Isolation and characterization of photosystem II core complexes with high oxygen evolution activity from spinach using the non-ionic detergent 6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside

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Abstract

Two different kinds of oxygen evolving photosystem II (PSII) core complexes were isolated in the present study by solubilization of PSII enriched thylakoid membranes from spinach with the non-ionic detergent 6-O-(N-heptyl-carbamoyl)-methyl-α-D-glucopyranoside (Hecameg) under different conditions. The PSII core complex isolated at higher ionic strength was similar to that isolated by using octyl-β-D-glucopyranoside (OGP) and lacked the 23 and 17 kDa extrinsic proteins of the oxygen evolving complex but retained the 22 kDa PsbS protein. Solubilization of the PSII membranes with Hecameg at lower ionic strength allowed the isolation of another PSII complex that retained all the three extrinsic proteins (33, 23 and 17 kDa) of the oxygen evolving complex but was depleted of the 22 kDa PsbS protein. This complex exhibited high rates of oxygen evolution and was found to be more sensitive to DCMU indicating a better structural and functional integrity and may be treated as the minimal functional unit required for PSII photochemistry. The detergent Hecameg is relatively inexpensive and the methodology remains simple since it does not require any chromatography or density gradient ultracentrifugation.

Additional key words: Hecameg; oxygen evolution; photosystem II.

Introduction

PSII core complexes which are depleted of the bulk of Chl *a/b* binding proteins of the light harvesting complex, have provided an excellent system for the study of primary photochemistry and oxygen evolution in PSII. These PSII complexes have proved to be very useful as they exhibit the complete electron transport activity from H_2O to Q_B and retain all the proteins required for primary photochemistry of the PSII complex including the 23 kDa and 17 kDa extrinsic proteins of the water oxidation complex. These proteins reduce the physiological requirement of Ca^{2+} and Cl^- and, along with the 33 kDa extrinsic protein, stabilize the Mn complex which stores the oxidizing equivalents required for photosynthetic water oxidation.

A number of biological detergents, such as octyl glucoside (Ikeuchi and Inoue 1986, Ghanotakis *et al.* 1987), heptyl thioglucoside (Enami *et al.* 1989), octyl thioglucoside (Mishra and Ghanotakis 1993) and dodecyl maltoside (Haag *et al.* 1990, Hankamer *et al.* 1997), are currently being used for isolation of the oxygen evolving PSII core complexes. Although these detergents are efficient in solubilizing the PSII membranes, and thus allow the isolation of various PSII sub-complexes, their cost remains relatively high. Hecameg is a synthetic, well defined glucose based non-ionic detergent molecule, providing consistent and reliable results. It is soluble in aqueous buffers at 4 °C or room temperature (>100 mg cm^{-3}), does not absorb at 280 nm and is stable for weeks at 4 °C. Its critical micellar concentration is 19.5 mM which allows its easy removal by dialysis. It dissociates

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Abbreviations: Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCMU – 3-(3',4'-dichlorophenyl)-1,1'-dimethylurea; Hecameg – 6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside; LHC – light-harvesting complex; Mes – 2-(*N*-morpholino) ethanesulfonic acid; OGP – 1-O-*n*-octyl-β-D-glucopyranoside; PAGE – polyacrylamide gel electrophoresis; PSII – photosystem II; PVDF – polyvinylidene difluoride; SDS – sodium dodecyl sulfate.
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aggregated proteins without denaturing them and helps breaking biological membranes. It also does not interfere with biological activities of proteins including enzymes, antigens and receptors.

The present communication describes a simple method for isolation of the oxygen evolving PSII core complexes from spinach. Solubilization of the PSII membranes with Hecameg at different ionic strengths allows the preparation of PSII core complexes either with only the 33 kDa or with all the three extrinsic (33 kDa, 23 kDa and 17 kDa) polypeptides associated with the oxygen evolving complex.

Materials and methods

Isolation of PSII enriched membranes: Thylakoid membranes were isolated from spinach purchased from the local market as described earlier (Mishra and Singhal 1991). The thylakoids were suspended in 50 mM Mes, pH 6.0, 15 mM NaCl, 5 mM $MgCl_2$ and 1 mM sodium ascorbate. Triton X-100, from a 25 % stock, was added drop wise to this suspension to yield a detergent to Chl ratio of 25:1 (w/w). After 30 min of gentle stirring in dark on ice, the suspension was centrifuged at 40 000 × *g* for 30 min to sediment the PSII membranes. These PSII membranes were resuspended in 0.4 M sucrose, 50 mM Mes, pH 6.0 and 10 mM NaCl at 1 mg(Chl) cm^{-3} and sedimented by centrifugation at 40 000 × *g* for 30 min. The resulting pellet was used for solubilization with OGP or Hecameg. This washing was performed to keep the residual Triton X-100 concentration to minimum and a constant level. This was done to maintain reproducibility of the conditions during subsequent solubilization of the PSII membranes with either OGP or Hecameg.

Preparation of Hecameg/HS-PSII core complex: An oxygen evolving PSII core complex depleted of the light harvesting complex and the extrinsic 23 kDa and 17 kDa proteins, called the 'Hecameg/HS-PSII core complex', was isolated by the method described earlier by Ghanotakis *et al.* (1987) with some modification but using the same molarity of Hecameg instead of OGP. In details, the PSII membranes at 2.5 mg Chl cm^{-3} were resuspended in 0.4 M sucrose, 50 mM Mes, pH 6.0 and 10 mM NaCl. It was mixed with an equal volume of a solution containing 1.0 M sucrose, 50 mM Mes, pH 6.0, 0.8 M NaCl, 10 mM $CaCl_2$, and 70 mM Hecameg. The mixture was incubated for 10 min at 4 °C in dark. Upon incubation, two volumes of a solution containing 1.0 M sucrose, 50 mM Mes, pH 6.0, 0.4 M NaCl, and 5 mM $CaCl_2$ was added. This mixing was followed by 5 min of incubation on ice and 90 min of centrifugation at 40 000 × *g* at 4 °C to sediment the LHC detached from the PSII core complex. The supernatant at this stage contained the PSII core complex in a medium that had approximately 0.4 M NaCl. Hence this complex was called 'Hecameg/HS-PSII core complex'. The complex present in the supernatant was sedimented by addition of an equal volume of 20 % (v/v) PEG (M_r 6 000) and subsequent centrifugation at 40 000 × *g* for 30 min at 4 °C.

Preparation of OGP-PSII core complex: Another oxygen evolving PSII core complex, depleted of the major light harvesting chlorophyll (Chl)-protein complex, was isolated by solubilization of PSII membranes with OGP instead of Hecameg essentially using the method described above. This preparation is called 'OGP-PSII core complex'.

Preparation of Hecameg-PSII core complex: Isolation of the 'Hecameg-PSII core complex' retaining all the three (33, 23, and 17 kDa) extrinsic proteins of the oxygen evolving complex was achieved by solubilization of the PSII membranes with Hecameg at lower ionic strength (10 mM NaCl as compared to 0.4 M NaCl in the case of Hecameg/HS- PSII core complex and OGP-PSII core complex). For the isolation of this complex, the PSII membranes, in 0.4 M sucrose, 50 mM Mes, pH 6.0 and 10 mM NaCl at 1 mg Chl cm^{-3}, were incubated with 30 mM Hecameg for 10 min, on ice in dark, and were subsequently centrifuged at 40 000 × *g* for 30 min. The green supernatant was diluted with 1.2 volumes of 0.4 M sucrose, 50 mM Mes, pH 6.0, 10 mM NaCl, and 5 mM $MgCl_2$. $MgCl_2$ was included in this mixture to facilitate the aggregation of the LHC, which was recovered by a second centrifugation at 40 000 × *g* for 30 min at 4 °C. The pellet was washed once with 0.4 M sucrose, 50 mM Mes, pH 6.0 and 10 mM NaCl, and was resuspended in the same medium. Approximately 12–15 % of the total Chl in the PSII membranes was recovered in the final pellet.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting: SDS-PAGE was carried out on 13 % polyacrylamide gels in the presence of 6 M urea according to Laemmli (1970), but using 0.6 M Tris in the resolving gel. The proteins in the gels were either stained with Coomassie brilliant blue R-250 or transferred on to PVDF membranes and probed with antisera raised against CP 24 and the 22 kDa PSII polypeptide (PsbS) as described earlier (Mishra and Ghanotakis 1993). The identification of protein bands has been based on relative electrophoretic mobility of proteins with respect to standard molecular weight markers and/or use of antibodies.

Measurement of oxygen (O_2) evolution rate: Light saturated rates of O_2 evolution, in the presence of 0.4 mM DCBQ and/or 2 mM $K_3Fe(CN)_6$ were determined on

a Clark type O_2 electrode equipped with a fibre optic guide tube (*DW2/LS2 Hansatech*, King's Lynn, Norfolk, England). The light intensity (PAR) within the chamber during measurements was approximately 1800 μmol m^{-2} s^{-1}. 5 μM DCMU or 10 mM $CaCl_2$ were added wherever indicated.

Chl concentrations in the various preparations were determined according to Arnon (1949) using a *UV-Vis* spectrophotometer (*Model 117, Systronics*, New Delhi, India).

Results and discussion

The polypeptide composition of the PSII membranes and the PSII core complexes prepared by using OGP and Hecameg is shown in Fig. 1. It is apparent that the protein composition of the 'Hecameg/HS-PSII core complex' (*lane 3*) was similar to that of 'OGP-PSII core complex' (*lane 2*) isolated by using OGP. The major polypeptides of these two complexes are the 47 kDa and 43 kDa Chl-binding proteins, the D1 and D2 reaction center polypeptides, Cyt b_{559}, the 33 kDa extrinsic protein, the 29 kDa Chl binding protein and the 22 kDa (the *psbS* gene product) and 10 kDa protein. On the other hand, the 'Hecameg-PSII core complex' that was isolated at lower ionic strength (*lane 4*), retained the 23 kDa and 17 kDa extrinsic polypeptides of the oxygen evolving complex while the 22 kDa and 10 kDa PSII membrane proteins were removed.

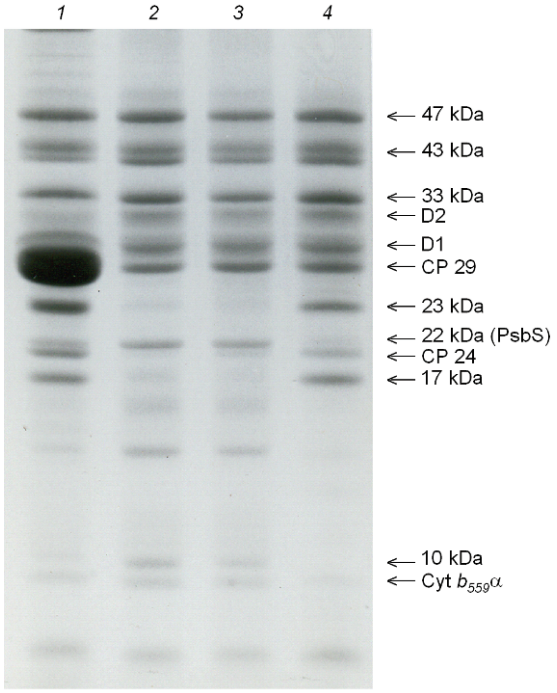

Fig. 1: SDS-PAGE pattern showing the polypeptide compositions of PSII membranes (*lane 1*), the 'OGP-PSII core complex' (*lane 2*), the 'Hecameg/HS-PSII core complex' isolated at high ionic strength (*lane 3*) and the 'Hecameg-PSII core complex' isolated at low ionic strength (*lane 4*). The samples were run on a 13 % polyacrylamide gel in the presence of 6 M urea and staining was performed with Coomassie brilliant blue R-250.

As shown in Fig. 1, the 'Hecameg-PSII core complex' isolated at low ionic strength (*lane 4*) also retained a protein that appears just under the position of the 22 kDa band in the gel. This protein was present in the Hecameg/HS-PSII core complex (*lane 3*) and the OGP-PSII core complex (*lane 2*) but absent in the 'Hecameg PSII- core complex' (*lane 4*). This protein showed immunoreactivity with antiserum raised against the CP 24 (Fig. 2), which indicates that this polypeptide is closely associated with the PSII complex. CP 24 is an important component of the PSII light harvesting antenna that plays an important role in determining the structure and function of the PSII light harvesting antenna providing the linker for the association of the light-harvesting complex (LHC) trimers into the PSII complex allowing specific organization that is necessary both for maximum quantum efficiency and for photoprotective dissipation of excess excitation energy (Dunahay and Staehelin 1986, Kovacs *et al.* 2006). Spangfort *et al.* (1989) have shown that a 20 kDa polypeptide is the Chl binding component of CP 24, which could explain the appearance of this protein under the position of 22 kDa band in the gels. The room temperature absorption spectra of the PSII core complexes isolated by using Hecameg at either high or low ionic strength were similar to the spectrum of the PSII core complex isolated by using OGP (data not shown). Fig. 2 also shows the anti-22 kDa immunoblots of PSII membranes and PSII core complexes isolated by solubilization of PSII membranes with OGP and with Hecameg at high and low ionic strengths. It is evident that the 22 kDa protein was present in the PSII core complexes isolated either with OGP or Hecameg at high ionic strength, but the PSII core

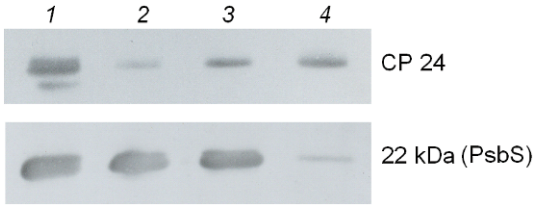

Fig. 2: Western blot analysis of PSII membranes (*lane 1*), the 'OGP-PSII core complex' (*lane 2*), the 'Hecameg/HS-PSII core complex' isolated at high ionic strength (*lane 3*) and the 'Hecameg-PSII core complex' isolated at low ionic strength (*lane 4*). The proteins, after being resolved on SDS-PAGE, were transferred on to PVDF membranes and were probed with antisera raised against CP 24 and the 22 kDa PSII protein.

Table 1. Light saturated oxygen evolution rates of the PSII core complexes prepared using OGP and Hecameg at high and low ionic strengths. [1]All activities, expressed in μmol(O_2) mg^{-1}(Chl) h^{-1}, were assayed on a Clark type electrode at 25 °C in a medium containing 0.4 M sucrose, 50 mM Mes, pH 6.0 and 10 mM NaCl in the presence of 0.4 mM DCBQ and/or 2 mM $K_3Fe(CN)_6$. Additions of 10 mM $CaCl_2$ and 5 μM DCMU were preformed during assay wherever indicated. The values shown in the table are mean ± standard deviation of three different sets of experiments. [2]HS stands for high ionic strength.

Additions	Rate of O_2 evolution[1] PSII core complex preparation		
	OGP	Hecameg/HS[2]	Hecameg
DCBQ	580 ± 19	470 ± 23	1 270 ± 36
DCBQ+DCMU	225 ± 10	175 ± 5	335 ± 17
DCBQ+$CaCl_2$	960 ± 52	930 ± 46	1 285 ± 51
DCBQ+$CaCl_2$+DCMU	310 ± 18	325 ± 13	345 ± 19
$K_3Fe(CN)_6$	260 ± 27	255 ± 30	460 ± 33
$K_3Fe(CN)_6$+DCMU	135 ± 5	160 ± 12	195 ± 6
$K_3Fe(CN)_6$+ $CaCl_2$	585 ± 22	570 ± 28	680 ± 25
$K_3Fe(CN)_6$+ $CaCl_2$+DCMU	300 ± 11	320 ± 17	300 ± 29
DCBQ+ $K_3Fe(CN)_6$	585 ± 21	430 ± 26	1 240 ± 51
DCBQ+ $K_3Fe(CN)_6$+DCMU	210 ± 9	150 ± 14	415 ± 14
DCBQ+ $K_3Fe(CN)_6$+ $CaCl_2$	1 030 ± 30	940 ± 52	1 280 ± 53
DCBQ+ $K_3Fe(CN)_6$+ $CaCl_2$+DCMU	325 ± 13	355 ± 15	440 ± 28

complex isolated by using Hecameg at lower ionic strength was significantly depleted of this species. It may be possible that under conditions of high ionic strength, the 22 kDa membrane protein is released from the PSII complex, but may not associate with the LHC and remains in a monomeric form which does not sediment with LHC. This protein may subsequently reassociate with the PSII core complex during its sedimentation. The reason for depletion of the 22 kDa protein in Hecameg-PSII core complex in not clear, but may be this protein remains in monomeric form that may preferentially associate with the LHCII at lower ionic strength and is sedimented with the major LHC during the preparation leaving the PSII core complex depleted of it. This is supported by the observation that the monomeric PsbS preferentially associates with LHCII while the dimeric PsbS preferentially associates with the PSII core and the predominance of monomeric and dimeric forms depends upon the pre-treatment conditions (Bergantino *et al.* 2003).

The PSII antenna contains a number of proteins of relatively low abundance, which have been implicated as playing roles in the regulatory processes (Jansson 1999). The PsbS is one of these proteins that have been believed to play a key role in non-photochemical quenching. Non-photochemical quenching is the process of energy dissipation, induced under excessive light conditions which provide photoprotection to the thylakoid membrane from the potentially damaging effects of the excessive absorbed radiant energy (Horton *et al.* 1996, Niyogi 1999). On the other hand, the PsbS protein was also found to be widely distributed in the thylakoid membrane and associated with a number of different complexes (Teardo *et al.* 2007). It has also been shown elsewhere that a PsbS-deficient mutant displayed alterations in PSII function that were not related to non-photochemical quenching (Peterson and Havir 2001). Moreover, a recent study confirms that PsbS specifically controls the association between LHCII and the PSII core complex (Kiss *et al.* 2008). So it seems likely that though this protein may be present in a number of PSII complexes prepared by different methods, it is unlikely to play a direct role in the primary photochemistry of PSII and water oxidation.

The light-saturated rates of O_2 evolution of the various PSII core complexes, prepared using OGP at high ionic strength and Hecameg at both high and low ionic strengths, are presented in Table 1. It is clear that the O_2 evolution rates of the PSII complexes isolated by using OGP and Hecameg at high ionic strength, which lacked the 23 and 17 kDa extrinsic proteins, were comparable. On the other hand, the 'Hecameg-PSII core complex' which retains all three extrinsic polypeptides, exhibited relatively higher O_2 evolution activity. The optimal O_2 evolution activity of the PSII complexes isolated at high ionic strength, either with OGP or Hecameg (OGP-PSII and Hecameg/HS-PSII core complexes), required non-physiological levels of $CaCl_2$, because of the loss of the 23 kDa and 17 kDa extrinsic polypeptides which act as concentrators of the inorganic cofactors Ca^{2+} and Cl^- (Ghanotakis *et al.* 1984a, 1984b). No such requirement of $CaCl_2$ was observed for the 'Hecameg-PSII core complex' that was isolated at low ionic strength. This observation indicates that both the 23 kDa and 17 kDa extrinsic polypeptides are not only present with the 'Hecameg-PSII core complex' but also functionally bound at their native sites. The Hecameg PSII core complex was also found to be more sensitive to DCMU than the other two complexes that lacked the 23 and 17 kDa proteins. In the presence of DCMU and with DCBQ as the electron acceptor, the rate of O_2 evolution

by 'Hecameg-PSII core complex' was inhibited by 74 % whereas the inhibition was only 61 % and 63 % in the case of OGP-PSII core and Hecameg/HS-PSII core complexes respectively. This indicates that the Q_B-binding site in the Hecameg PSII core complex' was not altered during isolation. It may be mentioned at this point that Kashino *et al*. (1996) using heptyl thioglucoside-extracted PSII core complex preparations from spinach and cyanobacteria have demonstrated that chloro- or methyl-substituted benzoquinones accept electrons at two sites, Q_B and PQ and removal of the membrane structure or light harvesting Chl *a/b* protein complexes have little effect on the characteristics of the Q_B site in both the preparations. DCBQ that is used as electron acceptor in the present study is a chloro-substituted benzoquinone, and hence accepts electrons and supports O_2 evolution. It, therefore, seems likely that the 'Hecameg PSII core complex' is much less perturbed during the isolation and maintained better structural and functional integrity on both donor as well as acceptor sides of PSII. The 'Hecameg-PSII core complex' is functionally similar to the PSII core complex isolated by Enami *et al.* (1989). The main difference between the two complexes is that although the 'Hecameg-PSII core complex' retains the CP 24 species, it is depleted of the 22 kDa and 10 kDa polypeptides which are present in the preparation of Enami *et al.* (1989). In addition, the isolation of the 'Hecameg-PSII core complex' is very simple since it required no ultracentrifugation.

Hecameg has been developed by Plusquellec *et al.* (1989). Its chemical structure, molecular mass, aggregation number, and critical micellar concentration (CMC) are similar to those of OGP (Plusquellec *et al.* 1989, Frindi *et al.* 1992, Ruiz *et al*. 1994), and it is likely to be able to substitute for it under many circumstances. Hecameg appears as an economically interesting substitute to the more expensive octyl glucopyranoside, octyl thioglucoppyranoside and dodecyl maltoside for membrane protein purification. It has been applied to the purification of mycoplasma surface antigens (Plusquellec *et al.* 1989, Brenner *et al.* 1995), the sarcoplasmic Ca^{2+}-ATPase (Ruiz *et al.* 1994), and to the crystallization of beef heart cytochrome bc_1 (Lee *et al.* 1995). Proteins that are sensitive to OGP are not good candidates for purification with Hecameg, as indicated by experiments with Ca^{2+}-ATPase (Ruiz *et al.* 1994). Hecameg has also been the detergent of choice for preparation of cytochrome b_6f complex. It has yielded highly active preparations from the green alga *Chlamydomonas rheinhardtii* (Pierre *et al.* 1995, 1997), as well as from spinach (Dietrich and Kuhlbrandt 1999). In fact, *C. reinhardtii* cytochrome b_6f complex is solubilized by Hecameg somewhat more specifically than by OGP and purified preparations in Hecameg are stable for weeks provided lipids are also present. Dietrich and Kuhlbrandt (1999) have also reported that the most active cytochrome b_6f complex was actually the one isolated using Hecameg. Hecameg has also been utilized in concert with high calcium for purification of the actin-binding protein scruin from the sperm of the horseshoe crab *Limulus* (Sanders *et al.* 1996). More recently, Ravaud *et al.* (2006) have demonstrated that solubilization of ABC transporter *Bmr*A, that is involved in multidrug resistance, was indeed possible by Hecameg, though dodecyl-maltoside was more efficient for the preparation but much more expensive. In addition to extraction, purification and stabilization of natural and recombinant proteins, Hecameg has also been utilized as surfactant for chromatography, electrophoresis and ELISA analysis, extraction of other biomolecules (DNA and RNA) from proteous samples, study of protein structure, reconstitution or crystallization of membrane proteins, enzymes or antigens, liposomes preparation and sanitization of chromatography columns.

Hence the results presented in this communication demonstrate that the detergent Hecameg, which is a relatively less expensive non-ionic detergent, does not exert any adverse effect on the PSII components. It can not only substitute for OGP but could also help the isolation of a PSII core complex that retains all the three extrinsic proteins of the O_2 evolving complex and is minimally perturbed on the donor and acceptor sides. The PSII complexes isolated using Hecameg could also provide excellent materials for investigation of the physiological roles of the PsbS and the CP 24 species in PSII complex.

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