Biochimica et Biophysica Acta, 1060 (1991) 89–96

ADONIS 000527289100225K

BBABIO 43479

Preparation and characterisation of Photosystem II core particles with and without bound bicarbonate

Simon J. Bowden, Beverly J. Hallahan, Stuart V. Ruffle, Michael C.W. Evans and Jonathan H.A. Nugent

Department of Biology (Darwin Building), University College London, London (U.K.)

(Received 12 December 1990)

Key words: Photosystem II; Bicarbonate; EPR; Reaction center; Iron-quinone complex

By studying the electron paramagnetic resonance (EPR) signals of Q_A^--$Fe^{2+}TBTQ^-$ and the oxidised non-haem iron we have found that detergent solubilisation of BBY Photosystem II (PS II) preparations using standard methods, involving either the detergents *n*-octyl β-D-glucopyranoside (OGP) or *n*-heptyl β-D-thioglucoside (HTG) at pH 6.0, results in loss of bicarbonate binding. New preparations including a dodecyl maltoside (DM) prepared CP47, CP43, D1, D2, cytochrome *b*-559 complex are described which at pH 7.5 retain native bicarbonate binding. These preparations provide a new system for studies on the 'bicarbonate effect' because bicarbonate depletion can now be achieved without displacement by another anion. They are also a more suitable starting material for the isolation of Q_A retaining D1/D2 reaction centres because the detrimental changes to the Q_A binding region are avoided.

Introduction

The Photosystem II (PS II) reaction centre components responsible for primary charge separation are now known to be bound to the polypeptides D1 and D2. This location was originally proposed when the amino acid sequences revealed the close relationship between D1 and D2 and the L and M reaction centre subunits of *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* [1]. The first experimental evidence was provided by Nanba and Satoh [2] who treated PS II membranes with the detergent Triton X-100 and obtained a reaction centre complex containing D1, D2 and cytochrome *b*-559 polypeptides. The reaction centre complex was able to photoaccumulate reduced pheophytin (Ph^-) and electron paramagnetic resonance (EPR) studies showed the presence of the spin-polarised reaction centre triplet upon illumination at cryogenic temperatures [2–4]. These results confirmed earlier assumptions about the structure of the PS II reaction centre but the electron acceptor immediately after pheophytin, the primary quinone Q_A, was found to be absent in the Nanba and Satoh preparation [2–4]. This has lead to work aimed at reintroducing quinones back to the deficient reaction centre complex [5].

The PS II membrane preparation used as the basis for further studies was developed by Berthold, Babcock and Yocum [6] using Triton X-100 to digest thylakoid membranes. The PS II complexes isolated using this procedure (BBYs) contain a large number of polypeptides which make up the reaction centre plus the polypeptides associated with the light harvesting complex. Detergents other than Triton X-100 have been utilised by several groups to develop new types of PS II preparation, originally for more detailed investigations into the mechanism of water oxidation. PS II core complexes which exhibit high rates of oxygen evolution were isolated. Ikeuchi et al. [7] and Ghanotakis and Yocum [8] used octyl glucopyranoside (OGP) to remove the light harvesting complex (LHCII), leaving a particle that retains manganese and the 33 kDa extrinsic polypeptide. The method of Enami et al. [9] using *n*-heptyl thioglucoside (HTG) produces a similar loss of light harvesting polypeptides but all three extrinsic polypeptides (17, 23 and 33 kDa) are retained. Further

Abbreviations: TBTQ, tribromotoluquinone; EPR, electron paramagnetic resonance; PS II, Photosystem II; LHCII, light harvesting complex two; DMBQ, 2,6-dimethylbenzoquinone; OGP, *n*-octyl β-D-glucopyranoside; HTG, *n*-heptyl β-D-thioglucoside; DM, *n*-dodecyl-β-D-maltoside; PEG, poly(ethylene glycol); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Correspondence: J.H. Nugent, Department of Biology (Darwin Building), University College London, Gower Street, London, WC1E 6BT, U.K.

advances have been made by Dekker et al. [10] who reported a method using dodecylmaltoside (DM) to digest OGP-prepared core particles to give a CP47, D1, D2, cytochrome *b*-559 complex and then a D1, D2, cytochrome *b*-559 reaction centre. Nanba and Satoh have recently isolated a reaction centre which consists of a D1,D2 heterodimer by digesting D1, D2, cytochrome *b*-559 complexes with OGP to remove the cytochrome and the *psbI* gene product [11].

Structural predictions of electron acceptor binding indicate that the Q_A binding site is created by the loop between transmembrane helices four and five of D2 and that the secondary quinone Q_B is bound to a similar site on D1 [12]. Q_A is a single electron carrier whilst Q_B is a two electron gate that receives electrons from Q_A before dissociating from PS II in the quinol form. Two other factors known to influence this region are the non-haem iron atom bound by ligands provided from both D1 and D2 and the bicarbonate ion which also provides at least one ligand to the iron [13–15]. The non-haem iron exerts a magnetic influence over Q_A and Q_B which can be detected by major changes in the characteristics of the EPR signal from the semiquinone. The variations in *g*-value and lineshape of this signal depend on the presence or absence of bicarbonate. With bicarbonate present the native EPR signal is observed at $g = 1.9$ [17]. When bicarbonate is displaced at low pH values or by another small anion such as formate, the $g = 1.8$ signal is seen [17,18]. The non-haem iron displays an EPR signal if oxidised to the ferric state, either by potassium ferricyanide [19] or exogenous semiquinones [13,20]. This signal has peaks at $g = 8$ and 5.6 and the signal shape has been shown to be sensitive to the presence of herbicides and quinone analogues binding at the D1 Q_B binding site [13] and to trypsin treatment [21], which cleaves both D1 and D2 in the quinone binding regions [22].

EPR studies by Petersen et al. on the Dekker D1, D2, cytochrome *b*-559, CP47 complex [23] found Q_A to be absent. However, a split $g = 6$ signal was observed that differed greatly from the native signal arising from the oxidised non-haem iron. This split $g = 6$ signal was suggested to arise from decoupling of the iron from the quinone and disruption of the non-haem iron ligands [23]. They also reported that a PS II complex retaining CP43 retained Q_A, although only the $g = 1.8$ form of the EPR semiquinone-iron signal could be observed. These results suggest that significant structural damage is occurring to the quinone binding region of PS II during the dodecyl maltoside digestion.

Our experiments have been aimed at conserving the native state of the quinone binding region of PS II in small 'core' complexes. We have used the detergent methods of Ghanotakis [24], Enami [9] and Dekker [10] to develop new preparations which achieve this aim. These preparations exhibit the native EPR signals from Q_A and the non-haem iron indicating that the electron acceptor side of PS II is relatively undamaged.

Materials and Methods

BBYs were prepared at pH 6.5 from market spinach (*Spinacea oleracea*) using the method of Ford and Evans [25]. To ensure that the starting material for polypeptide removal was as homogeneous as possible all PS II preparations used in these studies were checked using EPR to determine the state of the electron acceptor region of PS II. Only preparations having no g = 1.8 (bicarbonate absent) and 100% $g = 1.9$ (bicarbonate present) Q_A^--Fe^{2+} type EPR signals were used (see Results, Fig. 1).

OGP core PS II particles were prepared using a method based on that of Ghanotakis, Demetriou and Yocum [24]. BBYs were resuspended in 50 mM Hepes-NaOH (pH 7.5), 0.4 M sucrose, 10 mM NaCl, 50 mM $NaHCO_3$ (where used, bicarbonate and formate were always the sodium salts) to a chlorophyll concentration of 2.5 mg/ml Chl. To this was added an equal volume of 50 mM Hepes-NaOH (pH 7.5), 1.0 M sucrose, 0.8 M NaCl, 50 mM $NaHCO_3$, 75 mM octyl glucopyranoside. The digestion mixture was incubated for 12 min on ice, in the dark, then diluted with 2 vol. 50 mM Hepes-NaOH (pH 7.5), 1.0 M sucrose, 0.4 M NaCl, 50 mM $NaHCO_3$, followed by 30 min centrifugation at $40\,000 \times g$. The supernatant was dialysed for 60 min against a buffer containing 50 mM Hepes-NaOH (pH 7.5), 50 mM NaCl, 50 mM $NaHCO_3$, then centrifuged at $40\,000 \times g$ for 60 min. The resulting pellet was resuspended in 20 mM Hepes-NaOH (pH 7.5), 15 mM NaCl, 5 mM $MgCl_2$, 50 mM $NaHCO_3$, 20% (v/v) glycerol and stored at 77 K. OGP PS II core particles were also prepared at pH 6.0 using the same buffers as above but Mes was substituted for Hepes and $NaHCO_3$ was omitted.

HTG core PS II particles were prepared using a method based on that of Enami et al. [9]. BBYs were resuspended to 2 mg/ml Chl with 2% (w/v) *n*-heptyl thioglucoside, 1.0 M sucrose, 40 mM Hepes-NaOH (pH 7.5), 40 mM $MgCl_2$, 10 mM NaCl, 20 mM $NaHCO_3$. This mixture was incubated on ice, in the dark, for 12 min then combined with 1.5 vol. 0.5 M sucrose, 40 mM Hepes-NaOH (pH 7.5), 20 mM $NaHCO_3$. The suspension was centrifuged at $40\,000 \times g$ for 45 min and the resulting supernatant mixed with 2 vol. 40 mM Hepes-NaOH (pH 7.5), 20 mM $NaHCO_3$ followed by a second centrifugation at $40\,000 \times g$ for 60 min. The pellet was resuspended in 40 mM Hepes-NaOH (pH 7.5), 10 mM NaCl, 20 mM $NaHCO_3$, 1 mM EDTA and centrifuged again for 10 min at $40\,000 \times g$. The final pellet was resuspended in 40 mM Hepes-NaOH (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 20 mM $NaHCO_3$, 20% (v/v) glycerol and stored at 77 K. HTG

PS II core particles were also prepared at pH 6.0 using Mes instead of Hepes and no $NaHCO_3$.

The methodology employed for dodecyl maltoside digestion of OGP PS II core particles was similar to that developed by Dekker et al. [10]. The OGP-prepared particles were resuspended in 0.5% (w/v) dodecylmaltoside, 20 mM Hepes-NaOH (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$, 1.5% (w/v) taurine, 50 mM $NaHCO_3$, to 0.5 mg/ml Chl then incubated for 60 min before being mixed with 2 vol. of the above buffer minus detergent. The suspension was passed through a fast flowing S-Sepharose ion-exchange column and then loaded onto a Q-Sepharose column. The S-Sepharose step is unnecessary if the starting material used is pH 7.5 prepared OGP PS II because these particles already lack the 22 and 10 kDa subunits removed by this step [10]. The Q-Sepharose column was washed extensively with 20 mM Hepes-NaOH (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$, 1.5% taurine, 50 mM $NaHCO_3$, 0.03% DM and 30 mM $MgSO_4$ until the eluate was colourless. The CP47, CP43, D1, D2, cytochrome *b*-559 (DM PS II) complexes were eluted using the previous buffer plus 100 mM $MgSO_4$. The detergent digestion and column steps were carried out in the dark at either 24 or 4 °C (see Results). To quickly obtain concentrated samples for EPR analysis we used a poly(ethylene glycol) (PEG) precipitation method modified from McTavish et al. [26]. 0.325 g PEG 3350 per ml was added to the PS II particles and the mixture stirred continuously for 30 min. The suspension was then centrifuged for 20 min at $40\,000 \times g$ and the pellet resuspended in 20 mM Hepes-NaOH (pH 7.5), 20 mM NaCl, 1.5% taurine, 50 mM $NaHCO_3$, 0.03% DM and stored at 77 K.

The polypeptide composition of the PS II complexes under investigation was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide gels containing 6 M urea according to the method of Chua [27]. The molecular weight markers were only used as a rough guide to the identity of protein bands. Assignments were also based on experience with and reference to the position of bands from samples of purified D1/D2/cytochrome *b*-559 reaction centres, purified extrinsic and chlorophyll binding polypeptides, where polypeptides were identified by antibody labelling.

Measurements of oxygen evolving capacity were made using a Clark type oxygen electrode in 20 mM Mes (pH 6.3), 5 mM $MgCl_2$, 15 mM NaCl and 10 mM $CaCl_2$ with 500 μM DMBQ as the artificial electron acceptor.

For studies on the pH sensitivity of the EPR signals, the OGP or HTG PS II complexes were first centrifuged at $40\,000 \times g$ then resuspended at the new pH and dark adapted for 60 min before freezing. If two buffer changes were involved the sample was resuspended in the first and incubated for 30 min before a second centrifugation and subsequent resuspension as above. Duplicate epr samples were dark adapted for 60 min before freezing to 77 K in the dark. Illumination was carried out either at 77 K in a silvered dewar or at 200 K in an unsilvered dewar using a 650 W light source for 10 min (for EPR conditions and sample concentrations see figure legends).

To observe the oxidised non-haem iron, samples were resuspended in a Hepes-NaOH (pH 7.5) buffer and treated with 5 mM potassium ferricyanide before being dark adapted for 45 min, on ice, then frozen to 77 K. Samples treated with 500 μM TBTQ were illuminated at room temperature in an unsilvered dewar for 30 s then dark adapted for 30 min before freezing. EPR spectrometry was performed at cryogenic temperatures using a Jeol RE1X spectrometer with 100 kHz field modulation and an Oxford Instruments liquid helium cryostat.

Results

Fig. 1 shows the SDS-PAGE analysis of the PS II preparations studied in this paper. The polypeptides indicated are the three chlorophyll proteins CP47, CP43 and CP29, the reaction centre subunits D1 and D2, the three extrinsic subunits of 33, 23 and 17 kDa, the 22 and the 10 kDa subunits and the 9 kDa cytochrome *b*-559 component. The polypeptide composition of each preparation is summarised in Table I along with the '*g*' values of the EPR signals observed in each case. Digestion with HTG or OGP at either pH 6.0 or 7.5 removes the LHCII polypeptides but the raised pH causes the loss of further subunits. OGP PS II prepared at pH 6.0

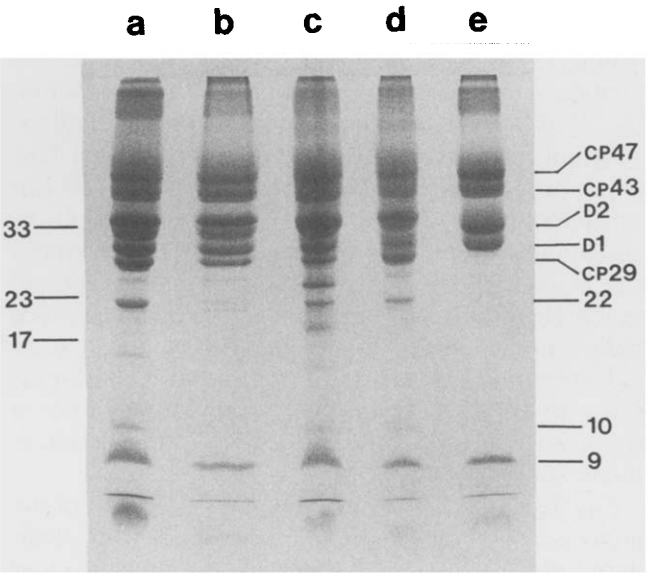

Fig. 1. SDS-PAGE analysis of (a) pH 6.0 OGP PS II, (b) pH 7.5 OGP PS II, (c) pH 6.0 HTG PS II, (d) pH 7.5 HTG PS II and (e) pH 7.5 DM PS II. Molecular mass in kDa.

TABLE I

A summary of the polypeptide composition of each PS II preparation and the g values of the EPR signals observed

Details of each preparation are given in Materials and Methods. ex, extrinsic polypeptide; CP, chlorophyll protein; ( ), partial removal; polypeptide sizes in kDa.

PS II preparation	Polypeptides in addition to the D1, D2, CP47, CP43, cyt. *b*559 core	EPR signal *g* values	
		Q_A^--Fe^{2+}	Fe^{3+}
BBY	CP29, 22, 10, LHCII, ex33, ex23, ex17	1.9	8.0 5.6
pH 6.0 OGP	CP29, 22, 10, ex33	1.8	6.2 5.75
pH 7.5 OGP	CP29, ex33	1.9	7.7 5.7
pH 6.0 HTG	CP29, 22, 10, ex33, ex23, ex17	1.8	– –
pH 7.5 HTG	CP29, 22, 10, ex33, (ex23)	1.9	7.8 5.7
pH 7.5 DM	core only	1.9	7.6 5.7 split $g = 6$

contains the 10 and the 22 kDa subunits (Fig. 1a) [24] but these are lost when the method is carried out at pH 7.5 (Fig. 1b). The major differences between the two different pH HTG PS II complexes are related to the extrinsic subunits. At pH 6.0 all three extrinsic polypeptides are retained (Fig. 1c) [9] but preparation at pH 7.5 causes the removal of the 17 and most of the 23 kDa extrinsic subunits (Fig. 1d).

Q_A and Q_B

Fig. 2 shows spectra from BBYs to demonstrate the quinone signals obtained in this type of preparation. Fig. 2a is a 77 K illuminated minus dark difference spectrum and has a peak near $g = 1.6$, a signal now known to arise from an interaction between the two semiquinones, Q_A^--$Fe^{2+}Q_B^-$ [28]. This shows that Q_B is still bound to some reaction centres in these samples. Fig. 2a also contains the $g = 1.9$ signal but this is more clearly seen in Fig. 2b. In Fig. 2b the sample was frozen under illumination to reduce Q_B to the quinol form and stored for 7 days while Q_A^- decayed by backreaction with D^+ [29]. The difference spectrum shown is the $g = 1.9$ Q_A^--Fe^{2+} signal formed on subsequent illumination at 77 K.

Fig. 3a is the 77 K illuminated EPR spectrum of the quinone region in oxygen evolving PS II core complexes prepared at pH 6.0 using the detergent OGP (OGP PS II). The EPR signal from Q_A^--Fe^{2+} is the $g = 1.8$ form, associated with the removal of bicarbonate from PS II [17]. If 50 mM formate is added to the

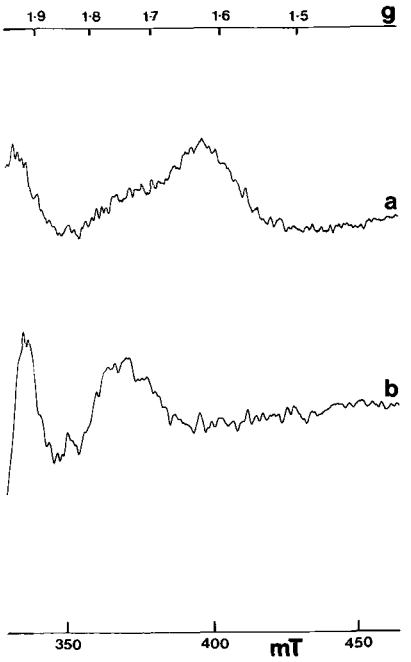

Fig. 2. EPR spectra of the iron-semiquinones in BBY PS II samples. (a) a 77 K illuminated minus dark difference spectrum and (b) a 77 K illuminated minus dark difference spectrum from a sample frozen under illumination and stored at 77 K for 7 days to allow Q_A^- decay. Sample concentration, 8 mg/ml Chl. EPR conditions: temperature, 5 K; microwave power, 10 mW; modulation width, 1.25 mT. The spectra shown are the averages of three scans.

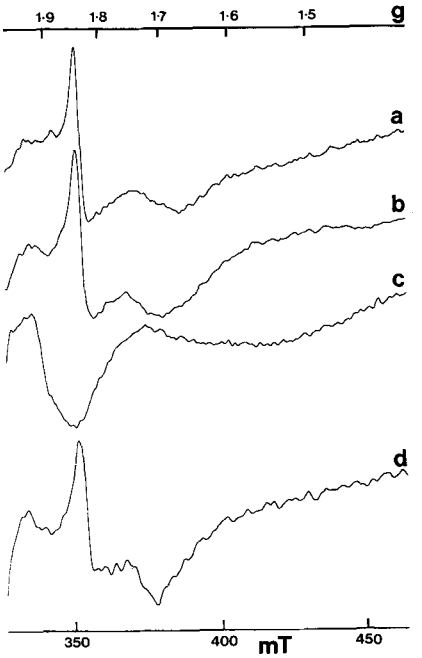

Fig. 3. EPR spectra of the Q_A^--Fe^{2+} signals in OGP PS II. (a) 77 K illuminated pH 6.0 OGP PS II; (b) as (a) +50 mM formate; (c) 77 K illuminated pH 7.5 OGP PS II+50 mM bicarbonate; and (d) as (c) but bicarbonate is replaced with 50 mM formate. Sample concentration, 2 mg/ml Chl. EPR conditions as Fig. 2 except (d) where instrument gain is halved.

pH 6.0 OGP PS II sample (Fig. 3b) the $g = 1.84$ peak is broadened slightly and the trough near $g = 1.7$ is narrowed. Formate addition does not increase the amplitude of the signal. The detergent treatment at pH 6.0 clearly alters PS II so that bicarbonate is removed. HTG PS II prepared at pH 6.0 showed identical EPR characteristics.

When OGP or HTG PS II prepared at pH 6.0 is resuspended in a pH 7.5 buffer containing 50 mM bicarbonate, it is possible to restore the $g = 1.9$ signal in some, but not all centres (data not shown). However if the preparation of OGP PS II is carried out entirely at pH 7.5 with 50 mM bicarbonate present then the Q_A^--Fe^{2+} $g = 1.9$ signal is retained (Fig. 3c) and no $g = 1.8$ signal is observed. If bicarbonate is replaced with formate at pH 7.5 then the conversion to a $g = 1.8$ Q_A^--Fe^{2+} occurs (Fig. 3d). The signal in Fig. 3d has a broader $g = 1.84$ peak and a larger amplitude than those in Fig. 3a, b.

PS II core particles prepared using HTG behave in an identical manner, only retaining the native Q_A EPR signal if the preparation is performed at pH 7.5 with 50 mM bicarbonate present. The pH sensitivity and reversibility of bicarbonate binding are demonstrated in Fig. 4. If pH 7.5 HTG PS II are placed in a pH 6.0 buffer with no bicarbonate present, then the EPR

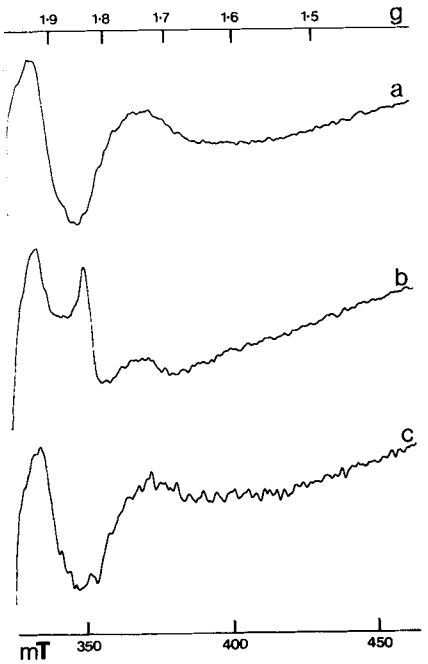

Fig. 4. The pH reversibility of the Q_A^--Fe^{2+} EPR signal from pH 7.5 prepared HTG PS II. (a) 77 K illuminated spectrum of pH 7.5 HTG PS II in pH 7.5 + 20 mM bicarbonate buffer; (b) 77 K illuminated spectrum of pH 7.5 HTG PS II resuspended in pH 6.0 buffer, no bicarbonate; and (c) 77 K illuminated spectrum from pH 7.5 HTG PS II incubated for 30 mins in pH 6.0 minus bicarbonate buffer, then resuspended in pH 7.5 + 20 mM bicarbonate buffer. Amplitude of spectra were adjusted to a sample concentration of 2 mg/ml Chl for comparison. EPR conditions as Fig. 2.

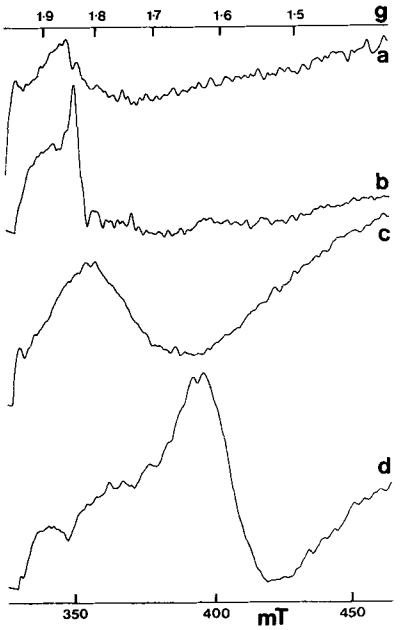

Fig. 5. The effect of 500 μM TBTQ addition on the iron-semiquinone EPR signals from OGP PS II. (a) Dark spectrum of TBTQ treated pH 6.0 OGP PS II, (b) 77 K illuminated spectrum of TBTQ treated pH 6.0 OGP PS II, (c) and (d) are as (a) and (b) respectively but using pH 7.5 OGP PS II. Sample concentration, 2 mg/ml Chl. EPR conditions as Fig. 2.

spectrum is altered from the $g = 1.9$ signal (Fig. 4a) to one containing both $g = 1.9$ and 1.8 components (Fig. 4b). This conversion is completely reversible for at least 30 min if the sample is then returned to the pH 7.5 buffer with bicarbonate present (Fig. 4c).

Further evidence that the EPR behaviour exhibited by the pH 7.5 bicarbonate HTG and OGP PS II is indicative of a more native electron acceptor region was obtained by using the Q_B analogue TBTQ. This compound is thought to bind to the Q_B binding site as a stable semiquinone [28,30]. Fig. 5a is the dark spectrum of a pH 6.0 OGP PS II sample with 500 μM TBTQ added. A broad signal with a peak near $g = 1.85$ is observed. The 77 K illuminated (Fig. 5b) spectrum shows a characteristic $g = 1.8$ $Q_A^-Fe^{2+}$ signal. No significant change in the amplitude of the $g = 1.8$ signal compared to the untreated pH 6.0 sample (Fig. 3a) is observed but the size of the $g = 1.7$ trough is decreased. The $g = 1.6$ signal attributable to an interaction between Q_A and Q_B semiquinones is absent. Previous studies on the $g = 1.6$ signal have also described the inability to observe a signal in formate treated samples of PS II membranes [15,31]. Fig. 5c is the dark spectrum from TBTQ treated pH 7.5 OGP PS II. The Q_B analogue induces a broad signal around $g = 1.7$ attributed to TBTQ-Fe^{2+} [28,32]. When Q_A^- is formed by 77 K illumination, both Q_A^- and $TBTQ^-$ are present and a large signal is generated near $g = 1.6$ (Fig. 5d) from the interaction betweeen the two iron-semiquinones. HTG PS II prepared at pH 7.5 behaves in an

identical manner, so HTG and OGP pH 7.5 preparations have similar quinone binding properties to BBYs.

Photoreduction of the pheophytin at 200 K in the presence of chemically reduced Q_A^--Fe^{2+} produces an EPR signal at $g = 2$ termed the 'split pheophytin signal' [16]. The signal obtained in pH 7.5 OGP PS II is similar to that observed by Vermaas and Rutherford in BBY samples [17]. They found that in formate inhibited BBYs the splitting of the signal decreases and we have found that this is also the case in pH 6.0 OGP PS II (data not shown). This change is a further consequence of bicarbonate removal [17].

The next step used in Refs. 10 and 24 for the removal of polypeptides from OGP PS II involves digestion by the detergent dodecyl maltoside (DM). We have prepared DM PS II core complexes using pH 7.5 buffer containing 50 mM bicarbonate at 4 °C and room temperature. Under these conditions a complex containing CP47, CP43, D1, D2, cytochrome *b*-559 is obtained (see Fig. 1e and Table I). Our analysis of this complex has shown that it is possible to induce EPR signal II (D^+) if the samples are frozen under illumination (data not shown). This photooxidation of D^+ does not require the addition of ferricyanide, which was required to obtain D^+ in the CP47, D1, D2, cytochrome *b*-559 complex of Petersen et al. [23]. A more significant difference is that illumination at 200 K generates the $g = 1.9$ Q_A^--Fe^{2+} bicarbonate signal (Fig. 6a). A small amount of $g = 1.8$ signal is present in DM PS II but otherwise the indication is that native Q_A and bicarbonate binding are preserved. TBTQ was also added and Fig. 6b is the 200 K illuminated minus dark difference spectrum showing the large $g = 1.6$ signal of Q_A^--Fe^{2+}-Q_B^- which demonstrates that the Q_B site is also intact.

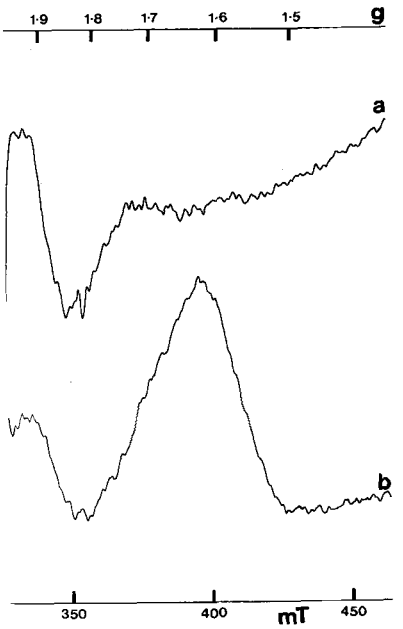

Fig. 6. The iron-semiquinone EPR signals observed in pH 7.5 DM PS II. (a) 200 K illuminated spectrum from untreated pH 7.5 DM PS II and (b) 200 K illuminated minus dark difference spectrum from 500 μM TBTQ treated pH 7.5 DM PS II. Sample concentration, 1.25 mg/ml Chl. EPR conditions as Fig. 2.

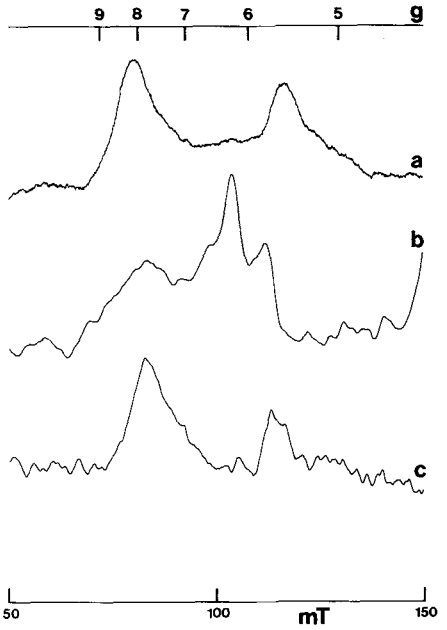

Fig. 7. Ferricyanide oxidised non-haem iron EPR difference spectra. (a) The dark minus 77 K illuminated difference spectrum from BBYs treated with 5 mM potassium ferricyanide; (b) as (a) but pH 6.0 OGP PS II; and (c) as (a) but pH 7.5 OGP PS II. Sample concentration, 2 mg/ml Chl. EPR conditions as Fig. 2.

The non-haem iron

The Q_A EPR signals obtained from OGP and HTG PS II show that preparation at pH 6.0 removes bicarbonate from the reaction centre without the requirement for a replacement anion such as formate. Several authors [1,13,14,33] have proposed that bicarbonate provides one or two ligands to the non-haem iron. Fig. 7a is the Fe^{3+} dark minus 77 K illuminated difference spectrum recorded from BBYs oxidised with ferricyanide and shows the characteristic peaks at $g = 8.0$ and 5.6. The dark minus 77 K illuminated difference spectrum from pH 6.0 OGP PS II oxidised with ferricyanide (Fig. 7b) has an altered spectrum with two peaks at $g = 6.2$ and 5.75. The abnormal peaks are obscuring a smaller signal with a $g = 8$ peak that is probably due to the minority of reaction centres that still have bicarbonate bound. Fig. 7c is the dark minus 77 K illuminated difference spectrum from ferricyanide oxidised pH 7.5 OGP PS II and is almost identical to the normal spectrum from PS II membranes seen in Fig. 7a. A small '*g*' value change in the positioning of the peaks to $g = 7.7$ and 5.7 occurs.

Investigation of the oxidised non-haem iron in DM PS II complexes (Fig. 8) revealed two different types of

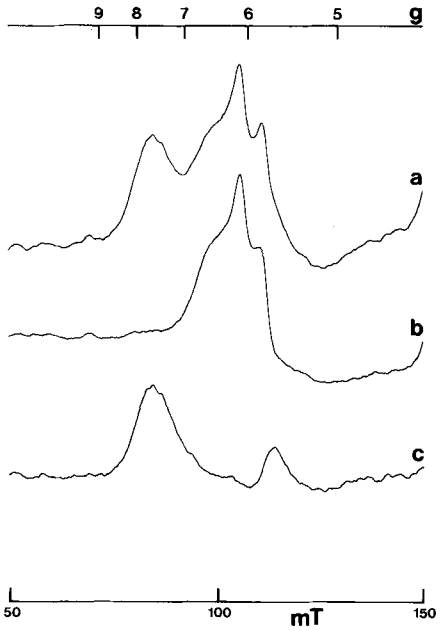

Fig. 8. The EPR signals observed in the $g = 5$–8 region in pH 7.5 DM PS II treated with 5 mM potassium ferricyanide. (a) dark; (b) after 200 K illumination; and (c) the dark minus 200 K illumination difference spectrum. Sample concentration, 1.25 mg/ml. EPR conditions as Fig. 2.

spectrum. In the absence of ferricyanide a prominent signal with two peaks at $g = 6.15$ and 5.85 is present in the dark that is unchanged by 200 K illumination (see central peaks of Fig. 8b). This is a similar signal to that observed by Peterson et al. [23] in the CP47, D1, D2, cytochrome *b*-559 complex. Following incubation of DM PS II with ferricyanide, an additional signal at $g = 7.6$ (Fig. 8a) is observed which is completely removed by 200 K illumination (Fig. 8b). The dark minus light difference spectrum (Fig. 8c) has two peaks from oxidised non-haem but the '*g*' values are altered so that the peaks are closer together at $g = 7.6$ and 5.7.

Discussion

Bicarbonate is a major component of the electron acceptor complex of PS II. It is required for normal electron transfer and protonation and possibly provides a ligand or ligands to the non-haem iron [13,14,28,32–34]. The effects of bicarbonate have been studied in preparations depleted of bicarbonate by formate treatment. We have shown that the detergent removal, at pH 6.0, of the light harvesting complex from PS II membrane preparations causes the loss of bicarbonate. Complete rebinding of bicarbonate, measured by conversion of the $g = 1.8$ Q_A^--Fe^{2+} EPR signal in the pH 6.0 OGP or HTG PS II particles to the $g = 1.9$ signal, have been unsuccessful. This behaviour shows that there are irreversible changes to the quinone binding region which occur after bicarbonate removal and which block bicarbonate readdition.

To maintain an intact electron acceptor region we have found it necessary to raise the pH and include bicarbonate at every stage in the preparation. These pH 7.5 particles display a $g = 1.9$ Q_A^--Fe^{2+} EPR signal that can be transformed reversibly into the $g = 1.8$ form of the signal using either a lower pH or formate replacement of bicarbonate [17]. The bicarbonate depleted samples show different characteristics to preparations where bicarbonate is displaced by formate. For example the non-haem iron in pH 6.0 OGP PS II can be oxidised by potassium ferricyanide although the EPR signal obtained is altered, reflecting bicarbonate loss. This contrasts with the observed behaviour if formate is added to PS II membranes to remove bicarbonate where non-haem iron oxidation by ferricyanide is inhibited, due to the formate induced E_m increase of the Fe^{2+}/Fe^{3+} couple [13,20,28].

The changes in the quinone regions and the properties of the non-haem iron explain the oxygen evolving characteristics of pH 6.0 OGP PS II obtained by Ghanotakis [8,24] and Ikeuchi et al. [7]. They found that the rates of oxygen evolution with potassium ferricyanide remained high compared with PS II membranes, because ferricyanide can oxidise the non-haem iron, but rates using the Q_B analogue DCBQ were comparatively low and sensitivity to DCMU was reduced. This is indicative of damage occurring at the Q_B binding site which we have shown to occur. Detergent isolation at pH 6.0 results in preparations that retain the highest rates of oxygen evolution. Therefore, although OGP or HTG PS II prepared at pH 6.0 is optimised for study of water oxidation, modifications are required to retain native quinone and bicarbonate binding.

We have obtained a CP47, CP43, D1, D2, cytochrome *b*-559 complex from dodecylmaltoside digested (pH 7.5) OGP PS II that exhibits the $g = 1.9$ Q_A^--Fe^{2+} signal and can be oxidised with ferricyanide to produce an Fe^{3+} EPR spectrum similar to that seen in BBYs (Fig. 7a). The iron spectrum also contains the split $g = 6$ component identified by Petersen et al. [23] in the Dekker [10] CP47, D1, D2, cytochrome *b*-559 complex. They have assigned this signal to non-haem iron uncoupled from the quinone(s) and were unable to obtain any ferricyanide induced oxidation of the iron in their preparation. The split $g = 6$ signal is observed in the dark without ferricyanide treatment and cannot be photoreduced by 200 K illumination. This means that in order for this to be the oxidised non-haem iron, the E_m of the Fe^{2+}/Fe^{3+} couple would have to be lower than that of Q_A, which is approx. +25 mV at pH 7.5 [28]. The E_{m7} of the non-haem iron Fe^{2+}/Fe^{3+} couple in BBYs is +400 mV [13]. Treatments which effect the non-haem iron ligands, such as formate addition, normally raise the E_m. Therefore, the split

$g = 6$ signal may not be due to the non-haem iron so an alternative explanation is that the signal originates from damaged cytochrome *b*-559 in which the iron atom has become high spin. However, if the origin of this signal is the non-haem iron then our preparation contains two populations of reaction centres which would show that the integrity of the acceptor side is damaged by DM digestion. Further experiments are necessary to quantitate the yields of the Fe^{3+} EPR signals in this preparation and to determine the source of the $g = 6$ signal.

Quantitative analysis of the quinone content in the Dekker CP47, D1, D2, cytochrome *b*-559 complex [23] revealed that removal of the CP43 subunit occurs in parallel with quinone loss from the reaction centre. It is proposed that the two events are related by the temperature dependence observed for both CP43 removal [10] and quinone loss [35]. Our results suggest that disruption of the non-haem iron ligands occurs as early as OGP digestion of BBYs at pH 6.0 so that quinone binding is destabilised even before DM is used in further purification steps. Therefore the preparations are predisposed towards Q_A loss from the reaction centre before CP43 is removed.

Acknowledgements

The authors would like to thank the U.K. Science and Engineering Research Council for financial support and Prof. W. Oettmeier for providing TBTQ.

References

1 Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
2 Nanba, O. and Satoh. K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
3 Okamura, M.Y., Satoh, K., Isaacson, R.A. Feher, G. (1987) In Progress in Photosynthesis Research (Biggins, J., ed.), Vol. pp. 1, pp. 379–381, Martinus Nijhoff, Dordrecht.
4 Nugent, J.H.A., Telfer, A., Demetriou, C. and Barber, J. (1989) FEBS Lett. 255, 53–58.
5 Chapman, D.J., Gounaris, K. and Barber, J. (1988) Biochim. Biophys. Acta 933, 423–431.
6 Berthold, D.A., Babcock, G.T. and Yocum C.F. (1981) FEBS Lett. 134, 231–234.
7 Ikeuchi, M., Yuasa, M. and Inoue, Y. (1985) FEBS Lett. 185, 316–322.
8 Ghanotakis, D.F. and Yocum, C.F. (1986) FEBS Lett. 197, 244–248.
9 Enami, I., Kamino, K., Shen, J.R., Satoh, K. and Katoh, S. (1989) Biochim. Biophys. Acta 977, 33–39.
10 Dekker, J.P., Bowlby, N.R. and Yocum, C.F. (1989) FEBS Lett. 254, 150–154.
11 Tang, X.S., Fushimi, K. and Satoh, K. (1990) FEBS Lett. 273, 257–260.
12 Trebst, A. (1987) Z. Naturforsch 42c, 742–750.
13 Diner, B.A. and Petrouleas, V. (1987) Biochim. Biophys. Acta 895, 107–125.
14 Diner, B.A. and Petrouleas, V. (1990) Biochim. Biophys. Acta 1015, 141–149.
15 Nugent, J.H.A., Corrie, A.R., Demetriou, C., Evans, M.C.W. and Lockett, C.J. (1988) FEBS Lett. 235, 71–75.
16 Klimov, V.V., Dolan, E. and Ke, B. (1980) FEBS Lett. 112, 97–100.
17 Vermaas, W.F.J. and Rutherford, A.W. (1984) FEBS Lett. 175, 243–248.
18 Nugent, J.H.A., Diner, B.A. and Evans M.C.W. (1981) FEBS Lett. 124, 241–244.
19 Itoh, S., Tang, X.S. and Satoh, K. (1986) FEBS Lett. 205, 275–281.
20 Zimmerman, J.-L. and Rutherford, A.W. (1986) Biochim. Biophys. Acta 851, 416–423.
21 Bowden, S.J., Demetriou, C., Lockett, C.J. and Nugent, J.H.A. (1989) In Current Resesarch in Photosynthesis (Baltscheffsky, M., ed.), Vol. 1, pp. 519–522, Kluwer, Dordrecht.
22 Trebst, A., Depka, B., Kraft, B. and Johanningmeier, U. (1988) Photosynthesis Res. 18, 163–177.
23 Petersen, J., Dekker, J.P., Bowlby, N.R., Ghanotakis, D.F., Yocum, C.F. and Babcock, G.T. (1990) Biochemistry 29, 3226–3231.
24 Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
25 Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160, 159–163.
26 McTavish, H., Picorel, R. and Seibert, M. (1989) Plant Physiol. 89, 452–456.
27 Chua, N.M. (1980) Methods Enzymol., 69, 434–446.
28 Corrie, A.R., Nugent, J.H.A. and Evans, M.C.W. (1991) Biochim. Biophys. Acta 1057, 384–390.
29 Nugent, J.H.A., Demetriou, C. and Lockett, C.J. (1987) Biochim. Biophys. Acta 894, 534–542.
30 Renger, G., Messinger, J. and Fromme, R. (1989) Z. Naturforsch. 44, 423–430.
31 McDermott, A.E., Yachandra, V.K., Guiles, R.D., Cole, J.L., Dexheimer, S.L., Britt, R.D., Sauer, K. and Klein, M.P. (1988) Biochemistry 27, 4021–4031.
32 Hallahan, B.J., Ruffle, S.V., Bowden, S.J. and Nugent, J.H.A. (1991) Biochim. Biophys. Acta, in press.
33 Van Rensen, J.J.S., Tonk, W.J.R. and De Bruijn, S.M. (1988) FEBS Lett. 226, 347–351.
34 Govindjee and Blubaugh D.J. (1988) Photosynthesis Res. 19, 85–128.
35 Diner, B.A., de Vitry, C. and Popot, J. (1988) Biochim. Biophys. Acta 934, 47–54.