CHANGES IN ELECTROPHORETIC MOBILITY OF A CHLOROPLAST MEMBRANE POLYPEPTIDE ASSOCIATED WITH THE LOSS OF THE OXIDIZING SIDE OF PHOTOSYSTEM II IN LOW FLUORESCENT MUTANTS OF *SCENEDESMUS*

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1. Introduction

The exact mechanism of how the photosynthetic process derives electrons and oxygen from water is not known. Precise kinetic analyses of early events in oxygen evolution and of fluorescence associated with PS-II have yielded significant information useful in modeling this reaction [1–5]. Techniques which either alter chloroplast membrane integrity and/or remove manganese ions disturb the capacity for water photolysis [2,6–13]. These techniques also result in a loss of the high potential form of cytochrome *b*-559 [13], a membrane component hypothesized to function in a cyclic electron flow around photosystem II (PS-II) but whose precise role, if any, in water photolysis is not clear [14]. Recent efforts combining detergent-polyacrylamide gel electrophoresis and mutational analysis of chloroplast membrane polypeptides have shown the existence of two or more chlorophyll-protein complexes and their respective apoproteins associated with PS-II [15–18]. However, specific biochemical evidence regarding intermediates, cofactors, enzymes, etc., involved in water photolysis remains obscure.

In this paper we demonstrate that specific low fluorescent yield, non-photosynthetic mutants of the green alga, *Scenedesmus obliquus* D_3, which lack oxygen evolving capacity but retain an otherwise functional PS-II reaction center (plus antennae chlorophyll), are affected primarily in their capacity for binding and/or accumulating manganese. Two separate mutant phenotypes, one showing permanent loss of photosynthesis and strongly altered Chl/Mn^{2+} ratios and the other variable photosynthetic capacity and Chl/Mn^{2+} ratios alterable by temperature and by growth medium concentration of manganese, were utilized for this study. Evidence for the possible involvement of a chloroplast membrane polypeptide of apparent MW-34 kd in the participation of manganese in PS-II is presented.

Abbreviations: AA, ascorbic acid; Chl, chlorophyll; CP, chlorophyll-protein complex; DPIP, 2-6-dichlorophenol indophenol; DPC, diphenylcarbazide; HF, high fluorescent; kl, kilodalton; LF, low fluorescent; MV, methyl viologen; LiDS-PAGE, lithium dodecyl-sulfate polyacrylamide gel electrophoresis; PS, photosystem; WT, wild type

2. Materials and methods

The algal strains employed in this study were the wild-type of *Scenedesmus obliquus*, strain D_3, and two low fluorescent mutants blocked on the oxidizing side of PS-II (LF-1 and LF-2). Characterization, maintenance and growth of the various algal strains were done according to techniques previously described [19].

Chloroplast membrane fragments for gel electrophoresis studies were prepared by the methods described earlier [17]. Except for minor modifications the procedures for solubilization of chloroplast membranes and the LiDS-PAGE were identical to those of Delepelaire and Chua [18]. For MW calibration a 10% acrylamide gel, rather than the 7.5–15% gradient gel, was prepared and samples run with protein markers obtained from Sigma Chemical Co. (St. Louis, MO 63178). Following electrophoresis

the gel slab was photographed to register the position of the chlorophyll-protein complexes, and subsequently fixed and stained in a solution of 0.2% Coomassie brilliant blue (R-250), acetic acid (7%) and methanol (25%). Photographic records of the slabs were made following destaining.

The photochemical activities of chloroplast particles prepared from the various algal strains were measured as previously described [19,20]. Evaluation of the content and redox state of cytochrome *b*-559 in chloroplast membranes from the different mutants was made by chemical difference spectra analysis with an Aminco DW-2 spectrophotometer according to the techniques previously summarized and by modifications implied by Horton and Croze [21].

Manganese analysis of isolated chloroplast membranes was made by either atomic absorption or by neutron activation analysis. Thylakoid membranes were prepared as previously indicated with precautions taken to exclude extraneous manganese. The final membrane pellet was suspended in deionized water and chlorophyll concentrations determined.

For neutron activation analysis, aliquots were dispended into polyethylene vials. These, and appropriate manganese standards, were heated to dryness, sealed and the contents activated by exposure to thermal neutrons in a TRIGA nuclear reactor. Manganese concentrations were determined by comparing the corrected gamma ray photon peak size at 846 KeV (arising from decay of ^{56}Mn as measured with a lithium drifted germanium semiconductor detector system) to a standard curve which was linear over the range examined.

3. Results

3.1. *General photosynthetic and chloroplast reaction characteristics*

In table 1 the general photochemical characteristics of the strains employed in this study are summarized. The mutant LF-1 lacks the ability to use water as an electron source but retains rates comparable to the WT for those reactions using alternate

Table 1
Rates of photosynthesis, photoreduction and specific photochemical reactions of whole cells and chloroplast particles of WT and select mutants of *Scenedesmus* as influenced by growth temperature

Strain designation	Photosynthesis[a] (PS-I + PS-II)	Photoreduction[b] (PS-I)	H_2O-DPIP[c] (PS-I + II)	DPC-DPIP[c] (PS-II)	DPIP-MV[d] (PS-I)
Wild type					
20°C	55	20	63	ND	310
28°C	53	19	52	21	293
34°C	50	19	72	ND	390
LF-1					
20°C	2	20	0	26	393
LF-2					
20°C	44	ND	52	ND	261
28°C	24	19	21	ND	233
34°C	3	ND	3	33	262
34°C + Mn[e]	42	ND	ND	ND	ND

[a] Rates of photosynthesis and photoreduction given as μmol O_2/mg Chl/h and μmol CO_2/mg Chl/h as determined at saturating levels of red light with wavelength greater than 620 nm. Temperature for rate determination 25°C

[b] Photoreduction measured in an atmosphere of 4% CO_2 + 96% H_2 following a 4 h adaptation period. 50 μl cells (packed cell volume) added to 3 ml 0.05 M PO_4 containing 5 μM DCMU

[c] Rates of photochemical reactions given as μmol DPIP reduced/mg Chl/h at saturating light intensities and at wavelengths greater than 620 nm

[d] PS-I catalyzed reduction of methyl viologen measured polarographically as μmol O_2 consumed/mg Chl/h at 25°C. ND = not determined

[e] Reactivation of photosynthesis in LF-2 (34°C) accomplished by adding cells to 5 mM PIPES buffer, pH 6.8, containing 1 mM $MnCl_2$

electron donor systems. LF-2, when grown at the nonpermissive temperature (34°C) exhibits a severe reduction in PS-II dependent oxygen production, but retains WT levels of the other reactions as noted above for LF-1. That the two mutants possess a functional PS-II reaction center is shown by the DPC-DPIP reaction values which are greater than those noted for TRIS inhibited [11] WT membranes. Additionally, the variable yield fluorescence which is absent in LF-1 and LF-2 (34°C) can be restored to whole cells (or isolated membranes) by providing the PS-II electron donor system, hydroquinone/ascorbate [11] (data not shown). The data of table 1 also demonstrate the temperature-dependent nature of the expression of the mutant syndrome in LF-2 when grown on standard medium. The mutant characteristic of LF-1 is not similarly affected by growth.

3.2. *Manganese and cytochrome analyses*

Data of table 2 reveal that the Mn content of thylakoid membranes from LF-1 and LF-2 (34°C) is much less than that of the WT. The dramatic shift in the Chl/Mn ratio in LF-2 from 183 at 20°C to 905 at 34°C suggests that the loss of photosynthesis in these cells occurs at the site of manganese involvement in PS-II. That this is indeed the case was substantiated by the finding that addition of excess Mn^{2+} (1 mM vs. the 10 μM $MnCl_2$ normally present in the growth medium) to cells of LF-2 grown at 34°C caused restoration of photosynthetic activity within minutes. Addition of excess Mn^{2+} to cells of LF-1 failed to influence their photosynthetic activity. Simple transfer of cells of LF-2 from 34°C to 20°C did not result in immediate recovery of activity nor did transfer of cells from 20°C to 34°C cause sudden inactivation of photosynthetic activity. Growth of the cells through several cell cycles was required for manifestation of the mutant syndrome (data not shown).

Analysis of the cytochrome content of thylakoids of LF-1 and LF-2 (34°C) revealed that both mutants show a similar difference from the WT pattern. Each shows a greatly reduced amount of the high potential (390 mV) form of cytochrome *b*-559 while retaining nearly normal levels of total *b*-type cytochromes as measured by ferri/ferro and ferri/dithionite difference spectra (table 2).

Restoration of PS-II activity in LF-2 either by growth at 20°C or by addition of excess Mn^{2+} results in an increase in the level of the high potential form of cytochrome *b*-559 to that observed for WT chloroplasts. This increase in measured concentration of *b*-559 parallels the restoration of photosynthesis in whole cells, the photochemical activity of isolated chloroplast membranes dependent upon water as the electron donor and the manganese content of the membranes. Furthermore, this restoration occurs within minutes in LF-2 (34°C) following the addition of 1 mM $MnCl_2$ to the suspending buffer.

Table 2

The influence of growth temperatures on Chl/Mn, Chl/cytochrome *b*-559$_{HP}$ and Chl/total b-type cytochrome ratios of chloroplast membranes of WT and select low fluorescent mutants of *Scenedesmus*

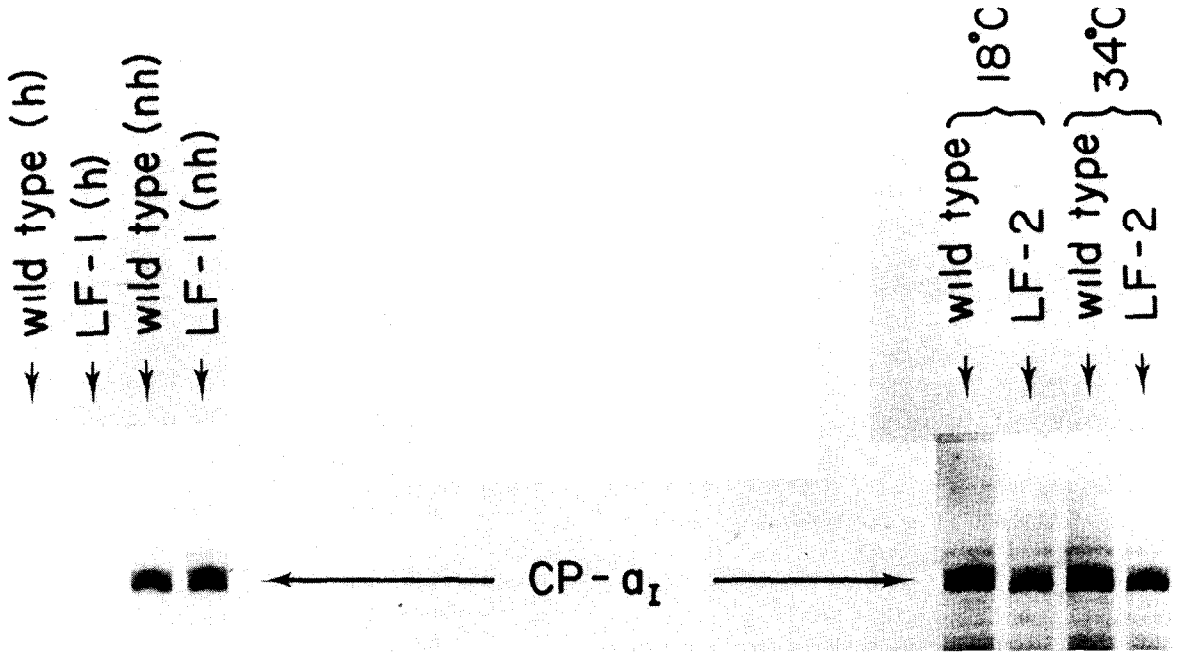
Strain designation	Chl/Mn	Chl/cytochrome *b*-559	Chl/total *b* cytochrome
Wild type			
20°C	135	720	196
28°C	80	548	192
34°C	186	633	184
LF-1			
28°C	248	2252	233
LF-2			
20°C	183	800	200
28°C	512	939	189
34°C	905	4468	279
LF-2 (34°C)			
+ Mn	ND	593	206

Reactivation of photosynthesis in LF-2 (34°C) accomplished as indicated in table 1. Cytochrome determinations made by difference spectra analysis as outlined in Section 2

3.3. *Polyacrylamide gel electrophoresis of chloroplast membranes*

Fig. 1A presents data showing the characteristic polypeptide pattern seen when purified chloroplast thylakoids are solubilized and electrophoresed in the presence of lithium dodecylsulfate on a 7.5–15% linear polyacrylamide gel slab according to the technique of Delepelaire and Chua [18]. Heated samples of chloroplast membranes from WT and LF-1 were placed in slots 1 and 2 and unheated samples in slots 3 and 4. The unheated samples show five chlorophyll containing areas: these are CP-a_I, two chlorophyll proteins derived from PS-II, CP-$a_{II\text{-}1}$ and CP-$a_{II\text{-}2}$, the chlorophyll *a*/*b*-protein complex containing several distinct pigmented bands, CP-a/b, and the detergent-chlorophyll complex. Heating dis-

sociates these complexes giving rise to the well-known increase in the associated apoproteins. In the Coomassie blue stained gel, as presented in fig.1A, these are represented by the increase in staining at approximate MW of 60 kd ($CP\text{-}a_I$), 52 and 48 kd ($CP\text{-}a_{II\text{-}1}$ and $CP\text{-}a_{II\text{-}2}$) (see [18]) and in the 23–30 kd region derived from CP-a/b. Comparison of the polypeptide patterns of the unheated WT and LF-1 demonstrates that all of the bands normally associated with PS-II, i.e., the apoproteins of apparent MW of 52 and 48 kd and the two chlorophyll-protein complexes $CP\text{-}a_{II\text{-}1}$ and $CP\text{-}a_{II\text{-}2}$ are present in LF-1. Of para-



mount importance, however, is the change noted in the apparent MW of the 34 kd band of the WT to 36 kd in LF-1. In the heated samples these bands are reduced in intensity but are still evident and the difference between WT and LF-1 is retained. Examination of the polypeptide pattern obtained from thylakoids of LF-2 cultures grown at 20°C and 34°C (fig.1B) shows that all components of the membranes including those of PS-II and the 34 kd band are unchanged from the WT. Comparison studies on mutant strains totally deficient in PS-II [17,19,20,22] and PS-I (P-700) show that all PS-II associated polypeptides and CP complexes, including the 34 kd band are absent in the former phenotype but not the latter (data not shown).

4. Discussion

Previous studies by Epel and colleagues [24–26] demonstrated that genetic alteration of the oxidizing side of PS-II resulted in mutant strains with a low fluorescence yield and decreased levels of the high potential form of cytochrome *b*-559. It was not possible then to explain why only a fraction of the 'high' potential form should be altered but from the current result of Horton and associates [21,23] it is now recognized that the midpoint oxidation-reduction potential of cytochrome *b*-559 varies from ~+383 mV to +77 mV, with an intermediate value of +240 mV, being detectable. According to Horton and Croze [21], the amount of the highest potential form is correlated with the activity of PS-II and the manganese concentration in hydroxylamine treated chloroplasts. Earlier findings [27] already demonstrated the relationship between PS-II activity and the presence of the high potential form of *b*-559. Our findings, as summarized in tables 1 and 2 for the two LF mutants of *Scenedesmus*, indicate a similar correlation.

Electrophoresis of thylakoid membranes by the milder technique introduced by Delepelaire and Chua [18] revealed for the first time a non-pigmented component of PS-II which appears to be involved on the water side of PS-II. This component, of apparent MW of 34 kd in the WT, shows an altered electrophoretic mobility resulting in an apparent MW of 36 kd in LF-1. Although Delepelaire and Chua state than an additional pigmented band, possibly related to PS-II, was sometimes seen by them in the approximate position of the 34 kd, we have not. Because of the proximity of this polypeptide to the heavily pigmented area of the CP-a/b, the presence of an additional colored band could be overlooked. However, a mutant lacking chlorophyll *b* and the CP-a/b (Kϕ-9 of *Scenedesmus*), but possessing excellent PS-II activity, has only the CP-$a_{II\text{-}1}$, CP-$a_{II\text{-}2}$ and the non-pigmented polypeptide of 34 kd (data not shown). Also the retention of traces of the 34 and 36 kd bands in heat treated samples of WT and LF-1 (fig.1A) argues against this protein being complexed with pigments in *Scenedesmus*.

Although the amount of high potential cytochrome *b*-559 is greatly reduced in LF-1 the published molecular weights of the oligomeric and monomeric forms of *b*-559 [28,29] and of cytochrome *b*-563 [30] are not in agreement with the observed value for the newly resolved band from *Scenedesmus* chloroplast membranes. Since the total *b*-type cytochrome content of LF-1 is not significantly different from that of the wild type, it seems apparent that the 34 kd polypeptide does not arise from the *b*-cytochromes.

In the strain LF-2, which is a temperature dependent for the expression of the mutant syndrome, it was noted (fig.1B) that all of the chlorophyll-protein complexes, their apoproteins and the 34 kd polypeptide of PS-II were present and apparently unaltered even at the non-permissive temperatures (34°C). The finding (table 2) that oxygen-evolving capacity can be restored to cultures grown at 34°C by addition of excess Mn^{2+} and that such addition also restores the level of the high potential form of cytochrome *b*-559 suggests that this type of mutation involves either recognition or permeability of manganese by the chloroplast outer membrane. Growth at low temperature at normal levels of manganese or addition of excess manganese to 'inactivated' cultures overcomes this limitation and PS-II activity can be retained or recovered. Since transfer of cultures grown at 18°C to 34°C does not result in immediate inactivation of oxygen evolving capacity indicates that the manganese accumulated within the chloroplast is retained in a functional mode; it is only growth (and the resulting dilution of the available manganese) which causes loss of the activity.

We have stressed in earlier publications [17,19,22] that mutants of *Scenedesmus* lacking PS-II activity and possessing high and invariant fluorescence yield were deletion-type mutants and that the resulting

effect upon PS-II was pleiotropic. The absence of all polypeptides known to be associated with PS-II provides further support for this interpretation. Since these mutants contain cytochrome *b*-559 [22], but only in a low potential form, the absence or diminution of the 34 kd band cannot be correlated with this cytochrome as indicated earlier.

Comparison of the data obtained from LF-1 and LF-2 and from other types of mutants suggests that the 34 kd band may represent a chloroplast factor involved in the binding of manganese. The point mutation indicated in LF-1, which results in an increase in the apparent MW of this polypeptide (from 34 to 36 kd as determined with a LiDS-10% acrylamide gel) might have altered the manganese binding capacity of this chloroplast component. Since no alteration of the apparent MW of this band occurred in LF-2 and activity of PS-II is restored by excess Mn^{2+}, the tentative interpretation that the 34 kd band is derived from a manganese-binding protein of thylakoid membranes appears logical. Further experiments to define more closely the characteristics of this component are in progress.

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