A high redox potential form of cytochrome c550 in Photosystem II from Thermosynechococcus elongatus

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Cytochrome c550 (cyt c550) is a component of Photosystem II (PSII) from cyanobacteria, red algae and some other eukaryotic algae. Its physiological role remains unclear. In the present work, measurements of the midpoint redox potential (Em) were performed using intact PSII core complexes preparations from a histidine-tagged PSII mutant strain of the thermophilic cyanobacterium Thermosynechococcus elongatus. When redox titrations were done in the absence of redox mediators, an Em value of +200 mV was obtained for cyt c550. This value is about 300 mV more positive than that previously measured in the presence of mediators (Em = -80 mV). The shift from the high-potential form (Em = +200 mV) to the low-potential form (Em = -80 mV) of cyt c550 is attributed to conformational changes, triggered by the reduction of a component of PSII which is sequestered and out of equilibrium with the medium: most likely the Mn4Ca cluster. This reduction can occur when reduced low potential redox mediators are present or under highly reducing conditions even in the absence of mediators. Based on these observations, it is suggested that the Em of +200 mV obtained without mediators could be the physiological redox potential of the cyt c550 in PSII. This value opens the possibility of a redox function for cyt c550 in PSII.

In all photosynthetic oxygen-evolving organisms, the primary steps of light conversion take place in a large pigment-protein complex named PSII, which drives light-induced electron transfer from water to plastoquinone with the concomitant production of molecular oxygen (for review see Ref. 1). The reaction center (RC) of PSII is made up of two membrane-spanning polypeptides, D1 and D2, which bind four chlorophylls, two pheophytins, two quinones, Qa and Qb, a non-heme iron atom and a cluster made up of four manganese ions and one calcium ion. In green algae and higher plants, three extrinsic proteins are associated to RC in water-splitting active PSII complexes: 23 kDa, 16 kDa and 33 kDa proteins, while in cyanobacteria, red algae and some other eukaryotic algae, cyt c550, 12 kDa and 33 kDa proteins are found. The 3-D structure of PSII confirmed that cyt c550 binds on the lumenal membrane surface in the vicinity of the D1 and CP43 (2-6).

Cyt c550, encoded by the psbV gene, is a monoheme protein with a molecular mass of ≈15 kDa and an isoelectric point between 3.8 and 5.0 (7, 8). The recent resolution of the 3-D structure of the soluble form of cyt c550 from three cyanobacteria, Synechocystis PCC 6803 (9), Arthrospira maxima (10) and Thermosynechococcus (T.) elongatus (11) has confirmed a previously proposed bis-histidine coordinated heme that is very unusual for monoheme c-type cytochromes (8, 11, 12). Crystal structures of both isolated and PSII-bound forms of cyt c550 show that the protein presents a hydrophobic inner core typical of monoheme cytochromes c, with three helices forming a nest for the prosthetic group and a fourth helical segment in the N-terminal domain protecting the heme from solvent, indicating that the heme structure is not very different from most c-type cytochromes (13).

The exact physiological role of cyt c550 is
unclear. Extensive research has established that it does not participate in the main photosynthetic reactions despite its close proximity (22 Å) to the water oxidation complex. Cyt c550 is thus suggested to play the same role as the other (albeit cofactor-less) extrinsic proteins. By stabilising the neighbouring proteins and protecting the Mn cluster from external reductants, it stabilizes the oxygen-evolving complex (14, 15). Studies of phenotype of the cyt c550-less mutant (ΔPsbV) of *Synechocystis* PCC 6803 have shown that both the cyt c550 and the 12 kDa protein stabilize the binding of the Ca\(^{2+}\) and Cl\(^{-}\) ions which are essential for the oxygen-evolving activity of PSII, in a manner analogous to the extrinsic 17 and 24 kDa polypeptides of higher plants (14, 16, 17). The fact that cyt c550 can be isolated as a soluble protein (7, 8, 18-20) suggests that other functions not directly related to PSII are possible for this protein. Several non-photosynthetic roles have been suggested for cyt c550. In fact, a function related to anaerobic disposal of electrons from carbohydrates reserves, or fermentation to sustain an organism during prolonged dark and anaerobic conditions have been proposed (19, 21, 22).

According to Shen and Inoue (23), cyt c550 can accept electron from ferredoxin II in the presence of sodium dithionite, and is proposed to remove excess electrons in anaerobically grown cells.

The midpoint redox potential (\(E_m\)) of cyt c550 is one of the key parameters for elucidating the biological role of this cytochrome. An \(E_m\) value at pH 7.0 (\(E_{m7}\)) of -260 mV was the first to be reported for purified cyt c550 from *Anacystis nidulans* (20). Cyt c550 from *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* were found to be reducible by sodium dithionite (\(E_{m7} = -420\) mV), but not by sodium ascorbate (\(E_{m7} = +58\) mV) (7). Later, \(E_{m7}\) values from -280 mV to -314 mV were obtained for purified cyt c550 from the same species (12) and an \(E_{m3}\) of -250 mV from *Synechocystis* sp. PCC 6803 (8). In previous work, we determined an \(E_{m6}\) value of -240 mV for the soluble form of cyt c550 from the thermophilic cyanobacterium *T. elongatus* after its extraction from PSII (24). Such low redox potentials are well below the range normally expected for a monoheme c-type cytochrome and seem incompatible with a redox function in PSII electron transfer. Using an electrochemical technique, a value 150 mV more positive (\(E_{m7} \approx -100\) mV) was measured for \(E_m\) of cyt c550 from *Synechocystis* sp. PCC 6803 adsorbed to an electrode surface (13). This higher value was attributed to the exclusion of water from the site due to the protein binding to the electrode (13). The \(E_m\) for cyt c550 associated with PSII was not established until our group was able to measure it using intact PSII core complexes preparations from *T. elongatus*. Using potentiometric redox titrations, a significantly higher \(E_m\) value was obtained for cyt c550 when bound to PSII (\(E_{m6} = -80\) mV) compared with its soluble form after its extraction from PSII (\(E_{m6} = -240\) mV) (24). Moreover, while the \(E_m\) of the bound form is pH-independent, the \(E_m\) of the soluble form varies from -50 mV at pH 4.5 to -350 mV at pH 9-10 (24). The difference of \(E_m\) between the isolated and the PSII-bound forms of cyt c550 has been confirmed by theoretical calculations based on crystal structures of the isolated and PSII-bound forms (25). Some authors (13, 24, 26) have proposed that in conditions more native than isolated PSII core complexes, it is possible that the \(E_m\) of cyt c550 may be even higher than -80 mV, and thus a redox function in the water oxidation complex could be conceivable. Therefore, the precise determination of the redox potential of this protein is of fundamental importance to the understanding its function.

One of the most standard techniques for determining redox potentials of proteins is the redox potentiometry. It involves measuring the ambient redox potential (\(E_a\)) while simultaneously determining the concentration of the oxidized and reduced forms of the protein using a spectroscopic technique. Meaningful results will be obtained only if chemical equilibrium is achieved between the various species in solution and electrochemical equilibrium is established at the electrode solution surface (27, 28). Unlike many small inorganic and organic redox couples, most redox proteins do not establish stable potentials because the heterogeneous charge-transfer (electrochemical) rates are low. A predominant reason for this is that the redox centre is often shielded by protein and so does not gain proper contact with the electrode surface. Redox mediators are required to act as go-betweens between the measuring electrode and the biological redox couple and thus to get rates of the electron transfer between electrode, mediator and biological component rapid enough to achieve a true equilibrium (i.e., one where all redox
complexes in the biological electron transfer system are at the same $E_m$) (29). Therefore, in most redox titrations of proteins, equilibrium is ensured by the addition of a cocktail of redox mediators which establishes rapid (heterogeneous) electrochemical equilibrium with the electrode and rapid (homogeneous) electron transfer with the protein without chemically modifying it in any way.

It has been reported that the $E_m$ of Q$_A$ in PSII-enriched membranes was affected by the presence of redox mediators at low ambient potentials. As consequence of this, a change in the redox potential from -80 mV (active form) to +65 mV (inactive form) has been determined in the potentiometric titrations performed on PSII membranes. This effect was attributed to the loss of the very high potential Mn$_4$Ca cluster due to reductive attack by the mediators and the sodium dithionite itself under some conditions (30). This was confirmed by the observation that the low potential, active form of the Q$_A$/Q$_A^-$ couple, could be regenerated when the Mn cluster was reconstituted (31). The binding and debinding of the Mn$_4$Ca cluster and even of the Ca$^{2+}$ ion is considered to be associated with conformational changes that are manifest far from the binding site itself (30, 31).

Based on these observations and taking account that in most of redox titrations of cyt c550 bound to PSII, equilibration was ensured by the addition of a cocktail of redox mediators (24, 26), it seemed possible that $E_m$ of cyt c550 when bound to PSII could suffer from this unexpected technical difficulty. The presence of these mediators could have led to the reduction of the Mn cluster, the consequent loss of the Ca$^{2+}$ and Mn$^{2+}$ ions and associated conformational changes in the protein. The $E_m$ value obtained for the $E_m$ of cyt c550 may not reflect the fully intact form of the PSII-bound cytochrome.

The main objective of this work has been to re-evaluate the redox potential of cyt c550 associated with PSII considering the effect of redox mediators. To check for the latter possibility, redox titration experiments were performed using highly active and intact core complexes preparations of PSII from T. elongatus testing the presence and absence of redox mediators and different redox mediators.

**EXPERIMENTAL PROCEDURES**

*Strain and standard culture conditions—* WT and His-tag CP43 mutant T. elongatus cells were grown in a DTN-medium (32). Cultures were carried out in 3 l flasks in a rotary shaker (120 rpm) at 45 °C under continuous illumination from fluorescent white lamps (100 μE m$^{-2}$ s$^{-1}$) and CO$_2$-enriched atmosphere. For maintenance, the His-tag CP43 mutant cells were grown in the presence of chloramphenicol (Cm) (5 μg ml$^{-1}$) at 45 °C under continuous illumination from fluorescent white lamps (40 μE m$^{-2}$ s$^{-1}$).

*Construction of the plasmid for His-tag CP43 T. elongatus mutant*—For constructing a plasmid for expression of His-tagged psbC in T. elongatus, the genome region containing the psbD1, psbC and tlr1632 genes was amplified by PCR. Genomic DNA of T. elongatus as template and the primers CP43a (5'-ATGACGATCGCCGAGAATGGACGA(3')) and CP43b (5'-GCAATCCAATGATGGACCTTGACGGGGAGAGAGG-3') were used. The amplified region was digested by KpnI and BamHI and it was cloned in a pBluescript KS+ plasmid also previously digested by the same restriction enzymes. Then, by site directed mutagenesis, the bases coding for six histidines were added in the 3' terminal of the psbC gene using the synthetic oligonucleotides CP43 Histag a (5'-CCTTCGTAGCAGGCCAGCCT-TGATCCACCATCATCATCATTTAGGTT-3') and CP43 Histag b (5'-TTAATGTTGACCTGATCAATGCTTTATGTT-GATGGTGATGGTGAAGATGGCGCATC-GAGGGGAG-3'). A Smal site was created between CP43 Histag a and b using the synthetic oligonucleotides CP43SmaIa (5'-ATGACGATCGCCGAGAATGGACGA(3')) and CP43SmaIb (5'-GCAATCCAATGATGGACCTTGACGGGGAGAGAGG-3'). A Cm resistance cassette was introduced in the Smal site. The Cm resistance cassette was obtained by amplification by PCR of a 1.1 kb fragment of the plasmid pBC SK+ Cm$^8$ using the synthetic oligonucleotides Cam1.1a (5'-GCTTCGTACGGGATAATAGCTAACTT-CGC-3') and Cam1.1b (5'-GCTCCAGGGGA-GAGGCCCTGAGCA-3'). Finally a Cm resistance cassette was introduced in the Smal site. The Cm resistance cassette was obtained by amplification by PCR of a 1.1 kb fragment of the plasmid pBC SK+ Cm$^8$ using the synthetic oligonucleotides Cam1.1a (5'-GCTTCGTACGGGATAATAGCTAACTT-CGC-3') and Cam1.1b (5'-GCTCCAGGGGA-GAGGCCCTGAGCA-3'). The construction obtained, pCH-Cm, was a plasmid of 7 kb. In order to increase the chances for T. elongatus transformation, this plasmid was digested by the restriction enzyme EcoRI deleting the psbD gene and the beginning of the psbC gene. The 5 kb
plasmid obtained, named pCH-5.1, was used to transform WT *T. elongatus* cells.

*Transformation of T. elongatus cells and genetic analysis of mutants* The pCH-5.1 plasmid containing the His-tagged *psbC* gene and the Cm resistance cassette was introduced into WT *T. elongatus* cells by electroporation according to (32) with slight modifications as described in (26), creating the His-tag CP43 strain (WT'). After electroporation, cells were rapidly transferred to 2 ml of DTN medium and incubated for 48 hours in a rotary shaker at 45 °C under low light conditions. Then, the cells in 0.1-0.2 ml aliquots were spread on agar plates containing Cm (2 µg ml⁻¹) and incubated at 45 °C, under dim light and humidified atmosphere. After 2-3 weeks transformants emerged as green colonies; then these colonies were spread at least twice on agar plates containing 5 µg ml⁻¹ Cm. Genomic DNA was isolated from *T. elongatus* cells essentially as described by Cai and Wolk (33). Total segregation of the mutation was checked by PCR amplification of the genome region containing the *psbC* gene and the gene for Cm resistance. The PCR gave a fragment of 3.6 kb containing the *psbC* and the Cm resistance gene, instead of a fragment of 2.5 kb observed in the WT therefore demonstrating the complete segregation of the mutant. Confirmation of the presence of the His-tag was done by sequencing of the amplified DNA fragment.

**PSII Core Complexes Preparation**—PSII core complexes were prepared from cells of *T. elongatus* as described by Kirilovsky *et al.* (26). The PSII core complexes preparations were resuspended in 40 mM MES, pH 6.5, 15 mM CaCl₂, 15 mM MgCl₂, 10% glycerol and 1 M glycinebetaine at about 2-3 mg of Chl ml⁻¹ and stored in liquid N₂. The preparations used in this work had an oxygen evolution activity of 2700-3200 µmol O₂ ng Chl⁻¹ h⁻¹.

**Redox Potential Measurements**—Potentiometric redox titrations were carried out basically as described in Roncel *et al.* (24). For titrations, samples contained PSII core complexes (30–50 µg Chl ml⁻¹) were suspended in 2.5-ml buffer containing 40 mM MES, pH 6.5. When indicated, a set of the following eight redox mediators was added: 10 µM *p*-benzoquinone (*Eₐₗ = +280 mV*), 20 µM 2,5-dimethyl-*p*-benzoquinone (*Eₐₗ = +180 mV*), 20 µM *o*-naphthoquinone (*Eₐₗ = +145 mV*), 2.5 µM *N*-methylenazinium methosulfate (*Eₐₗ = +190 mV*), 10 µM *N*-methylenazinium ethosulfate (*Eₐₗ = +80 mV*), 10 µM *N*-methylenazinium ethosulfate (*Eₐₗ = +55 mV*), 20 µM duroquinone (*Eₐₗ = +10 mV*) and 30 µM 2-methyl-*p*-naphthoquinone (*Eₐₗ = 0 mV*). Some redox titrations were carried out in the absence of these redox mediators or in the presence of DAD only. Experiments were done at 20 °C under argon atmosphere and continuous stirring.

Reductive titrations were performed by first oxidizing the samples to *Eₐₗ* = +450 mV with potassium ferricyanide and then reducing it stepwise with sodium dithionite. For oxidative titrations, the samples were first reduced to *Eₐₗ* ≈ -350 mV with sodium dithionite and then oxidized stepwise with potassium ferricyanide. In both cases, after the additions of potassium ferricyanide or sodium dithionite, the absorption spectrum between 500 and 600 nm and the redox potential of the solution were simultaneously recorded by using, respectively, an Aminco DW2000 UV-Vis spectrophotometer (USA) and a Metrohm Herisau potentiometer (Switzerland) provided with a combined Pt-Ag/AgCl microelectrode (Cirson Instruments, Spain). Differential spectra of cyt b559 and cyt c550 in PSII core complexes were obtained by subtracting the absolute spectra recorded at each *Eₐₗ* during titrations from the spectra of the fully oxidized state of each cytochrome (reductive titrations) or from the spectra of the fully reduced state of each cytochrome (oxidative titrations). The absorbance differences at 559-570 nm for cyt b559 and 549-558 nm for cyt c550 obtained from these spectra were normally converted into percentages of reduced cytochrome and plotted versus solution redox potentials. The *Eₐₗ* values were then determined by fitting the plots to the Nernst equation for one-electron carrier (*n*=1) with 1 or 2 components as needed, and using a non-linear curve-fitting program (Origin 6.0, Microcal Software).

**EPR measurements**—EPR spectra were recorded using a Bruker Elexys 500 X-band spectrometer equipped with a standard ER 4102 resonator and an Oxford Instruments ESR 900 cryostat. Instrument settings were: microwave frequency 9.4 GHz, modulation frequency 100 kHz. All other...
RESULTS

Effect of redox mediators—Initially potentiometric redox titrations of the isolated PSII core complexes preparations in the presence of a mixture of eight redox mediators (see Materials and Methods), covering the potential range between +430 and 0 mV, were performed. This mixture excluded five mediators with negative redox potential (anthraquinone-2-sulfonate, anthraquinone-2,6-disulfonate, anthraquinone-1,5-disulfonate, 2-hydroxy-p-naphthoquinone and anthraquinone) which were used in our previous work (24). Figure 1 shows a representative potentiometric titration of PSII core complexes from T. elongatus at pH 6.5 under these conditions. Differential absorption spectra in the α-band region of the cytochromes were obtained by subtracting the absolute spectrum recorded at +455 mV from those recorded during the course of the redox titration (Fig. 1A). This figure clearly shows that PSII core complexes contain two different components with absorption maxima in the α-band at 559 and 549 nm, which are progressively reduced during the course of titration. The component with an absorption maximum in the α-band at 559 nm that appeared between +455 mV and +210 mV can be assigned to cyt b559, whereas the component appearing between +210 mV and -295 mV can be assigned to cyt c550 as has been already described (24). Differential spectra in Fig. 1A reveals that both cytochromes can be sequentially titrated observing the change of the α-band of both cyt b559 and cyt c550. Consequently, it was first possible to determine the redox potential of cyt b559 by measuring the relative content of cyt b559 from the absorbance difference between 559 and 570 nm (Fig. 1A). A plot of the percentages of reduced cyt b559, obtained from these difference spectra, versus $E_m$ could be fitted to a Nernst equation for two $n=1$ components (Fig. 1C). It clearly indicated the existence of two different cyt b559 components with $E_m$ values of +389 mV (accounting for approximately 85% of the total amount of protein) corresponding to the high potential form (HP form) and +246 mV (approximately 15% of the total amount of protein) corresponding to the intermediated potential form (IP). These values are similar to those obtained in measurements on PSII core complexes of T. elongatus where reductive titrations were carried out in the presence of low-potential mediators (24). When most cyt b559 was reduced at $E_n$ of +210 mV, changes in the α-band of cyt c550 could be clearly observed and consequently its $E_m$ determined without any interference from cyt b559. Differential spectra of cyt c550 (Fig. 1B) were obtained by subtracting the spectrum recorded at +210 mV (cyt c550 almost fully oxidized and cyt b559 fully reduced) from each spectrum performed at different ambient redox potential (between +210 and -295 mV). The relative content of cyt c550 was calculated from the absorbance difference between 549 and 538 nm. Then, the percentages of reduced cyt c550 versus $E_n$ were plotted and an $E_m$ value of -20 mV was calculated by fitting the experimental points to the Nernst equation for one $n=1$ component (Fig. 1D). This $E_m$ value was significantly higher than those described to date for cyt c550 associated with PSII (24, 26).
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The $E_m$ of the cyt b559 and cyt c550 was measured in the absence of redox mediators other than sodium dithionite and potassium ferricyanide. Complete reductive potentiometric titration at pH 6.5 of cyt b559 and cyt c550 in PSII core complex was performed with the sample previously oxidized with potassium ferricyanide to an initial redox potential of approximately +450 mV (Fig. 2). Difference absorption spectra in the $\alpha$-band region of cytochromes obtained during the course of the redox titration between +450 mV and -45 mV are shown in Fig. 2A. Fig. 2C shows a plot of the percentages of reduced cyt b559 versus solution redox potential indicating the presence of two different forms of cyt b559 with $E_m$ values of +392 mV (HP form) and +222 mV (IP form), each representing about 85% and 15% of the total amount of protein, respectively. This result was similar to those found in the titrations carried out both in the absence (see Fig. 1C) and presence of low potential redox mediators (24). However, the plot of percentages of reduced cyt c550 obtained from the difference absorption spectra of the cyt c550 during the course of the redox titration (Fig. 2B) versus solution redox potential clearly showed that cyt c550 had a significant higher $E_m$ value (+200 mV) (Fig. 2D) than that obtained in the presence of low potential redox mediators (+80 mV) (24) and in the presence of other somewhat higher potential redox mediators (-20 mV) (see Fig. 1D). Similar $E_m$ value for cyt c550 was obtained if the reductive potentiometric titration of PSII core complex was started from ambient redox potential of the reaction mixture without previous addition of potassium ferricyanide (data not shown).

It has been observed that to get a well-defined $E_m$ most of proteins need the presence of mediators with redox potentials within ±30-60 mV of the redox centre $E_m$ value and that for a single redox centre, one mediator is ordinarily sufficient (27, 28). To verify the new value of $E_m$ obtained for cyt c550 in the absence of mediators (Fig. 2D), reductive potentiometric titrations were also performed in the presence of a single redox mediator (Fig. 3). We selected the mediator DAD (see Material and Methods) with an $E_m$ of +220 mV which is very close to the new value obtained for cyt c550 bound to PSII (Fig. 2). In these conditions, a value of $E_m$ of +215 mV for cyt c550 was measured very similar to that obtained in the absence of redox mediators (Fig. 2D). Thus, these results show that cyt c550 in the absence of redox mediators (other than potassium ferricyanide and sodium dithionite) or with only DAD has an $E_m$ value of about 200 mV higher than that obtained with the mixture of the eight mediators (see Material and Methods) (Fig. 1D). These results are consistent with those obtained in redox titrations of Q$_A$ in spinach PSII membranes where it was observed that the addition of redox mediators at low ambient potentials led to a shift of the $E_m$ for Q$_A$ from -80 mV to +65 mV (30).

**Hysteresis**—In order to increase confidence that titrations have been successfully performed at equilibrium, it is a common practice to performed redox titrations in both oxidative and reductive sequences and identical results should be obtained. Oxidative potentiometric titrations were carried out in the same conditions as Fig. 1 and 2. Fig. 4A shows the result of an oxidative potentiometric titration of cyt c550 in PSII core complexes preparations in the absence of redox mediators. In this case, cyt c550 was previously reduced by adding excess sodium dithionite and once reached an $E_m$ near -370 mV, the oxidative titration was performed by adding small amounts of potassium ferricyanide. Difference absorption spectra in the $\alpha$-band region of cyt c550 recorded during oxidative titrations in these conditions are shown (Fig. 4A, inset). From plots of percentage of reduced cyt c550 versus ambient redox potential obtained from these spectra, it was possible to adjust the oxidative titration curve with a Nernst equation with $n$=1 (Fig. 4A). Fig. 4A shows the presence of one component with $E_m$ value of -220 mV, a value very similar to that described for the soluble form of cyt c550 (8, 12, 20, 24, 26). One striking feature of this result is that the oxidative titration curve showed considerable differences from those performed in the reductive direction (see Fig. 2D). The $E_m$ determined by a reductive or oxidative potentiometric titration is usually identical in most of the biological and non-biological systems. However, cyt c550 bound to PSII exhibited anomalous redox chemistry, i.e. hysteresis was observed in the reductive and oxidative redox titrations in which an $E_m$ of +200 mV and -220 mV were obtained, respectively.

To test if the absence of redox mediators was responsible for the differences between reductive and oxidative titrations, an oxidative titration in the
presence of eight redox mediators spanning the range between 0 to +300 mV (see Material and Methods) was done. Fig. 4B shows difference spectra obtained during the course of this titration (inset) and a plot of percentage of reduced cyt c550 versus ambient redox potential obtained from these spectra. A very similar low redox potential ($E_m \approx -215$ mV) for cyt c550 could be calculated.

The above results suggest the possible existence of at least two states (A and B) corresponding to cyt c550 with substantially different $E_m$. An $E_m$ of +200 mV can be determined by reductive titrations in PSII preparations after oxidation with potassium ferricyanide. The preparation was initially oxidized with potassium ferricyanide to $E_h \approx +450$ mV and a reductive titration was performed, obtaining a value of $E_m \approx +200$ mV for cyt c550 (Fig. 5, curve 1). After reducing completely the sample and reaching to an $E_h \approx -300$ mV, oxidative titration was performed by re-oxidation of the sample with potassium ferricyanide (Fig. 5, curve 2) and a value of $E_m \approx -300$ mV was obtained. After complete reoxidation of the sample ($E_h \approx +410$ mV), a second reductive titration was held showing a similar reductive titration curve to the first one and a slightly lower value of $E_m \approx +100$ mV (Fig. 5, curve 3). It seems therefore that exist two extremes states for cyt c550 in PSII core complexes preparations that could be inter-convertible.

**Effect of incubation with sodium dithionite**—The experiments described above have shown that after addition of excess sodium dithionite and without mediators, the $E_m$ obtained in the oxidative titration of cyt c550 is significantly more negative than the $E_m$ from the reductive titration. To clarify the origin of this phenomenon, the effect of incubation with sodium dithionite on PSII and on the association of cyt c550 to PSII were studied.

PSII complexes were reduced with an excess of sodium dithionite (2 mM) and incubated for 10 min or 1 min and then reoxidised with potassium ferricyanide. Illumination of such samples at 200 K did not result in formation of the S2 Mn multiline signal (Fig. 6A,b and 6B,b) indicating that sodium dithionite reduced the Mn cluster. Illumination of the samples at room temperature followed by dark adaptation generated a state that gave rise to a S2 multiline signal upon illumination at 200 K (Fig. 6A,c and 6B,c). This indicates that the reduced state formed by sodium dithionite reduction can be reoxidized by light to form the usual S1 and S2 states. In the sample incubated with an excess of sodium dithionite for 10 minutes (Fig. 6A,c), the extent of the multiline seen is around 30% of that seen in the unreduced control sample (Fig. 6A,a).

The illumination treatment (3 flashes) means that the S0, S1 and S2 states sum up to around 30% of centres. Other experiments directly monitoring S0 (not shown) indicate that S0 makes only a small contribution at this illumination time, i.e. the 30% giving rise to the S2 signal arise from S1 and S2 (or its formal equivalent S1 TyrD). The other centres are presumably in more reduced forms of the cluster. Measurements of O2 evolution showed that in this sample the O2 evolution activity was 50% of that in untreated samples. The difference between the activity and the centres giving rise to the S2 signal presumably reflects centres that were further reduced than S2 but remained rapidly oxidizable and functional. The remaining centres are presumably either irreversibly damaged or require the low quantum yield assembly processes characteristic of photoactivation. We observed that reduction by dithionite also generates the typical Mn2+ signals in a small fraction of centres (Fig. S1A). This Mn2+ signal does not diminish when sample was illuminated after reoxidation with potassium ferricyanide. Ferricyanide is known to precipitate Mn2+ ions preventing them from undergoing oxidation by the reaction centres (34). The small Mn2+ signal thus presumably represents a small fraction of centres where the cluster is destroyed by the dithionite treatment. The Mn2+ signal was larger in the presence of a mediator (indigodisulfonate, $E_m = -125$ mV) indicating greater PSII damage (Fig. S1B).

Shorter incubation times (1 min) with sodium dithionite also showed no S2 formation upon reoxidation with potassium ferricyanide. However nearly 80% of S2 formation was seen after 3 flashes and dark adaptation (Fig. 6B,c). This indicates that all the centres were in the S0, S1 or
S\textsubscript{3} state after the dithionite treatment. O\textsubscript{2} evolution in such sample was around 70%.

The EPR experiments show that treatment with excess sodium dithionite results in over-reduction of the Mn cluster and that this can be photooxidised again when the ambient potential is returned to a range that allows reaction centre photochemistry to occur. Future experiments of this kind should allow us to define which states are formed at given times of incubation and to correlate this more precisely with the binding and redox state of cyt c550.

To test for the release of cyt c550 by sodium dithionite treatment reduced samples were precipitated by PEG and difference spectra were taken of the supernatant and the pellet (Fig. 7). These were compared to supernatants and pellet from unreduced samples. In the unreduced samples cyt c550 was entirely associated with the PSII (the pellet) (spectrum 1, Fig. 7A), with no cyt c550 present in the supernatant (spectrum 2, Fig. 7A). However, in the sample incubated with sodium dithionite, the appearance of a typical spectrum cyt c550 in the supernatant (spectrum 2, Fig. 7A) and the corresponding decrease of this cytochrome in the spectrum of the precipitate (spectrum 1, Fig. 7B), showed that incubation with sodium dithionite had caused the dissociation of a significant fraction of the cyt c550 from PSII.

**DISCUSSION**

Redox titrations of cyt c550 performed on PSII core complexes from *T. elongatus* in the absence of low potential redox mediators showed an *E\textsubscript{m}* value for this heme protein that is higher than was obtained previously. This *E\textsubscript{m}* value of +200 mV is about 300 mV more positive than the previously determined when mediators were present (*E\textsubscript{m}* = -80 mV) (24). A similar value was obtained in titrations carried out with DAD as a mediator, the potential of which (*E\textsubscript{m}* = +220 mV) is quite similar to that determined for cyt c550. The redox potential of the sample during the titration was shown to be reliable under these conditions as demonstrated by i) the similar *E\textsubscript{m}* values obtained for the cyt b559 in the presence or absence of mediators and ii) the correspondence of the *E\textsubscript{m}* values for cyt b559 with those reported in the literature. The good fits of the data sets to one-electron Nernst curves and the large number of measurements allow further confidence in the *E\textsubscript{m}* value obtained for cyt c550.

When titrations were begun at low potentials the values for the *E\textsubscript{m}* were shifted to low potentials, typical of the cyt c550 free in solution (26). This effect was specific to the cyt c550 and did not affect the cyt b559 in the same sample. Thus there was no technical problem in terms of establishing and measuring correct potentials.

The redox potential shift induced by low potentials is reminiscent of earlier reports on the redox potential of Q\textsubscript{A} that were reported by Krieger *et al.* (30) and Johnson *et al.* (31). The Mn\textsubscript{Ca} cluster has a very high potential even in the most reduced form of the enzyme cycle. It is protected from reductive attack from the medium by being buried inside a large protein complex, with access channels for substrate and products. However, reductants have access to the cluster when highly reducing conditions are used, when mediators are used or when extrinsic polypeptides are removed. The reduction of the cluster leads to the weaker binding of the metal ions of the cluster to the site and eventually to their release (35, 36). Reduction of the cluster reverses the assembly process known as photoactivation which is considered to involve protein conformational changes (37-40). We suggest that these conformational changes are responsible for the increased solvent access and weaker binding of the cyt c550 in the presence of mediators giving *E\textsubscript{m}* values of -80 or -20 mV. Under very reducing conditions, the cyt c550 even completely detaches from the PSII (Fig. 7).

Intriguingly when an over-reduced PSII preparation is allowed to become slowly oxidised during the course of a redox titration and then the reductive titration is repeated, the high potential *E\textsubscript{m}* value is recovered. In the context of the explanation given above for the low potential shift, this indicates that the cyt c550 rebinds tightly, the protein must have returned to its original conformation and hence the Mn\textsubscript{Ca} cluster also must have returned to its functional state.

Reassembly of the Mn\textsubscript{Ca} cluster by binding of free Mn\textsuperscript{2+} ions into the Mn-less PSII, photoactivation (37-40), is a complex process by which Mn\textsuperscript{2+} ions are bound and oxidised one at time by successive turnovers of reaction centre photochemistry. A Ca\textsuperscript{2+} ion is also incorporated into the cluster and conformation changes in the protein seem to occur. This process takes place.
with a relatively low quantum yield. When this is done in vitro, high concentrations of Mn ions, very high concentrations of Ca\(^{2+}\) ions and the presence of an artificial electron acceptor are required for photoactivation to occur efficiently. Under the conditions of the titrations used here, the medium conditions are clearly not appropriate for this type of photoactivation to occur. Nevertheless, it is still possible to entertain the idea that the cluster “debinds and rebinds” if we propose that the reduced Mn\(_4\)Ca cluster is not released from the site upon reduction. The Mn would have to be reduced to a level where the natural geometry of the functional site is lost, and hence the changes in the protein would take place, but the Mn and Ca ions would not be released into the medium. This is similar to the situation encountered by Mei and Yocum (41) in which reductants were allowed access to the Mn by removal of the 23 kDa polypeptide. Some Mn\(^{2+}\) was seen by EPR but was not available to chelators and the enzyme was rapidly activated by illumination. In the present work the weak measuring beam used for measuring the spectra during the course of the titration could have been sufficient to reoxidize the Mn ions and re-impose the protein conformation required to induce the high potential form of the cyt c550.

To test the feasibility of this idea we did an EPR study that showed a) the Mn\(_4\)Ca cluster was indeed reduced by sodium dithionite and upon reoxidation of the electron acceptors in the dark the Mn\(_4\)Ca remained in an over-reduced state and b) the over-reduced state could be efficiently reoxidized by flash illumination. The reoxidized PSII showed water oxidation activity in a large proportion of centres. With longer times of incubation in sodium dithionite the proportion of centres that could be reactivated by illumination diminished. Whether this effect is due to loss of Mn or to the requirement for low quantum yield photoactivation will be the subject of future work. Nevertheless, the EPR experiments are consistent with the proposition that the cyt c550 environment reflects conformational changes that are controlled by the redox state of the Mn\(_4\)Ca cluster. Further experimentation should allow this to be tested more directly.

The change in the PSII structure associated with the reduction of the Mn\(_4\)Ca cluster could lead to a greater accessibility of the heme to the aqueous medium and consequently to a total or partial release of cyt c550 from PSII. It seems likely that the increase in solvation energy that occurs when moving the heme out of the low dielectric of the protein environment into the high dielectric of water stabilizes the oxidized state more than the reduced state making the midpoint potential more negative (42-44).

These results lead us to suggest that the \(E_m\) of cyt c550 in PSII “in vivo” may be \(+200\) mV, at least under certain conditions. This opens the possibility of a redox function for this protein in electron transfer in PSII. The nearest redox cofactor is the Mn\(_4\)Ca cluster (22 Å) (6). This long distance means that electron transfer would be slow (ms-s time scale) relative to the charge separation events in the reaction centre. However, this rate remains potentially significant relative to the lifetime of the reversible charge accumulation states in the enzyme (tens of seconds to minutes) (45). Some kind of protective cycle involving a soluble redox component in the lumen may be envisioned. Before we enter into speculation an experimental verification that cyt c550 does indeed donate electrons to the \(S_2\) and or \(S_3\) states is required.

**CONCLUSIONS**

We conclude that earlier redox titrations of cyt c550 in PSII probably reflected the situation in which the Mn\(_4\)Ca cluster is chemically reduced. Conformational changes associated with this resulted in the downshift of the \(E_m\) due to solvent access to the heme. Thus, the \(E_m\) of \(+200\) mV obtained for cyt c550 without mediators and with only DAD as a mediator is probably relevant to the most functional form of the enzyme. This \(E_m\) value of about \(+200\) mV opens the possibility of a redox function for cyt c550 in the PSII as an electron donor to the Mn\(_4\)Ca cluster perhaps in some sort of protective cycle. This proposed role has yet to be demonstrated experimentally.
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**FOOTNOTES**

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The abbreviations used are: cyt b559, cytochrome b559; cyt c550, cytochrome c550; $E_m$, midpoint redox potential; $E_h$, ambient redox potential; PSII, photosystem II; QA, and QB, the primary and secondary quinone acceptors of the reaction centre of PSII.

**FIGURES LEGENDS**

**Fig.1.** Reductive potentiometric titrations of cyt b559 and c550 in PSII core complexes in the presence of a mixture of eight redox mediators covering the potential range between +430 and 0 mV.
(A, B) Difference absorption spectra in the α-band region of cyt b559 and cyt c550. The spectra were obtained by subtracting absolute spectra recorded during the course of the redox titration between +455 and -80 mV minus the spectrum recorded at +455 mV (A) and the spectra recorded between +210 mV and -300 mV minus the absolute spectrum recorded at +210 mV (B). For simplification, only a set of selected spectra are included in panels A and B.
(C, D) Plot of the percentages of reduced cyt b559 and reduced cyt c550 obtained from the absorbance differences at 559-570 nm and 549-538 nm versus ambient redox potentials, respectively. The solid curves represent the best fit of the experimental data to the Nernst equation in accordance with one-electron processes ($n=1$) for two components (C) with $E_m$ of +246 mV (20%) and +389 mV (80%) and for one component (D) with $E_m$ of -20 mV.

**Fig.2.** Reductive potentiometric titrations of cyt b559 and c550 in PSII core complexes without redox mediators in the presence of 25 µM potassium ferricyanide.
(A, B) Difference absorption spectra in the α-band region of cyt b559 and cyt c550. The spectra were obtained by subtracting absolute spectra recorded during the course of the redox titration between +430 and -80 mV minus the spectrum recorded at +430 mV (A) and the spectra recorded between +220 mV and -80 mV minus the absolute spectrum recorded at +230 mV (B). For simplification, only a set of selected spectra are included in panels A and B.
(C, D) Plot of the percentages of reduced cyt b559 and reduced cyt c550 obtained from the absorbance differences at 559-570 nm and 549-538 nm versus solution redox potentials, respectively. The solid curves represent the best fit of the experimental data to the Nernst equation in accordance with one-electron processes ($n=1$) for two components (C) with $E_m$ of +222 mV (20%) and +392 mV (80%) and for one component (D) with $E_m$ of +200 mV.
Fig. 3. Reductive potentiometric titration of cyt c550 in PSII core complexes with 20 μM DAD and 25 μM of potassium ferricyanide.

(A) Difference absorption spectra in the α-band region of cyt c550. The spectra were obtained by subtracting absolute spectra recorded during the course of titration minus the absolute spectrum recorded at +240 mV. For simplification, only a set of selected spectra are included in panels A and B. (B) Plots of the percentages of reduced cyt c550 obtained from the absorbance differences at 549-538 nm versus solution redox potentials. The solid curve represents the best fits of the experimental data to the Nernst equation in accordance with one-electron processes (n=1) for one component with $E_m$ of +215 mV.

Fig. 4. Oxidative potentiometric titrations of cyt c550 in PSII core complexes in the absence and presence of redox mediators.

(A, B) Plots of the percentages of reduced cyt c550 obtained from the absorbance differences at 549-538 nm versus solution redox potentials in PSII core complexes in the absence and in the presence of the mixture of eight redox mediators covering the potential range between +430 and 0 mV, respectively (see Material and Methods section). The solid curve represents the best fits of the experimental data to the Nernst equation in accordance with one-electron processes (n=1) for one component with $E_m$ of -215 mV and -220 mV, respectively. Insets: Difference absorption spectra in the α-band region of cyt c550 obtained by subtracting the absolute spectrum recorded at -330 mV from those recorded during the course of the redox titration with potassium ferricyanide in the absence or in the presence of redox mediators, respectively. For simplification, only a set of selected spectra are included.

Fig. 5. Reversibility of the potentiometric titrations of cyt c550 in PSII core complexes in the absence of redox mediators.

The plot represent titrations curves corresponding to further cycles of reduction and oxidation (up to three) in the same PSII membranes preparations. The percentages of reduced cyt c550 were plotted versus solution redox potentials in the first reductive titration (curve 1), in the first oxidative titration (curve 2) and in the second reductive titration (curve 3). Each curve was fitted to the Nernst equation in accordance with one-electron processes (n=1) for one component.

Fig. 6. Effect of sodium dithionite on the formation of S$_2$ multiline EPR signal.

PSII complexes were incubated 10 min (panel A) or 1 min (panel B) in the absence (a) and in the presence of 2 mM of sodium dithionite (b,c). All spectra were difference spectra after 200 K illumination (light minus dark). (a) Untreated PSII; (b) PSII complexes reduced by sodium dithionite and reoxidized by potassium ferricyanide; (c) sample b was defrozen and then was dark adapted at room temperature for 30 min, illuminated by a series of 3 flashes and finally dark adapted for 30 min. Instrument settings: microwave power, 20 mW; modulation amplitude, 25 gauss; temperature, 8.5 K.

Fig. 7. Effect of sodium dithionite on the association of cyt c550 to PSII.

Difference absorption spectra of cyt b559 and cyt c550 were recorded from the pellet (spectrum 1) and the supernatant (spectrum 2) obtained by precipitation of PSII core complexes. The spectra were obtained by subtracting the absolute spectrum at -430 mV (the spectrum of reduced cyt b559 and cyt c550) minus at +430 mV (the spectrum of oxidized cyt b559 and cyt c550) in PSII core complexes untreated (A) and treated (B) with 2 mM of sodium dithionite during 30 min.
Figure 2

A

B

C

D

Absorbance

Reduced cytochrome (%)

Ambient redox potential (mV)

Wavelength (nm)

$E_m = +200$ mV

$E_m = +222$ mV

$E_m = +392$ mV

Cyt $b_{559}$

Cyt $c_{550}$

$\Delta A = 0.002$

$\Delta A = 0.005$
Reduced cyt (550%)

**Figure 3**

**A**
- Absorbance
- Wavelength (nm)

**B**
- Reduced cyt (%) vs. Ambient redox potential (mV)
- $E_m = +215$ mV
Figure 4

Reduced cyt c550 (%)

$E'_m = -220 \text{ mV}$

$E'_m = -215 \text{ mV}$

Ambient redox potential (mV)
Figure 5

Reduced cyt c550 (%)

Ambient redox potential (mV)
Figure 6
