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Characterization of a 160 kD Photosystem II reaction center complex isolated from photoinhibited *Dunaliella salina* thylakoids *

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Abstract

Photoinhibition in the green alga *Dunaliella salina* is accompanied by the formation of inactive Photosystem II reaction centers. In SDS-PAGE analysis, the latter appear as 160 kD complexes. These complexes are structurally stable, enough to withstand re-electrophoresis of excised gel slices from the 160 kD region. Western blot analyses with specific polyclonal antibodies raised against the D1 or D2 reaction center proteins provided evidence for the presence of both of these polypeptides in the re-electrophoresed 160 kD complex. Incubation of excised gel slices from the 160 kD region, under aerobic conditions at 4 °C for a prolonged period of time, caused a break-up of the 160 kD complex into a ~52 kD D1-containing and ~80 and ~26 kD D2-containing pieces. Western blot analysis with polyclonal antibodies raised against the apoproteins of CPI (reaction center proteins of PS I) did not show cross-reaction either with the 160 kD complex or with the ~52, ~80 and ~26 kD pieces. The results show the presence of both D1 and D2 in the 160 kD complex and strengthen the notion of a higher molecular weight D1- and D2-containing complex that forms upon disassembly of photodamaged PS II units.

Abbreviations: Chl-chlorophyll; PS II – Photosystem II; D1 – the 32 kD reaction center protein of PS II, encoded by the chloroplast *psbA* gene; D2 – the 34 kD reaction center protein of PS II, encoded by the chloroplast *psbD* gene; CPI – the 82 and 83 kD reaction center proteins of PS I, encoded by the chloroplast *psaA* and *psaB* genes; HL – high light; LL – low light

Introduction

The process of oxygenic photosynthesis is catalyzed by Photosystem II, a chloroplast thylakoid membrane macrocomplex consisting of about 25 transmembrane and peripheral proteins. The holocomplex functions as a H_2O-to-plastoquinone oxidoreductase (Barber 1989). This strongly endergonic reaction becomes thermodynamically possible upon consumption of light energy. The conversion of excitation energy to chemical form occurs within the reaction center proteins of PS II. These proteins, known as D1 and D2, bind all the co-factors and electron-transport carriers essential for the H_2O-to-plastoquinone oxidoreductase reaction (Nanba and Satoh 1986).

The specialized function of PS II, and the generation of highly oxidizing intermediates within the reaction center proteins, make PS II susceptible to a light-dependent damage. The molecular target of this irreversible photodamage is a co-factor (probably the reaction center Chl) in D1 (Cleland et al. 1986; Aro et al. 1993; Barber 1995). In spite of more than two billion years of evolution, oxygen-evolving photosynthetic organisms apparently could not avoid this photodamage to D1. Nature, however, devised a repair mechanism which entails the degradation of the damaged D1 and its replacement by a de novo synthesized

* This publication is dedicated to the memory of the late Professor Daniel Arnon, whom the first author will fondly remember for his many accounts of past scientific discovery and debate.

D1 protein (Mattoo et al. 1984; Mattoo and Edelman 1987). This cycle of photodamage and repair (Guenther and Melis 1990; Adir et al. 1990), operates constantly during the course of daily photosynthesis.

The rate of photodamage depends on the light intensity during plant growth (Baroli and Melis, in preparation). However, the rate of the enzymatic repair process is limited (Kim et al. 1993; Aro et al. 1993). Under low and moderate light intensities, the frequency of photodamage does not exceed the capacity of chloroplast for repair. Under high light intensities, however, photodamage occurs faster than the repair, resulting in accumulation of inactive PS II centers in the thylakoid membrane (Kyle et al. 1984; Smith et al. 1990). This condition is known as photoinhibition (Powles 1984); it causes a decline in the rate of photosynthesis and a loss in plant growth and productivity (Kok 1956; Powles 1984).

In the green alga *Dunaliella salina,* evidence was presented that, under photoinhibition conditions, photodamaged PS II reaction centers accumulate in the thylakoid membrane and appear in SDS-PAGE as 160 kD complexes (Melis 1992; Kim et al. 1993). The structural stability and polypeptide composition of the 160 kD complex was the limited objective of the present study.

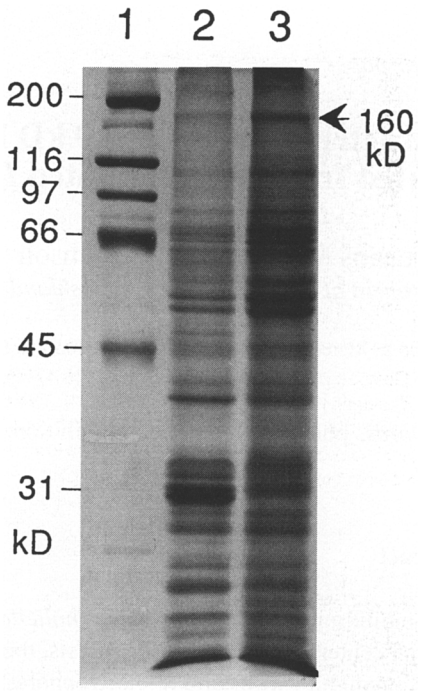

Fig. 1. Coomassie stained profile of SDS-PAGE resolved thylakoid membrane proteins. Lane 1, molecular weight markers. Lane 2, thylakoid membrane proteins (10 nmol Chl loaded) from low-light-grown *Dunaliella salina.* Lane 3, thylakoid membrane proteins (3.3 nmol Chl loaded) from high-light-grown cells. Note the presence of the 160 kD protein in the HL-grown sample.

Materials and methods

Plant material, growth conditions, thylakoid membrane isolation, SDS-PAGE and Western blot analysis procedures were described in earlier publications from this laboratory (Smith et al. 1990; Kim et al. 1993). Electroelution of protein bands from excised gel slices was implemented with a *BIO RAD* model 422 electroeluter. Specific polyclonal antibodies for D1, D2, and CPI (PS I) were raised in rabbit in this laboratory.

Results and discussion

Growth of oxygen-evolving photosynthetic organisms under light-intensities greater than that required to saturate photosynthesis causes photoinhibition. This adverse phenomenon entails accumulation of photodamaged PS II reaction centers in the thylakoid membrane of chloroplasts. In the green alga *Dunaliella salina*, photodamaged PS II units may account for as much as 80% of the total PS II in the thylakoid membrane (Vasilikiotis and Melis 1994).

In *Dunaliella salina*, photodamaged reaction centers appear on SDS-PAGE as a higher molecular weight complex, migrating with an apparent size of 160 kD. These were shown to exist in a dynamic steady-state with the functional 32 kD form of D1 (Kim et al. 1993). The exact protein composition and function of the 160 kD complex in the PS II damage and repair cycle is not fully known. In this regard, it is important to determine whether D2 also participates in the formation of the 160 kD complex. Earlier Western blot analyses (Melis 1992; Kim et al. 1993) showed a distinct cross-reaction between anti-D1 antibodies and the 160 kD complex. However, a strong.and reproducible cross-reaction with anti-D2 antibodies had not been obtained.

The lack of reproducible cross-reaction between D2 antibodies and the 160 kD complex could be attributed to folding patterns of the 160 kD complex which could be such as to prevent easy access of the polyclonal antibodies to the D2 antigenic epitopes in the complex. To overcome this difficulty, we employed two

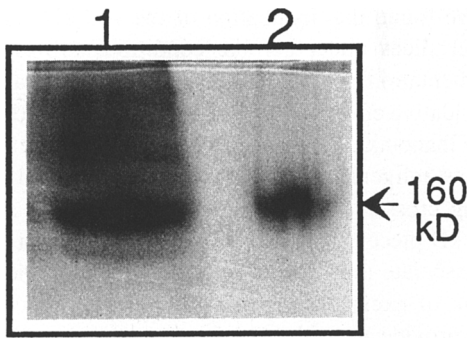

Fig. 2. Coomassie stained profile of SDS-PAGE resolved *D. salina* thylakoid membrane proteins that migrate to the 160 kD region. Lane 1, total thylakoid membrane protein from high-light-grown cells. Lane 2, re-electrophoresis of proteins from a gel slice, excised from the 160 kD region of HL-grown thylakoids.

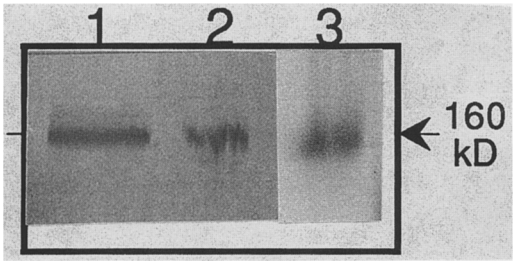

Fig. 3. Western blot analysis of *D. salina* thylakoid membrane proteins migrating to the 160 kD region. Lane 1, total thylakoid membrane protein from high-light-grown cells probed with antibodies against the D1 protein. Lane 2, re-electrophoresed proteins from a gel slice excised from the 160 kD region of HL-grown thylakoids, probed with antibodies against the D1 protein. Lane 3, re-electrophoresed proteins from a gel slice excised from the 160 kD region of HL-grown thylakoids, probed with antibodies against the D2 protein.

different experimental approaches designed to unfold and/or break-up the 160 kD complex.

Figure 1 shows Coomassie stained SDS-PAGE profiles of thylakoid membrane proteins isolated from LL-grown (100 μmol photons m^{-2} s^{-1}, lane 2) and HL-grown cells (2,000 μmol photons m^{-2} s^{-1}, lane 3). On a per Chl basis, there is considerably more LHC-II protein in the LL samples, migrating to about 31 kD, whereas a distinct and abundant 160 kD protein band is clearly evident in the HL sample (lane 3). In earlier work, we have shown that this 160 kD complex is recognized by specific polyclonal antibodies raised against the D1 protein (Melis 1992; Kim and Melis 1993). In the following, we employed band excision, re-electrophoresis and Western blot analysis to probe for the presence of D2 in the 160 kD complex.

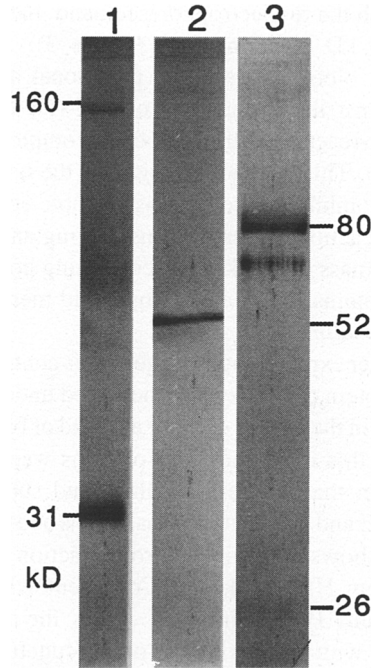

Fig. 4. Western blot analysis of *D. salina* thylakoid membrane proteins, probed with antibodies against the D1 or D2 proteins. Lane 1, total thylakoid membrane protein from high-light-grown cells, probed with D1 polyclonal antibodies. Lane 2, proteins of the 160 kD complex from HL-grown cells, incubated in gel slices under aerobic conditions, probed with anti-D1 antibodies. Lane 3, proteins of the 160 kD complex from HL-grown cells, incubated in gel slices under aerobic conditions, probed with anti-D2 polyclonal antibodies.

Figure 2 shows Coomassie stained SDS-PAGE profiles of proteins migrating to the 160 kD region. Lane 1 shows the 160 kD complex from HL thylakoid membranes. Lane 2 shows the result of an experiment in which a gel segment from the 160 kD region of HL-grown thylakoids was excised, placed in the well of lane 2 (Fig. 2) and re-electrophoresed together with control HL-thylakoids (Fig. 2, lane 1). The result of this band excision and re-electrophoresis experiment shows that the 160 kD protein complex apparently retains its structural integrity so that a protein band re-appears in the 160 kD position.

The protein bands shown in Fig. 2 were transferred to nitrocellulose and probed with specific polyclonal antibodies raised against either the D1 or D2 proteins. Figure 3 (lane 1) shows that D1-specific polyclonal antibodies cross-react with the 160 kD complex from isolated HL-thylakoids (Kim et al. 1993). The same antibodies also recognized the re-electrophoresed band that migrates to 160 kD (Fig. 3, lane 2). Importantly, antibodies raised against the D2 protein also cross

reacted with the re-electrophoresed band that migrates to the 160 kD position (Fig. 3, lane 3). Additional Western blots with specific polyclonal antibodies raised against the apoproteins of CPI (PS I) did not show cross-reaction with the 160 kD complex (results not shown). This analysis strengthens the notion that, under photoinhibition conditions, a stable complex of the PS II reaction center proteins, having an apparent molecular mass of 160 kD and containing both the D1 and D2 proteins, forms in the thylakoid membrane of *Dunaliella salina*.

In further experimentation, gel slices containing the 160 kD protein complex were incubated under aerobic conditions in the dark at 4 °C for a period of two weeks. Following this incubation, the proteins were electro-eluted from the gel, re-solubilized and subjected to SDS-PAGE and Western blot analysis as above. Figure 4 (lane 1) shows the antibody cross-reaction with total protein from HL-thylakoids. Consistent with earlier results (Melis 1992; Kim et al. 1993), the antibodies cross-react with the 32 kD form of D1 (functional form of D1) and with the 160 kD complex (photodamaged centers). Figure 4 shows that, upon incubation under aerobic conditions, the 160 kD complex disintegrated into a ~52 kD band that is recognized by D1 antibodies (lane 2) and into ~80 and ~26 kD bands that are recognized by D2 antibodies (lane 3). Further Western blot analyses with polyclonal antibodies raised against the apoproteins of CPI (PS I) failed to show cross-reaction with the ~52, ~80 or ~26 kD pieces (results not shown). This observation strengthens the notion that the hydrophobic CPI apoproteins do not aggregate with D1 and D2 to generate the 160 kD complex.

Solubilization of thylakoid membranes, or of the excised and electro-eluted 160 kD complex, in the presence of dithiothreitol, instead of β-mercaptoethanol, did not bring about dissociation of the 160 kD complex into the constituent D1 and D2 polypeptides (Baroli and Melis, unpublished).

In summary, the work provides further evidence that a higher molecular weight (~160 kD) complex in *Dunaliella salina* forms upon photoinhibition and contains both the D1 and D2 proteins. The 160 kD complex is stable enough to be re-electrophoresed from excised gel bands. Gel excision and re-electrophoresis apparently cause a partial unfolding, but not dissociation, of the proteins within the 160 kD complex, sufficient to permit a strong and reproducible cross reaction between anti-D2 antibodies and the 160 kD complex (Fig. 3, lane 3).

We found that incubation of the 160 kD complex in gel slices under aerobic conditions brings about fragmentation of the complex, probably as a result of oxidative effects on the D1 and D2 proteins (Mishra and Ghanotakis 1994). This fragmentation helped to expose antigenic epitopes of D2, as evidenced by the strong cross reaction between D2 antibodies and an ~80 kD piece (Fig. 4, lane 3). The ability to break-up or dissociate the 160 kD complex upon aerobic incubation of excised gel bands is interesting because it may provide us with a useful approach by which to test for the presence of other proteins in the 160 kD complex.

The results support the notion that, in *Dunaliella salina*, photodamaged D1 proteins are not automatically degraded but accumulate in the thylakoid membrane under photoinhibition conditions. As such, they can be detected and quantitated from the amount of the 160 kD complex forming under such conditions. This observation is an important step in our understanding of cell recovery from photoinhibition and it may turn out to provide us with a valuable tool in future studies of the PS II repair process.

Acknowledgements

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References

Adir N, Shochat S and Ohad I (1990) Light-dependent D1 protein synthesis and translocation is regulated by reaction center II: Reaction center II serves as an acceptor for the D1 precursor. J Biol Chem 265: 12563–12568

Aro E-M, McCaffery S and Anderson JM (1993) Photoinhibition and D1 protein degradation in peas acclimated to different growth irradiances. Plant Physiol 103: 835–843

Aro E-M, Virgin I and Andersson B (1993) Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Barber J (1989) Function and molecular biology of Photosystem two. Oxford Surveys of Plant Molecular & Cell Biology, Vol 6: 115–162

Barber J (1995) Molecular basis of the vulnerability of photosystem II to damage by light. Austr J Plant Physiol 22: 201–208

Cleland RE, A Melis and PJ Neale (1986) Mechanism of photoinhibition: Photochemical reaction center inactivation in system II of chloroplasts. Photosynth Res 9: 79–88

Guenther JE and Melis A (1990) The physiological significance of Photosystem II heterogeneity in chloroplasts. Photosynth Res 23: 105–110.

Kim JH, Nemson JA and Melis A (1993) Photosystem II reaction center damage and repair in *Dunaliella salina* (green alga) – analysis under physiological and irradiance-stress conditions. Plant Physiol 103: 181–189

Kok B (1956) On the inhibition of photosynthesis by intense light. Biochim Biophys Acta 21: 234–244

Kyle DJ, Ohad I and Arntzen CJ (1984) Membrane protein damage and repair: Selective loss of a quinone-protein function in chloroplast membranes. Proc Natl Acad Sci USA 81: 4070–4074

Mattoo A, Hoffman-Falk H, Marder J and Edelman M (1984) Regulation of protein metabolism: coupling of photosynthetic electron-transport to in vivo degradation of the rapidly metabolized 32-kDa protein of the chloroplast membranes. Proc Natl Acad Sci USA 81: 1380–1384

Mattoo AK and Edelman M (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein. Proc Natl Acad Sci USA 84: 1497–1501

Melis A (1992) Modification of chloroplast development by irradiance. In: Argyroudi-Akoyunoglou JH (ed) Regulation of Chloroplast Biogenesis, pp 491–498. Plenum Press, New York

Mishra NP and Ghanotakis DF (1994) Exposure of a Photosystem II complex to chemically generated singlet oxygen results in D1 fragments similar to the ones observed during aerobic photoinhibition. Biochim Biophys Acta 1187: 296–300

Nanba O and Satoh K (1987) Isolation of a Photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. Proc Natl Acad Sci USA 84: 109–112

Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol 35: 15–44

Smith BM, Morrissey PJ, Guenther JE, Nemson JA, Harrison MA, Allen JF and Melis A (1990) Response of the photosynthetic apparatus in *Dunaliella salina* (green algae) to irradiance stress. Plant Physiol 93: 1433–1440

Vasilikiotis C and Melis A (1994) Photosystem-II reaction center damage and repair cycle – chloroplast acclimation strategy to irradiance stress. Proc Natl Acad Sci USA 91: 7222–7226