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Granal Photosystem II complexes contain only the high redox potential form of cytochrome *b*-559 which is stabilised by the ligation of calcium

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Abstract

Photosystem-II-enriched membrane fragments obtained by detergent solubilisation of thylakoid membranes were found to contain almost exclusively the high redox potential form of cytochrome *b*-559 ($E_{m,6.0}$ = +353 mV), provided that the haem was maintained in the reduced state during the isolation procedure. A reducing potential was required during the isolation due to the instability of the oxidised form of the high-potential couple. Additional detergent treatment of such preparations converted all of the cytochrome to a low potential form ($E_{m,6.0}$ of around +100 mV). Dissociation of the 23 kDa extrinsic polypeptide, bound at the lumenal side of Photosystem II, had no effect on the redox state of the cytochrome provided that calcium remained in association with the Photosystem II complex. Removal of the 33 kDa extrinsic protein, in addition to the 23 kDa, resulted in the conversion of the haem to an intermediate ($E_{m,6.0}$ = +169 mV) redox form, independent of the presence of calcium. Considering that these preparations are derived from the granal regions of the thylakoid membranes, the data suggest that, in vivo, Photosystem II complexes in these regions contain only the high redox potential form of the cytochrome. The data further suggest that, in addition to the 33 kDa protein, ligation of calcium rather than the 23 kDa polypeptide is required for the stabilisation of this form of cytochrome *b*-559.

Keywords: Cytochrome *b*-559; High redox potential form; Photosystem II; Redox titration; Calcium; Thylakoid membrane

1. Introduction

The role of cytochrome *b*-559 (cyt *b*-559) in the photosynthetic process has been subject to considerable debate in the years since its discovery [1,2]. Numerous biophysical, biochemical and molecular biological studies have demonstrated a close relationship between cyt *b*-559 and the reaction centre of Photosystem II (PS II) and indeed it was shown to be present even in very highly resolved photochemically active PS II reaction centre preparations [3,4]. Cyt *b*-559 is an integral membrane protein composed of two polypeptides, the α and β subunits [5], both of which are required for the stable assembly of PS II [6]. However, its function in oxygenic photosynthesis remains unresolved. Recent suggestions have included participation in an electron cycle [7,8] or in a side path for electron transport through PS II [9,10] to provide protection against photoinhibition; stabilisation of oxygen evolution either during photoactivation [11] or by accepting protons during turnover of the S state cycle [12,13]; electron donation to a fatty acid desaturase enzyme [14] and scavenging of photogenerated superoxide radicals [15].

The redox properties of cyt *b*-559 are particularly unusual. This protein has been reported to occur in vivo in one of two interconvertible redox forms: a high mid-point potential (HP) form which diplays an E_m of about +380 mV, and a form with a lower mid-point potential (LP), displaying an E_m of +60 mV [1]. The E_m of HP cyt *b*-559 is one of the highest values reported for a *b*-type cytochrome and it has been suggested that this value is maintained by an extreme hydrophobicity in the microdomain surrounding the haem, in conjunction with the positive dipole potential arising from the parallel α-helices of the two subunit polypeptides [2,16]. Many of the factors that are known to switch the state of cyt *b*-559 from HP to

Abbreviations: Cyt *b*-559, cytochrome *b*-559; PS II, Photosystem II; HP, high redox potential form; LP, low redox potential form; IP, intermediate redox potential form; Mes, 2-[*N*-morpholino]ethanesulfonic acid; DM, dodecyl β-D-maltoside.

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LP involve damage to the structural integrity of the thylakoid membrane [1,17,18], while a switch in the reverse direction, from LP to HP, has been observed following reconstitution with extracted lipid components [19–21].

The conversion of HP cyt *b*-559 to lower E_m values is especially rapid in the presence of detergents [17], presumably as a consequence of their effect on the hydrophobicity of the haem domain. Preparations of PS-II-enriched membrane fragments obtained by solubilisation of thylakoid membranes with the non-ionic detergent Triton X-100 have been used in several recent investigations on this cytochrome [16,22–24]. In these preparations the components of the cyt b_6f complex are absent and thus analysis of the spectra is simplified. It has not been unequivocally demonstrated, however, that cyt *b*-559 in these preparations remains undamaged. The consensus from several investigations, based mostly on data obtained from three point titrations, is that about 45–70% of PS II complexes in such membrane fragments contain HP cyt *b*-559. Full redox titrations [22] indicated the presence of two populations of cyt *b*-559 in PS-II-enriched membranes with mid-point potentials of +380 mV and +140 mV. The high potential couple accounted for about 65% of the total cytochrome and was pH independent. The mid-point potential of the other couple varied by −72 mV per pH unit below pH 7.6, indicating that deprotonation of a group with a pK_a of 7.6 was coupled to the oxidation of the haem. By a combination of redox potentiometry and electron paramagnetic resonance, the presence of an intermediate potential (IP) cytochrome, in addition to HP and LP forms has also been reported in similar preparations, and it was shown that one-third of PS II complexes contained cyt *b*-559 with an E_m of +226 mV, at pH 6.0 [16]. These workers thus concluded that each native PS II complex contains one HP and one IP cyt *b*-559. Considering the pH dependence of the LP couple, it was further suggested that the IP cyt *b*-559 determined in [16] correlated with the LP form reported in [22]. In general, however, the extent to which these data are indicative of the properties of the cytochrome in its native environment, or are a reflection of detergent induced damage during isolation of the membranes is not clear.

The presence of HP cyt *b*-559 has been correlated with the capacity of thylakoid membranes to photo-oxidise water [1,2,11], but the correlation is not absolute in that there is evidence that oxygen evolution can proceed in vitro in the absence of HP cyt *b*-559 in calcium reactivated PS II fragments [25–27] and oxygen-evolving PS II core particles [28]. There is considerable evidence to suggest that cyt *b*-559 interacts with the extrinsic polypeptides associated with oxygen evolution. It has been suggested that there is a differential stability of the attachment of these extrinsic proteins depending on the redox state of the cytochrome haem [29]. Additionally, it has been shown that the redox state of the haem may be modulated by the presence of the 23 kDa extrinsic protein [16,30,31]. Conversion of the HP cyt *b*-559 to lower potential forms and concomitant inhibition of oxygen evolution has been reported to occur following the removal of the 23 kDa extrinsic protein, and the inevitable release of calcium, by NaCl treatment of membranes [30]. Various lines of experimental evidence have suggested that although the capacity for oxygen evolution can be restored by the addition of calcium, the recovery of the HP couple requires the rebinding of the 23 kDa protein [25,27,30–32].

It has been demonstrated that NaCl treatment of PS II membranes destabilises HP cyt *b*-559 and that the predominant forms of cyt *b*-559 in treated membranes are the IP form with an $E_{m,6.0}$ of +170 mV and the LP form with an $E_{m,6.0}$ of +3 mV [16]. It has thus been concluded that the redox state of the haem may be influenced by the binding of the 23 kDa protein.

We report here the isolation of PS-II-enriched membrane fragments containing almost exclusively HP cyt *b*-559. It is possible to obtain such preparations provided that reducing conditions are maintained throughout the isolation procedure, and we show that this requirement arises from a differential stability of the HP cyt *b*-559 depending on whether it is in the reduced or oxidised state. We further show that it is the presence of calcium rather than the 23 kDa protein that is required for the stabilisation of the HP redox form of cyt *b*-559.

2. Materials and methods

PS-II-enriched membranes were prepared from market spinach essentially by a procedure previously described [33]. We modified the above protocol by the inclusion of 5 mM sodium ascorbate in every solution used for isolation and storage of samples, thus maintaining the solutions at a mildly reducing ambient redox potential. The final resuspension solution contained 10% glycerol, 20 mM Mes (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$ (solution A) as well as 5 mM sodium ascorbate. The latter was removed prior to assays by repeated centrifugation and resuspension of the samples in solution A.

Cytochrome *b*-559 absorption spectra were taken using an Aminco DW2000 spectrophotometer in the split beam mode, scanning between 590 nm and 530 nm at a speed of 0.6 nm s^{-1}. Redox titrations of cyt *b*-559 were performed using a specially constructed, three-necked quartz cuvette similar to that described in [34]. The redox potential was measured using a combination platinum-silver/silver chloride electrode (Russel CMMPtRL) attached to a Philips 9158 pH meter. The electrode was calibrated before each titration against a saturated freshly prepared solution of quinhydrone at pH 7. The electrode was inserted into the cuvette, containing 3 ml of solution A with 0.1% (w/v) bovine serum albumin (solution B) plus redox mediators. The solution was bubbled extensively with oxygen-purged nitrogen (bubbled through an anaerobic solution of re-

duced methyl viologen) before the addition of PS-II-enriched membranes to a concentration of 100 μg chlorophyll ml^{-1}. The cuvette was then purged continuously with oxygen-free nitrogen through a side arm, while additions of oxidants and reductants were made through a second side arm. A reference cuvette contained identical concentrations of PS-II-enriched membranes and redox mediators and was maintained at a fixed potential and in an anaerobic state throughout. Anaerobic conditions were further ensured by the addition of glucose (5 mM), glucose oxidase and catalase (0.1 mg ml^{-1} of each) prior to the introduction of the samples. If maintained in the dark, this oxygen trap did not affect ambient redox potential. The whole assembly was kept in the dark and thermostatically controlled at 7°C.

Two types of titration were performed, since significant differences were found to occur depending on the initial direction of the titration. Oxidative titrations were performed by initially adjusting the potential of the sample and reference to between −150 mV and −200 mV with sodium dithionite. A baseline was then established between 590 nm and 530 nm with all haem in the reduced state. Reductive titrations, on the other hand, involved adjusting the potential to between 440 mV and 460 mV with potassium ferricyanide prior to recording the baseline. Cytochrome *b*-559 was subsequently reduced or oxidised by the addition of sodium dithionite (0.0025–0.25M) and potassium ferricyanide (0.0025–0.25 M), respectively. After each addition, the redox potential was allowed to stabilise such that the variation in potential during the recording of each spectrum did not exceed ±1 mV. Upon completion, a rapid titration was performed in the opposite direction to test reversibility. The following mediators were added to a final concentration of 20 μM: hydroquinone, 1,2-naphthoquinone, *N*-methylphenazonium methosulfate, 1,4-naphthoquinone and anthraquinone 6-sulfonate. Each titration was completed in about 4 h. Data analysis was achieved using the kaleidograph data fitting package on an Apple Macintosh computer by fitting the titrations to a linear combination of one to three Nernst curves for one-electron reduction processes as outlined in [35].

Where indicated, PS-II-enriched membrane fragments were further treated with detergent solutions prior to redox titrations as follows: Triton X-100 (0.5%, w/v, final concentration) was added to membrane fragments at 4°C, in the dark, and at a chlorophyll concentration of 2 mg ml^{-1}. The suspension was mixed and centrifuged at 15000 × *g* for 15 min. The pellet was resuspended in solution A, the centifugation step was repeated and the final pellet was resuspended in solution B. Dodecyl β-D-maltoside was added to PS II membrane fragments at a concentration of 0.05% (w/v) in solution B. Treatment of samples with NaCl was carried out as detailed in [36] and treatment with divalent cations was as in [37]. Chlorophyll concentrations were determined as previously described [38].

3. Results

We prepared PS-II-enriched membrane fragments while maintaining all solutions at mildly reducing conditions by the inclusion of ascorbate. The properties of cyt *b*-559 in PS-II-enriched membranes isolated in this way were examined by redox titrations, monitoring the absorbance at 559 nm as a function of ambient redox potential. Samples were washed and resuspended in ascorbate free medium prior to initiating the titrations in order to prevent the reductant from interfering with the measurements.

Typical reduced minus oxidised spectra of cyt *b*-559, recorded at various potentials, are shown in Fig. 1. It is apparent that the components of the cytochrome b_6f complex are absent in these preparations, thus confirming that the slight modifications introduced in the isolation procedure did not modify the properties of the membrane fragments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis also verified that the polypeptide composition of the preparations was indistinguishable from those previously reported (data not shown).

The results obtained from two titrations of PS-II-enriched membranes are shown in Fig. 2. After the addition of suitable mediators and allowing for equilibration under anaerobic conditions, the ambient potential was adjusted either with dithionite or with ferricyanide depending on the direction of the titration. Titrations were then performed in either oxidative or reductive direction as shown in Fig. 2A and B, respectively. Immediately after the titration was completed, a second more rapid titration was performed in the opposite direction in order to establish that the ob-

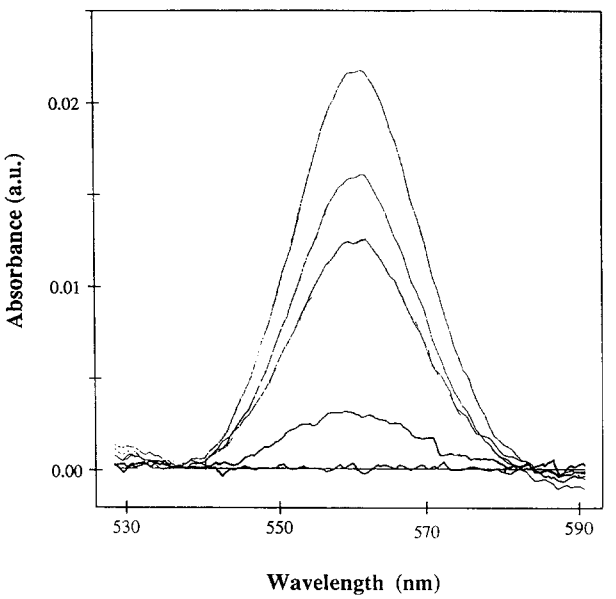

Fig. 1. Optical absorption difference spectra obtained during a redox titration of PS-II-enriched membrane fragments. Samples were oxidised with ferricyanide in order to obtain a background spectrum prior to recording reduced minus oxidised spectra. From top to bottom: 26 minus 443 mV; 105 minus 443 mV; 195 minus 443 mV; 389 minus 443 mV.

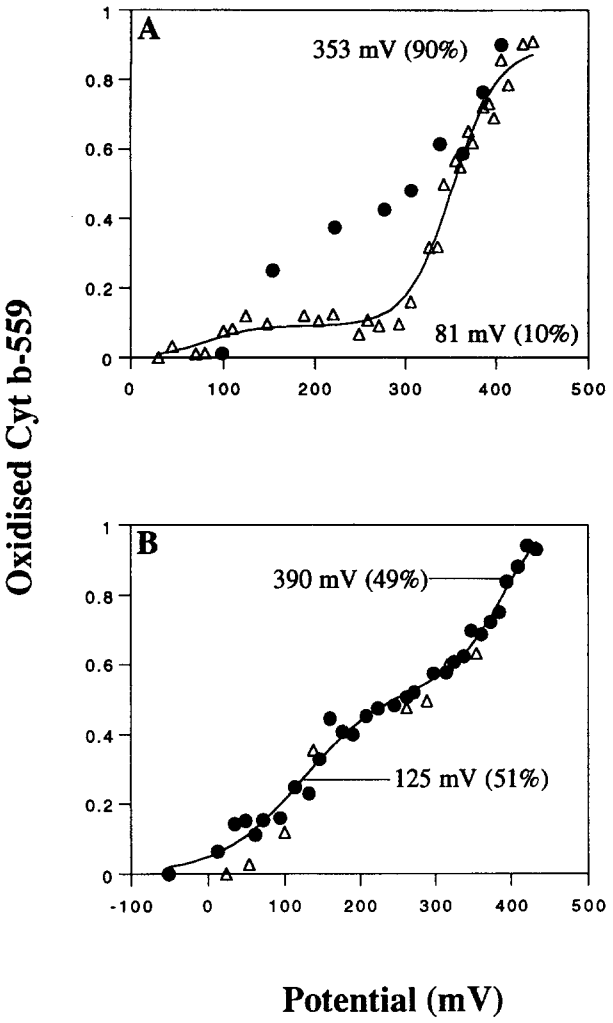

Fig. 2. Redox titrations of cyt *b*-559 in PS-II-enriched membrane fragments. (A) Oxidative titration (△) followed by a rapid reductive titration (●); (B) Reductive titration (●) followed by a rapid oxidative titration (△). Oxidised cyt *b*-559 is expressed as a proportion of the total cytochrome. The percentage of each component present is shown in parentheses.

served behaviour was reversible. The data for each titration was fitted to a linear combination of Nernst equations allowing for either 1, 2 or 3 components, depending on the best fit. The values given for the potentials were obtained by assuming that each component corresponded to a one-electron reduction. As an additional test of the data, the fitting process was repeated allowing this parameter to vary. For all titrations shown, introducing this additional flexibility in the data-fitting did not affect the outcome of the fitting process, indicating that each component of the titrations corresponded to one-electron redox species. Oxidative titrations of PS-II-enriched membranes isolated under reducing conditions revealed the presence of 90% of cyt *b*-559 in its HP form with an $E_{m,6.0}$ of +353 mV. Little variation was observed between different preparations and this form of the cytochrome invariably exceeded 80% of the total. The remainder of the cytochrome was present in the LP form with an $E_{m,6.0}$ of +81 mV. There was no indication for any cyt *b*-559 with an intermediate redox potential.

It is apparent, however, that the oxidative titration shown in Fig. 2A was not reversible. When measured in a reductive direction almost half of the total of cyt *b*-559 titrated with an $E_{m,6.0}$ of approximately +100 mV. This titration was not fitted to a combination of Nernst equations since the number of data points was too low for accurate analysis. Although we cannot discount that the observed hysteresis was indicative of a lack of equilibrium, we consider it unlikely for the following reasons: (a) this behaviour was entirely reproducible between different titrations irrespective of the length of time allowed for equilibration after addition of titrants, and (b) changing the mediator concentration from 20 μM to 40 μM had no discernible effect either on the mid-point potentials or the degree of apparent hysteresis (data not shown).

We consider it more probable that a proportion of HP cyt *b*-559 was destabilised under the oxidising conditions generated at the termination of the oxidative titration and became converted to the LP form. This conclusion is supported by the data obtained from the reductive titration shown in Fig. 2B. The sample used in this titration was taken from the same preparation of PS-II-enriched membranes that was used for the oxidative titration of Fig. 2A. The reductive titration, however, revealed the presence of HP cyt *b*-559 in only half of the PS II complexes. This titration was also fitted to a combination of two Nernst equations since there was no indication of a third, intermediate potential form of cyt *b*-559. This oxidative titration was entirely reversible and showed no signs of hysteresis. It did, however, correlate very closely with the rapid reductive titration shown in Fig. 2A. It thus appears that the apparent content of HP cyt *b*-559 in PS II membranes depends crucially on the direction in which the titration is performed. When the haem is maintained in a reduced state virtually all of cyt *b*-559 can be present in the HP form but after oxidation some HP cyt *b*-559 is invariably converted to lower potential forms.

The lability of HP cyt *b*-559 to detergent solubilisation is well established. It was therefore surprising that fragmentation of the thylakoid membranes by partial detergent solubilisation did not cause a more substantial destabilisation of the cytochrome. Damaging effects of detergents on cyt *b*-559 were tested and the results obtained are shown in Fig. 3. In light of the decreased stability of oxidised HP cyt *b*-559, as revealed in Fig. 2, the titrations shown in Fig. 3 were performed in an oxidative direction using PS II membranes isolated and stored under reducing conditions. Following additional exposure to the detergent, HP cyt *b*-559 became highly susceptible to denaturation. Cyt *b*-559 in PS-II-enriched membranes was titrated in a solution containing 0.05% dodecyl β-D-maltoside (w/v). The detergent was introduced after the samples had equilibrated under anaerobic conditions and after addition of a cocktail

of redox mediators which fixed the ambient potential at approx. +30 mV. Hence, any conversion of the cytochrome's potential can be related solely to the effect of the detergent on PS II complexes containing the reduced form of the haem. Under these conditions the membranes contained only the LP form of cyt *b*-559 with an $E_{m,6.0}$ of +96 mV (Fig. 3A). PS II membranes further treated with Triton X-100 (0.5% w/v), and subsequently resuspended in detergent free solution, were also found to contain only the LP form with an $E_{m,6.0}$ of +109 mV (Fig. 3B). Conversion to this state was not dependent on the length of exposure time to either of the detergents and an IP form of cyt *b*-559 was not detected.

The relationship between the extrinsic proteins, calcium and the redox state of cyt *b*-559 was examined by performing redox titrations of PS II membranes subjected to various treatments causing selective depletion of proteins and/or calcium. These treatments were carried out in

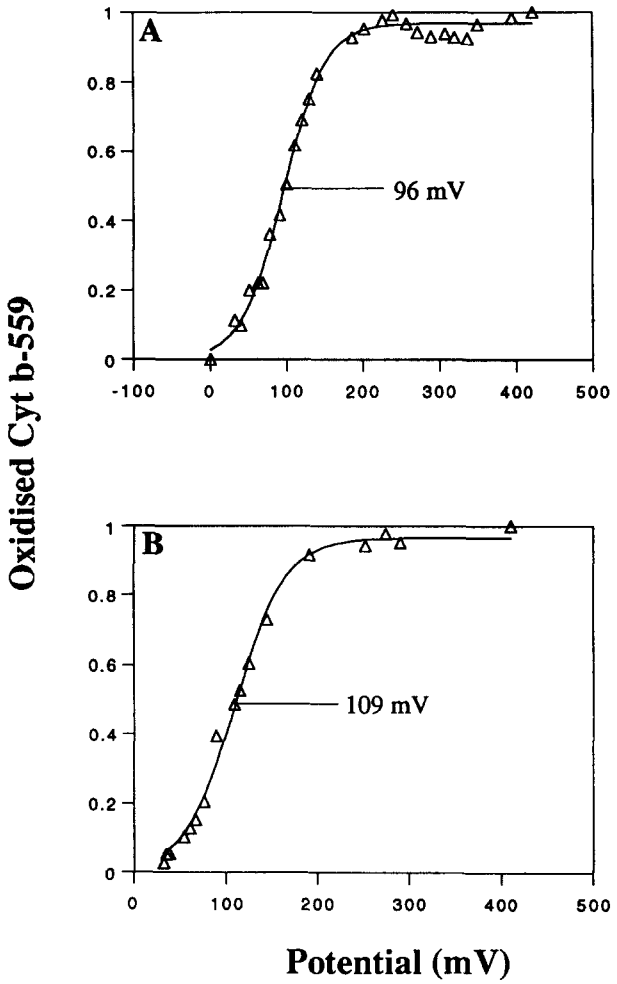

Fig. 3. Oxidative redox titrations of cyt *b*-559 in detergent-treated PS-II-enriched membrane fragments. (A) Titration performed in the presence of 0.05% (w/v) DM; (B) Titration of membrane fragments treated with 0.5% (w/v) Triton X-100 and finally resuspended in detergent-free solution as described in Section 2. Oxidised cyt *b*-559 is expressed as a proportion of the total cytochrome.

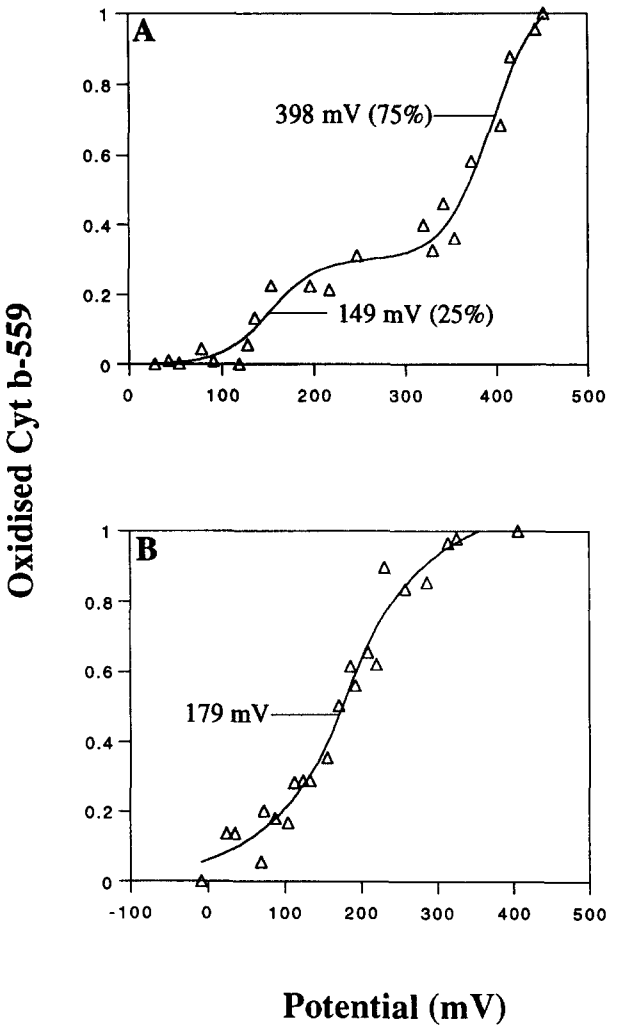

Fig. 4. Oxidative redox titrations of cyt *b*-559 in PS-II-enriched membrane fragments treated with: (A) 1.5 M NaCl in the dark, thus depleted of the 23 kDa extrinsic protein, and (B) 1.5 M NaCl, 50 mM EGTA under dim illumination, thus additionally lacking calcium. Oxidised cyt *b*-559 is expressed as a proportion of the total cytochrome. The percentage of each component present in (A) is shown in parentheses.

media containing 5 mM ascorbate to stabilise the HP redox form of the cytochrome and all titrations were initially performed in an oxidative direction. Redox titrations of PS II membranes washed with 1.5 M NaCl as to release the 23 kDa protein are shown in Fig. 4. The association of calcium with the protein depleted membranes was controlled by employing two different procedures for the NaCl treatment [36]. Thus, preparations depleted of the 23 kDa protein but retaining calcium were obtained by incubation in complete darkness in the presence of 10 mM calcium. Membranes depleted of both the extrinsic protein and calcium were prepared by incubation under dim illumination in the presence of 50 μM EGTA. As shown in Fig. 4A, removal of the 23 kDa protein, while calcium was maintained, had little effect on the redox state of the haem since the majority of the cytochrome was in the HP state. Calcium depletion, however, in addition to the removal of

the protein resulted in the conversion of the cytochrome to a form displaying an $E_{m,6.0}$ of +179 mV (Fig. 4B).

We further examined the redox state of the cytochrome in membrane preparations in which the extrinsic 33 kDa protein was also removed. Dissociation of the proteins was achieved either by incubation with $MgCl_2$ and EGTA, a treatment which will additionally release calcium, or by incubation with $CaCl_2$ followed by titration in a solution supplemented with 10 mM calcium. By the latter treatment the majority of the complexes are expected to retain the metal. The redox titrations obtained from samples treated as above are shown in Fig. 5A and B. It is apparent that all of the cytochrome is present in a homogeneous IP redox form irrespective of the method used to release the polypeptides or the presence of calcium. A summary of the redox mid-point potentials and the percentage distribution of the redox forms we obtained is presented in Table 1.

Table 1
Redox mid-point potentials (mV) of cytochrome *b*-559 in PS-II-enriched membranes

Sample	HP	IP	LP
Untreated-oxidative titration	353 (90%)	–	81 (10%)
Untreated-reductive titration	390 (49%)	–	125 (51%)
In the presence of 0.05% DM	–	–	96 (100%)
Treated with 0.5% Triton X-100	–	–	109 (100%)
Treated with NaCl in the dark	398 (75%)	–	149 (25%)
Treated with NaCl in dim light	–	179 (100%)	–
Treated with $MgCl_2$	–	167 (100%)	–
Treated with $CaCl_2$	–	169 (100%)	–

Errors were within 10 mV. The percentage distribution of redox forms is shown in parentheses.

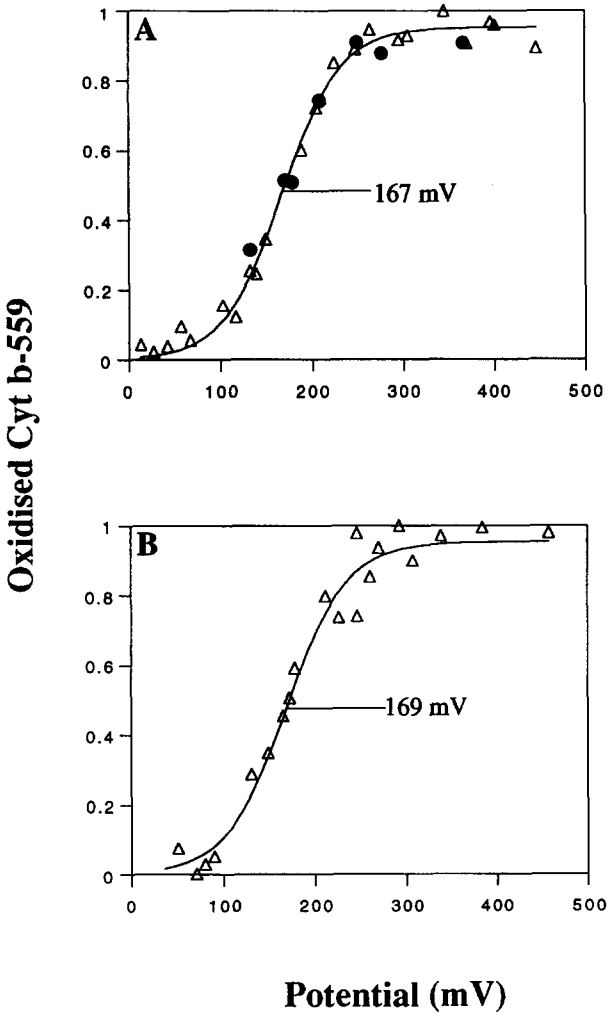

Fig. 5. Redox titrations of cyt *b*-559 in PS-II- enriched membrane fragments treated with divalent cations. (A) Oxidative titration (△) followed by a rapid reductive titration (●) of membrane fragments treated with 1 M $MgCl_2$, 50 mM EGTA; (B) Oxidative titration of membrane fragments treated with 1 M $CaCl_2$ and titrated in the presence of 10 mM $CaCl_2$. Oxidised cyt *b*-559 is expressed as a proportion of the total cytochrome.

4. Discussion

PS II enriched membrane fragments were prepared by detergent solubilisation and found to contain cyt *b*-559 almost exclusively in the HP redox form. The stabilisation of HP cyt *b*-559 in the membrane fragments reported here was made possible by the maintenance of mildly reducing conditions during their isolation and storage. Those samples in which the haem of cyt *b*-559 was allowed to become oxidised were found to contain only 50% of the HP form. This effect was apparent even in samples which although isolated under reducing conditions were oxidised by the addition of ferricyanide at the initiation of the titration, indicating that the conversion to low potential forms occurred rapidly once the haem of the cytochrome had been oxidised (Fig. 2B). The results of the reductive titrations are consistent with previous reported values for the proportion of HP cyt *b*-559 in PS II membranes, with roughly half of the complexes containing this form of the cytochome [16,22]. These analyses, however, were based on estimations of HP cyt *b*-559 after oxidation of the haem. This is necessary in order to observe EPR signals which arise exclusively from ferric ion. In the case of full redox titrations, however, these considerations do not necessarily apply. Nevertheless, for practical reasons, redox titrations are usually performed initially in an oxidative direction. This partly arises from the difficulty in establishing a stable redox potential between −100 mV and −200 mV, in order to record a baseline with the cytochrome fully reduced. With suitable mediators, we found this could be achieved reproducibly by careful redox poising with low concentrations of dithionite. Further problems arise with the use of ferricyanide as a titrant since it can give rise to membrane aggregation. We found the latter effect to be prevented by the addition of high concentrations of bovine serum albumin to the samples.

Under reducing conditions the removal of the 23 kDa extrinsic protein did not significantly affect the redox state of the cytochrome provided that calcium was maintained. Calcium is thought to dissociate from PS II after formation of the S_3 state [36] and little of this metal is lost if salt washed membranes are maintained in the dark [26]. The HP form of cyt *b*-559 was dominant under these conditions despite the absence of the 23 kDa protein. Removal of calcium however resulted in the conversion of the cytochrome into its intermediate potential form. These data are in conflict with the report that higher redox forms of the cytochrome are maintained through the interaction with the 23 kDa protein [16]. Rebinding of the 23 kDa polypeptide has been reported to restore the HP form of cyt *b*-559 in a proportion of salt washed PS II complexes. The possibility, however, that the restoration of the HP form depended upon a synergistic interaction between the rebinding of the protein and the concomitant re-association of calcium was not investigated. Interestingly, it has previously been observed that the inclusion of calcium led to a more pronounced conversion to the HP form upon binding of the 23 kDa protein [27]. The data presented here show that the extrinsic protein does not directly influence the redox state of the cytochrome. In contrast, the HP form is converted to lower potentials following loss of calcium. The presence of this latter polypeptide constitutes a steric barrier to the dissociation of calcium, as well as chloride, and previous data can be explained on this basis. It is possible that earlier reports which associated loss of the 23 kDa protein with the conversion to LP can be re-interpreted in terms of increased calcium depletion from salt washed membranes. Recent data have indicated that the interaction of the 23 kDa protein with lumenally exposed residues of integral proteins causes structural modifications to the manganese cluster in PS II, in agreement with proposals that this polypeptide can modify the conformation of integral proteins [39]. It is therefore suggested that calcium depletion is sufficient to convert HP to IP or LP but the combined reconstitution of both calcium and the 23 kDa protein is necessary in order to restore the HP redox state.

The additional removal of the 33 kDa extrinsic polypeptide associated with PS II resulted in the conversion of the cytochrome to the IP form. It is interesting that, although the structural modifications resulting from the loss of the extrinsic proteins destabilised the HP cyt *b*-559, these were insufficient to convert it to the LP redox form. Despite the presence of high calcium concentrations, the HP form of the cytochrome was not observed in the absence of the 33 kDa polypeptide. On the basis of limited sequence homology with the metal binding domain of known calcium binding proteins, the 33 kDa protein has been considered as a possible candidate to ligate the calcium atom associated with the oxygen evolution activity of PS II [40]. This raises the possibility that the loss of HP in the PS II complexes lacking the 33 kDa polypeptide was related to a disruption of the conformation of the native calcium binding site.

It has been previously reported that acidification of the thylakoid lumen caused a reversible decrease in the midpoint potential of cyt *b*-559 [41,42]. More recently [43,44] it has been demonstrated that, in the presence of a high ΔpH, the donor side of PS II is inactivated due to calcium release and PS II is down-regulated. This effect may constitute an additional mechanism to protect against photoinactivation of PS II, complementing the established zeaxanthin-dependent energy quenching processes [45]. Since the maintenance of HP cyt *b*-559 requires ligation of calcium, as shown in this work, we could speculate that under conditions that generate a high ΔpH, cyt *b*-559 would be in an IP redox form as part of a down-regulated PS II. The precise mechanism by which this new light-induced redox state contributes to energy dissipation remains to be ascertained. Although further investigations are clearly warranted in order to determine the possible functional significance of the apparent interaction between cyt *b*-559 and calcium in PS II, it is interesting to speculate that the function of cyt *b*-559 may be intimately related to its interaction with calcium.

On the basis of the results reported here, we suggest that PS-II-enriched membrane fragments contain the HP redox form of cyt *b*-559. Since the membrane fragments are derived from the appressed regions of the thylakoid membrane, we also conclude that the endogenous LP form of this cytochrome is excluded from such domains. Considering that reducing conditions would be ensured in the stroma of the chloroplast, we suggest that, in vivo, PS II localised in the granal membranes, in the dark, is exclusively in the high redox potential form. In order to fulfil the established stoichiometric requirements for the thylakoid membranes, PS II complexes located in stromal lamellae must be enriched in LP cyt *b*-559. This latter conclusion is in agreement with a number of studies on stromal lamellae vesicles separated from intact granal stacks either by mechanical fractionation or by aqueous two-phase partitioning [46–50]. It has not previously proven possible, however, to isolate by detergent treatment PS-II-enriched membranes of granal origin which retain only HP cyt *b*-559. Moreover, it is shown that the use of Triton X-100 to isolate PS-II-enriched membrane fragments, under the conditions stated here, does not apparently denature or otherwise modify the environment surrounding the haem. In addition, our data suggest that the HP form of the cytochrome requires ligation of calcium in order to be maintained rather than the presence of the 23 kDa protein.

Finally, we suggest that the apparent heterogeneity in the redox forms of cyt *b*-559 arises as a result of the heterogeneity of PS II itself, such that the HP form is associated exclusively with PS II centres located in the grana. It is therefore likely that the presence of significant amounts of IP and/or LP forms of cyt *b*-559 reported

previously in a great number of investigations on detergent-derived PS-II-enriched membrane fragments, PS II core and PS II reaction centre preparations, reflect damage to the protein during isolation.

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