* (2000) have produced a mutant of *Synechocystis* 6803 lacking the pgs A gene encoding a PG phosphate synthase required for PG synthesis. They found that cells deficient in PG showed a decrease in PSII activity

(but not PSI activity). Further studies using that mutant system may be useful in gaining better understanding of the role of PG in thylakoid bioenergetics.

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