Structural changes in photosystem II after treatment with the zero-length bifunctional cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide: an electron microscopic study

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Two-dimensional (2D) crystals of photosystem II (PS II) treated with various concentrations of the zero-length crosslinker 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) were analysed by electron microscopy in conjunction with crystallographic image processing. The preparations were characterized by SDS/PAGE and oxygen-evolution measurements, and the effectiveness of cross-linking was monitored by measuring the level of protection afforded against high concentrations of NaCl and CaCl₂, which normally remove extrinsic proteins from PS II.

We found that low concentrations of EDC (0.25%) increase the order of 2D crystals of PS II. Treatments with EDC concentrations higher than 0.5% did not improve the order of 2D crystals but induced gross structural changes, which were correlated with a decrease in oxygen evolution activity. Structural changes due to cross-linking did not affect packing or symmetry of the 2D crystals, further supporting the conclusion that PS II has a monomeric nature *in vivo*.

INTRODUCTION

In plants, photosynthetic water oxidation and oxygen evolution occur within a multi-protein/pigment thylakoid complex termed photosystem II (PS II) (reviewed in [1]). PSII consists of at least 22 individual polypeptides [2], and is considered to be organized into four distinct functional components: a reaction centre, a core light-harvesting complex (LHC), peripheral LHCs and an oxygen-evolution-enhancing (OEE) complex [3]. The reaction centre consists of two proteins with limited sequence similarities (D1 and D2, 32–34 kDa) and a heterodimeric cytochrome *b*₅₅₉ (13 kDa). The core LHC consists of chlorophyll *a*-binding proteins CP 43 and CP 47. Surrounding the core are several integral membrane LHC apoproteins (24, 25, 26, 27, 28 and 29 kDa) that serve to transfer light energy to the reaction core [4,5]. On the luminal side of the complex are three extrinsic proteins (16, 23 and 33 kDa) that enhance the oxygen-evolving capacity of PSII and form the OEE complex [6–8].

The zero-length water-soluble cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) catalyses the formation of a peptide bond between amino and carboxy groups [9]. Treatment of PS II with EDC has been previously used to identify polypeptides in close proximity. Treatment of PS II with 1% (w/v) EDC cross-links the OEE 33 kDa subunit with CP 47, creating a major cross-linked product of 80 kDa, and minor cross-linking of LHC proteins [9]. Higher concentrations of EDC, 2% and 3% EDC respectively, have also been used to totally immobilize the OEE 16 and 23 kDa proteins to unidentified polypeptides [10].

Another study showed that EDC treatments of PS II membranes produced main cross-linked products of 65 and 76 kDa [11]. Washing with CaCl₂ did not remove the OEE 33 kDa

protein after cross-linking, and cross-reaction with an antibody specific for CP 47 confirmed the presence of CP 47 in the cross-linked products. In addition, small amounts of unknown 57, 37 and 26 kDa products that reacted with the antibody to OEE 33 kDa were identified [11]. The reasons for the presence of different cross-linked products in the various studies are unresolved; however, EDC is known to be highly deliquescent and so actual concentrations used to cross-link PS II may vary.

The resolution and quality of structural information that can be derived from electron micrographs of two-dimensional (2D) crystals of proteins depends on the degree of order [12,13]. Because EDC is thought to stabilize PS II complexes, we considered the potential of EDC as a tool to improve the order of 2D crystals present in many of our preparations. Here we describe how low concentrations of EDC increase the quality of ordered 2D arrays of PS II.

MATERIALS AND METHODS

Membranes enriched in PS II were prepared from spinach (*Spinacia oleracea*) leaves as previously described in [14]. All membrane preparations were stored in resuspension buffer [20 mM Mes, pH 6.3, containing 15 mM NaCl, 5 mM MgCl₂ and 20% (w/v) glycerol] at –80 °C. Oxygen evolution was measured with a Clark type electrode as described in [14], and chlorophyll concentrations were determined by the methods described in [15]. Membrane preparations used for structural studies exhibited oxygen evolution rates in the range 300–350 μmol of O₂/h per mg of chlorophyll.

EDC cross-linking was performed as described in [9], with the

Abbreviations used: CP, chlorophyll *a*-binding protein; 2D, two-dimensional; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; FFT, fast Fourier transform; LHC, light-harvesting complex; OEE, oxygen-evolution-enhancing; PS II, photosystem II.

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following cross-linking buffer: 40 mM Mes/NaOH (pH 6.5)/0.4 M sucrose/20 mM EDTA/5 mM MgCl_2 /10 mM NaCl. EDC was freshly prepared, and all incubations were performed on ice.

Removal with NaCl of the 16 and 23 kDa OEE proteins was performed by the method described in [7], and removal with CaCl_2 of the 16, 23 and 33 kDa OEE proteins was performed as described in [16]. Salt solutions were prepared in the resuspension buffer previously described.

SDS/PAGE was performed with Tris/HCl polyacrylamide gradient gels [4–20% (w/v)] purchased from Bio-Rad Ltd. Samples containing 2 μg of chlorophyll were mixed with sample buffer [125 mM Tris-HCl (pH 6.8)/40% (v/v) glycerol/6% (w/v) SDS/0.05% Bromophenol Blue] and incubated at 60 °C for 5 min. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue in 40% (v/v) methanol/10% (v/v) acetic acid.

Collodium support films were placed on 300-mesh or 400-mesh copper grids [17] and then carbon-coated. Before use, grids were glow-discharged for 1 min at reduced pressure (approx. 13 Pa). Grids were then mounted by using the methods outlined in [18] and examined with a Philips CM10 electron microscope operated at 100 keV under low-dose conditions. Electron micrographs of 2D crystals of PS II and EDC-treated PSII were recorded at calibrated magnifications of $\times 27200$ and $\times 36500$ on Agfa Scientia 23 D 56 electron image film.

Electron micrographs were printed and then digitized at 64 μm increments with a Hewlett Packard Deskjet IIc flatbed scanner. Digitized images were analysed with the PC-based image analysis and processing packages CRISP and TRI-MERGE [19]. Suitable areas were boxed off from membranes and diffraction patterns were calculated. Lattice points were imposed and refined. Amplitudes and phases were extracted and different crystalline areas were merged. Some data were rotated through 180 ° along the a - b plane to align them correctly relative to reference data before merging. Only data sets with an individual average inter-image phase residual of less than 40 ° were merged. The quality of 2D arrays was assessed as follows. Entire 2D crystals were selected and the transforms calculated. Different amplitude thresholds were imposed and the number of reflections present in each fast Fourier transform (FFT) above this amplitude threshold was recorded. Amplitudes for each set of data were scaled relative to the maximum amplitude value determined for all data sets. The number of reflections above a given amplitude threshold was then plotted against the scaled amplitude threshold point to provide a graphical assessment of the degree of order.

Figure 1 The effect of a 10 min incubation at different concentrations of EDC on the oxygen evolution rate of PS II

RESULTS

Figure 1 shows the effect of increasing concentrations of EDC on the oxygen evolution rate of PS II. Treatments up to 0.5% EDC had no effects on oxygen evolution rate, which remained consistently between 300 and 320 μmol of O_2 /h per mg of chlorophyll. Increasing the EDC concentration to more than 0.5% caused the oxygen evolution rate to decrease; at 1% and 2% EDC, oxygen evolution rates were diminished by 20–30% and 75–85% respectively. Inspection of 2% EDC-treated preparations by electron microscopy revealed that membranes had aggregated extensively and 2D crystals could not be detected.

Cross-linking was confirmed by using SDS/PAGE (Figure 2). As the concentration of EDC increased, the intensities of all polypeptide bands decreased, suggesting that cross-linking occurred widely between the majority of PS II polypeptides. The disappearance of bands corresponding to cytochrome b_{559} (10 kDa), OEEs (16, 23 and 33 kDa), LHC apoproteins (24–29 kDa), D1 and D2 proteins (32 kDa), and CP 43 and CP 47 polypeptides was apparent (Figure 2). Cross-linking also produced a unique band with an approximate molecular mass of 40 kDa (asterisk in Figure 2), which decreased in favour of a high-molecular-mass species at higher EDC concentrations. In samples treated with 0.5%, 1% and 2% EDC, most of the sample did not migrate into the gel (stars in Figure 2), indicating that high-molecular-mass products (greater than 220 kDa) were induced by cross-linking.

Salt-washing assays were also used to determine the effectiveness of cross-linking [9,10] (results not shown). As expected, treatment with 1 M NaCl inhibited oxygen evolution rates by 60% but not in membranes treated with 1% EDC (0% inhibition). Similarly, 1 M CaCl_2 treatment inhibited oxygen evolution rates by 75%, but in membranes treated with 1% EDC the inhibition was only 30%.

Examination of negatively stained PS II membranes showed that approx. 5% of membranes contained 2D crystals of PS II. FFTs of micrographs of these crystalline areas contained 6th-order reflections (2,6) (Figure 3A). A projection map was calculated and overall structural features were in excellent

Figure 2 Coomassie Brilliant Blue-stained SDS/PAGE (4–20%) of PS II membranes treated with various concentrations of EDC

Lane 1, PS II membranes; lane 2, 0.25% EDC-treated PS II; lane 3, 0.5% EDC-treated PS II; lane 4, 1% EDC-treated PS II; lane 5, 2% EDC-treated PS II. Letters at the left indicate components as follows: A, cytochrome b_{559} (10 kDa); B, OEE 16 kDa; C, OEE 23 kDa; D, LHC apoproteins; E, D1 and D2 proteins; F, OEE 33 kDa; G, CP 43; H, CP 47. The stars in lanes 3, 4 and 5 show the existence of polypeptide aggregates with a mass in excess of 220 kDa. The asterisk shows the position of a cross-linking product with a mass of 40 kDa. The positions of molecular mass markers of 12, 17, 30, 44, 66 and 77 kDa are indicated by arrowheads at the left.

Figure 3 Electron micrographs of negatively stained PS II 2D crystals of untreated PS II membranes (A), 0.25% EDC-treated membranes (B) and 1% EDC-treated PS II membranes (C)

Diffraction patterns of areas from each crystal category are shown on the right of the micrographs. The 1% EDC-treated sample shows two distinct crystalline areas (1 and 2) and underlying membranes are highlighted by arrowheads. Scale bar, 100 nm.

agreement with previous data [18,20,21] (Figure 4a). Table 1 contains information used in the image analysis of all samples. The projection is dominated by information related to the luminal side of PS II and the map reveals four clearly defined domains surrounding a central cavity. The average unit-cell dimensions [$a = 17.0$ nm, $b = 19.0$ nm and $\gamma = 90^\circ$ ($P1$ plane group symmetry)] are similar to those previously reported ($a = 16.8$ nm, $b = 18.9$ nm and $\gamma = 91^\circ$) [20].

Figure 3(B) shows an area of negatively stained PS II membranes treated with 0.25% EDC. These membranes showed no obvious differences when compared with control membranes. Figure 4(b) shows the averaged projection map produced from 0.25% EDC-treated 2D crystals. No major structural changes are observed, and four clearly defined domains surrounding a central cavity are evident. The average unit cell dimensions ($a = 17.2$ nm, $b = 18.7$ nm and $\gamma = 90.3^\circ$) were similar to the control samples.

PS II membranes treated with 0.5% EDC were superficially similar to control membranes and contained similar amounts of 2D crystals. Figure 4(c) is the averaged projection map produced from 0.5% EDC-treated 2D crystals. No major structural

Figure 4 Projection maps of control PS II (a), 0.25% EDC-treated PS II (b), 0.5% EDC-treated PS II (c) and 1% EDC-treated PS II (d)

Common structural features have been labelled domains I–IV in accordance with [18,20,21]. Structural features in 1% EDC-treated PS II are labelled A and B. Unit-cell dimensions and resolution details are listed in Table 1.

changes are present; however, it should be noted the four luminal domains and central cavity are less defined.

PS II membranes treated with 1% EDC showed considerable differences from control membranes. Membranes appeared mainly as stacks of several membrane sheets and although 2D arrays were present, analysis of these areas was influenced by the underlying membrane layers (Figure 3C). The averaged projection map produced from 1% EDC-treated 2D arrays (Figure 4d) shows that gross structural changes are clear and the four luminal domains and central cavity are no longer present. PS II treated with 1% EDC possesses two central domains.

Because 0.25% EDC cross-linking produced no major structural or biochemical changes we quantitatively evaluated the quality of cross-linked PS II crystals (Figure 5). At amplitude threshold points greater than 6% of maximum, the numbers of reflections in control and 0.25% EDC-treated PS II FFTs are similar. However, at amplitudes less than 6% the number of reflections is much greater in the 0.25% EDC-treated samples. These results suggest that the degree of 2D order in entire PS II crystals treated with 0.25% EDC is significantly improved. Quantitative analysis of the 0.5% and 1% EDC-treated 2D arrays showed no significant difference in order from control samples (results not shown).

DISCUSSION

Previous cross-linking studies have been used to identify regions of PS II polypeptides in close proximity. The previous work of Enami et al. [9] showed that at 1% EDC the OEE 33 kDa protein was immobilized to the CP 47 protein of PS II with no inhibition of oxygen evolution. Even at 3% EDC only 20%

Table 1 Image-processing and crystallographic data

Sample	Control	0.25% EDC	0.50% EDC	1.00% EDC
Number of images	8	11	9	18
Number of measured reflections	339	303	216	345
Average interimage phase residual (degrees)	22.4	28.2	25.3	21.7
<i>a</i> (nm)	17.0	17.2	18.2	18.5
<i>b</i> (nm)	19.0	18.7	20.8	20.8
γ (degrees)	91.0	90.3	89.7	89.4
Highest resolution reflection detected (<i>h,k</i>)	2,6	2,7	2,6	1,6
Approximate resolution (nm)	3.0	3.3	3.0	3.3
<i>a</i> -to- <i>b</i> axis ratio	0.89	0.91	0.87	0.89
2-D plane group	<i>P</i> 1	<i>P</i> 1	<i>P</i> 1	<i>P</i> 1

Figure 5 Assessment of 2D order in PS II crystals

The data show the number of reflections present at various amplitude thresholds in control membranes and 0.25% EDC-treated membranes. Curves were fitted to data by using an exponential function.

inhibition of oxygen evolution was reported [10]. This study shows that large structural changes to PS II occur at 1% EDC, mirrored by a 20% inhibition of oxygen evolution. EDC cross-linking has been previously reported to inhibit activity directly by modifying carboxy groups that serve as ligands for Mn^{2+} at the Mn^{2+} -binding site [22,23]. The study of Preston and Seibert [23] noted a loss of Mn^{2+} at similar concentrations to this study (20 mM); however, in their study PS II membranes were denuded of the OEE by incubation with CaCl_2 and were cross-linked for 1 h. The small size of EDC might allow it access to the microcavity postulated to house the Mn^{2+} site [21], accounting for the slight decrease in oxygen evolution observed with the 1% EDC-treated sample.

Enami et al. [10] have previously reported that treatment with 1% EDC produced an 80 kDa (OEE 33 kDa and CP 47) cross-linked product and an LHC-containing complex of approx. 55 kDa. Bricker and Frankel [11] reported main cross-linked products of 65 and 76 kDa, containing the OEE 33 and CP 47 proteins and small amounts of unknown OEE-antibody-reactive 26, 37 and 57 kDa products [11]. SDS/PAGE of cross-linked samples in this study suggested that cross-linking was more extensive than previously reported. Although a cross-linked product of 40 kDa was apparent, cross-linking was widespread across all polypeptides present.

It is apparent from the projection map of 1% EDC-treated samples that the level of structural differences cannot be attrib-

uted to changes in the Mn^{2+} site alone. It is conceivable that treatment with 1% EDC decreased PS II subunit mobility by extensive inter-polypeptide connections, and this in turn distorted the PS II functional unit. However, we feel it is more likely that treatment with 1% EDC caused interthylakoid cross-linking, which in turn made the electron microscope data ambiguous. Thylakoid cross-linking has been previously reported with PS II-enriched membranes [24], although the cross-linker used in that study was the 1.1 nm long dithiobis(succinimidylpropionate), whose mode of action is distinctly different from that of EDC. Diffraction patterns of 1% EDC-treated 2D crystals were noisy, suggesting an additional contribution from underlying non-ordered membranes. This would support the possibility of two membranes cross-linking together. The projection map of the 1% EDC-treated sample also bears some similarities to the stromal view of PS II [20], possibly because membranes have cross-linked via their luminal faces. The different stain accessibility and distribution would then yield a different projection of PS II.

The structural data provided by cross-linked PS II also provide further support for the monomeric organization of PS II *in vivo*. Previous arguments relating to a monomeric or dimeric form of PSII have previously been discussed in detail [25]. The structural argument for a PS II dimer was based mainly on the pseudo-2-fold symmetry apparent in low-resolution models of PS II [26–29]. However, if PS II was in fact a dimer, structural changes by cross-linking with EDC would be related by an axis of 2-fold rotational symmetry in projection maps. This is clearly not so in the 0.5% EDC-treated sample, and treatment with 1% EDC produces a highly asymmetric structural projection.

Most significantly, 0.25% EDC treatment has a beneficial effect on the 2D order of PS II. Although the number of 2D arrays present in the 0.25% sample is not increased, the order is better. It has been previously proposed that 2D crystal formation is produced by the gradual depletion of lipids from PS II membranes, causing an increase in favourable protein packing [27]. The quantitative analysis of 0.25% EDC and control 2D array data would also suggest that PS II stability is an important factor. Cross-linking is not restricted to the OEE proteins as previously reported [9] and involves many more polypeptide species. EDC is a water-soluble cross-linker and so must act at luminally and stromally exposed polypeptide surfaces. Because no inhibition of oxygen evolution is observed, 0.25% EDC treatment must confer some stability on the PS II unit, and this manifests itself in better ordered arrays.

We have identified three main types of disorder present in PS II 2D crystal: mosaic, short-range and long-range disorder [30]. The 0.25% EDC treatment decreased mosaic and short-range

disorder, but over longer ranges, 2D arrays still had a tendency to be distorted. This might be due to uneven adsorption on the support film. The increased disorder in the 0.5 % and 1 % EDC-treated samples, relative to the 0.25 % EDC cross-linked samples, might be attributed to subtle hetero-cross-linking. This would decrease the homogeneity of the PS II population and expose itself in a decrease in 2D order. This is consistent with the observation that most of the disorder apparent in the 0.5 % and 1 % EDC samples was mosaic. At atomic resolution, cross-linking might present problems with subunit interpretation, but cross-linking at low concentrations of EDC is beneficial to structural studies of PS II at low resolution.

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