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Rapid Report

Redox potentials of cytochrome *b*-559 in the D1/D2/cytochrome *b*-559 reaction centre of Photosystem II.

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Redox titrations of a stable form of the Photosystem II (PS II) reaction centre, isolated from peas, have detected three redox forms of cytochrome *b*-559: a high-potential form (+430 mV), an intermediate-potential form (+180 mV) and a low-potential form (+25 mV) with relative amplitudes of 28%, 62% and 10%, respectively. These results contrast with the observations of Shuvalov et al. (Shuvalov, V.A., Heber, U. and Schreiber, U. (1989) FEBS Lett. 258, 27–31), who reported midpoint potentials much lower than these for cytochrome *b*-559 in isolated PS II reaction centres. Our data show that, if the reaction centre complex is handled appropriately, then cytochrome *b*-559 can have redox potentials quite close to those found in intact membrane systems.

Cytochrome *b*-559 has long been known to be an integral membrane protein of the Photosystem II (PS II) complex [1,2]. It is composed of two subunits, α and β, whose apparent molecular masses on SDS-PAGE are 9 kDa and 4 kDa, respectively, and which bind one haem [3]. Even the most simple photochemically-active PS II preparation, the isolated PS II reaction centre consisting of the D1 and D2 proteins [4,5], contains cytochrome *b*-559. Despite the close association of cytochrome *b*-559 with PS II, its role is still much debated. Possible functions of cytochrome *b*-559 include (i) its involvement in the electron transfer reactions on the oxidising side of PS II [1]; (ii) mediation of the assembly of the water splitting enzyme [3] and (iii) to protect PS II from photoinhibition, possibly via a cyclic pathway around PS II [6–9].

Despite the number of studies [10–15], there has been no concensus regarding the number of cytochrome *b*-559 haems per PS II reaction centre, although the most recent work does favour a 1:1 stoichiometry [14,15]. Despite this uncertainty, it is well-known that cytochrome *b*-559 can exist in a number of different redox forms. An interesting feature of this PS II haem protein is its unusually high (compared with most *b*-type cytochromes in other organelles) and variable redox midpoint potential (E_m). Its E_m values range from about +370 mV to about 0 mV, with the shift from high to low redox potentials often correlating with treatments which tend to inhibit PS II activity and/or alter membrane structure [2]. Cytochrome *b*-559 is thought to exist, in whole chloroplasts, in two or three forms known as 'high potential' (+370 mV) and 'low potential' (+60 to +80 mV) [2], and in some studies 'intermediate potential' (+240 mV) [16]. A recent study [11] of the redox properties of cytochrome *b*-559 in the isolated PS II reaction centre complex has, however, suggested much lower redox potentials for this cytochrome, namely +70 mV and −500 mV. These values are surprising and could imply that this form of isolated PS II complex is significantly modified from its native form(s) in vivo. For this reason we have carried out a careful study of the redox properties of cytochrome *b*-559 using stabilised preparations of the isolated PS II reaction centre, known to have high photochemical activity.

The isolated reaction centre of PS II, consisting of the D1 and D2 proteins, the apoproteins of cytochrome *b*-559 and the product of the *psbI* gene (the D1/D2 complex), can be, if care is not taken, labile when exposed to light. This is due both to its suscepti-

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Abbreviations: PS II, Photosystem II; E_m, redox midpoint potential; HP, high potential; IP, intermediate potential; LP, low potential.

bility to damage by singlet oxygen created by the recombination of the radical pair [17–19] and to an inbuilt light-activated protein cleavage [20]. This instability of the complex can be overcome by solubilising it in a low concentration (approx. 2 mM) of mild detergent, such as β-lauryl maltoside, and by removing all oxygen from the sample environment. Such stabilised reaction centres show high degrees of activity, with over 94% of the chlorophylls present being functionally coupled to electron transport [21,22].

In the present work, we report a redox study of cytochrome *b*-559 in the stabilised PS II reaction centre, isolated from peas. We observe three redox forms for cytochrome *b*-559 in this complex which have E_m values of +430 mV, +180 mV and +25 mV. These results contrast markedly with those reported by Shuvalov et al. [11] and are consistent with redox data obtained for this cytochrome using more intact PS II systems, including thylakoid membranes. We also report on the effect of high detergent concentrations on the redox potentials of cytochrome *b*-559 in the PS II reaction centre.

PS II reaction centre complexes were prepared as described in Ref. 23 and stored at −80°C.

Cytochrome *b*-559 absorption changes were measured at 559 nm using a Perkin-Elmer 554 spectrophotometer, after background correcting (see below).

Redox titrations of cytochrome *b*-559, in stabilised and active PS II reaction centres, were carried out at 4°C using a redox cuvette similar to that described by Dutton [24]. The redox potential of the medium was monitored by a microcombination Pt/Ag-AgCl electrode (Russell pH, CMPtRL) connected to a Philips PW9420 pH meter. The electrode was calibrated by measuring the potential of a saturated solution of quinhydrone at pH 7.0, prior to each experiment.

The protocol for resuspending the PS II reaction centres and carrying out the redox titrations was as follows. Redox mediators were added to 2 ml of buffer containing 50 mM Tris-HCl ((pH 8.0), subsequently referred to as 'sample buffer') in the redox cuvette, and the whole solution deoxygenated by bubbling, for 1 h, with a stream of oxygen-free nitrogen gas passed through a dithionite solution made up in the sample buffer. β-Lauryl maltoside was then added to the redox cuvette to give a final concentration of 2 mM (unless otherwise stated), followed by the addition of the PS II reaction centres to give a final chlorophyll concentration of 10 μg/ml. This solution was deoxygenated for a further 30 min with a much reduced bubbling rate to avoid frothing. Finally, the electrode was inserted into the sample while maintaining an anoxygenic atmosphere in and above the sample. The low bubbling rate in the sample and the flow of oxygen-free nitrogen gas over the sample were maintained during all redox titrations. Measurements were done by initially adjusting the potential of the sample to between +450 mV and +500 mV with potassium ferricyanide. The absorbance of the sample was then background corrected, between 500 nm and 600 nm, against a reference cuvette containing sample buffer with redox mediators at the same concentration as in the redox cuvette. Cytochrome *b*-559 was subsequently reduced with the appropriate reductant (see below). The potential was allowed to stabilise to within ±1 mV and the absorbance was then monitored at 559 nm. Both reductive and oxidative titrations were carried out. The reductive and oxidative titrants were solutions of sodium ascorbate, dithiothreitol and sodium dithionite (0.005–0.5 M) and potassium ferricyanide (0.005–0.5 M), respectively. These solutions were freshly prepared in deoxygenated sample buffer and the containers sealed.

The titration curves presented were each completed within 3–4 h, unless otherwise stated. Stable samples had titration midpoints reproducible to within ±5%. The red absorption maximum of the sample blue-shifted by 0.2–0.6 nm after each titration; this corresponds to an approx. 5% loss of radical pair forming activity of the PS II reaction centre [22].

The redox mediators used, at 10–20 μM, were: (hydroxymethyl)ferrocene (+405 mV); 2,3,5,6-tetramethylphenylene diamine (+220 mV); *N*-methylphenazonium methosulphate (+80 mV); *N*-ethylphenazonium ethosulphate (+55 mV); 1,4-naphthaquinone (+36 mV); anthraquinone 2-sulphonate (−225 mV) and benzyl viologen (−311 mV). These mediators show negligible absorbance in the 400–700 nm region at the concentrations used.

The concentration of cytochrome *b*-559 was calculated using a molar extinction coefficient of 17.5 mM^{-1} cm^{-1} [3,15].

The ambient potential of our PS II reaction centres in 50 mM Tris (pH 8.0) containing 2 mM β-lauryl maltoside and under anaerobic conditions (in the presence of redox mediators) was 200–220 mV.

Fig. 1 shows the redox titration curve of cytochrome *b*-559 in the PS II reaction centre. It displays three waves: a high potential (HP) component with a redox midpoint potential (E_m) of +430 mV, an intermediate potential (IP) component with an E_m of +180 mV and a low potential (LP) component with an E_m of +25 mV. The titration is fully reversible and each of the waves is well fitted by an $n = 1$ Nernst curve. The relative amplitudes of the HP, IP and LP forms are 28%, 62% and 10%, respectively. The pH dependence of these different redox forms, over the range pH 5.5–9.0, is not very clear, but the HP form does seem to be the least sensitive to pH. The steepness of the titration curves, however, is somewhat dependent on pH over this range (data not shown). The best fits to $n = 1$ Nernst curves were found to be at pH 8.0.

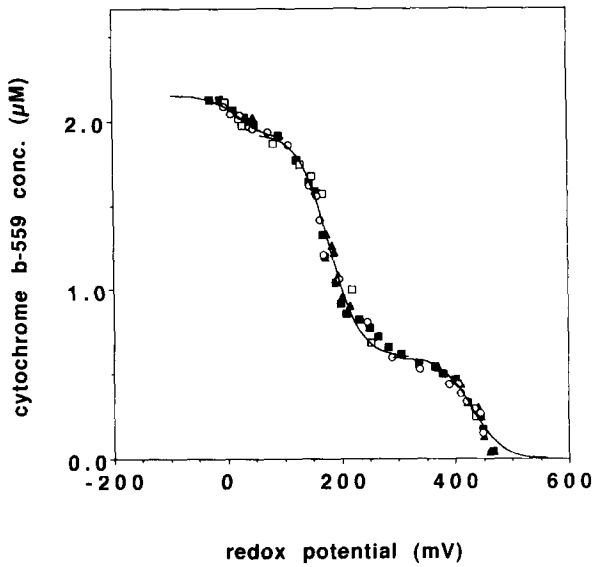

Fig. 1. Redox titration of cytochrome *b*-559 in the presence of 2 mM β-lauryl maltoside (■ and ▲ represent two separate reductive titrations and □ represents an oxidative titration) and in the presence of 1 mM β-lauryl maltoside (○). The curves through the points are $n = 1$ Nernst curves.

The concentration of the fully-reduced cytochrome *b*-559 was found to be 2.1 μM in the samples used here, giving a ratio of cytochrome *b*-559 to chlorophyll of 1:5.7.

Fig. 1 also shows that decreasing the concentration of detergent, present in the sample, to 1 mM, does not affect the cytochrome *b*-559 redox characteristics.

Fig. 2 shows the effect of increasing the solubilising detergent concentration (in this case, to 4 mM β-lauryl maltoside) on the redox titration curve for cytochrome *b*-559 and, also, how the time taken to complete the full titration can affect the final curve observed under these high detergent conditions. If the titration is done quickly (e.g., less than 2 h) then a titration curve, similar to that obtained in fig. 1, is observed (see Fig. 2, curve a), the main difference being a change in the amplitudes of the HP and IP forms: the proportion of the HP form is reduced to 18% while that of the IP form is increased to 72%. As longer times are taken to complete the titration (Fig. 2, curve b), the HP form is completely lost and the titration curve is dominated by more negative redox forms of cytochrome *b*-559. For the titration shown in Fig. 2, curve b, the redox waves display steeper gradients. The total concentration of cytochrome *b*-559 remains unaffected under these high detergent conditions, even though the potentials become more negative overall.

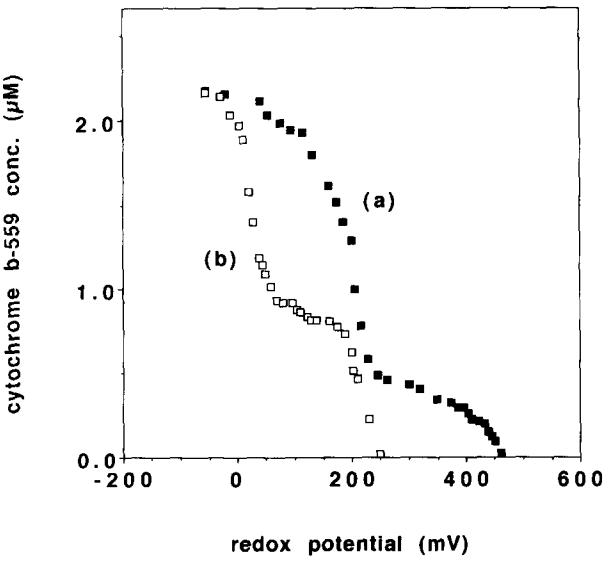

Fig. 2. Redox titration of cytochrome *b*-559 in the presence of 4 mM β-lauryl maltoside. Time taken to complete full titration; less than 2 h, curve a (■); between 3 and 4 h, curve b (□).

In this paper we report on the observation of three redox forms for cytochrome *b*-559 (Fig. 1), using stabilised PS II reaction centre preparations, the E_m values of which are +430 mV, +180 mV and +25 mV. These E_m values are similar to the high potential, intermediate potential and low-potential forms of cytochrome *b*-559 found in intact membrane systems [2,16]. They are, however, very different to the redox values reported by Shuvalov et al. [11], for cytochrome *b*-559 in the isolated PS II reaction centre complex, namely +70 mV and −500 mV. The reason for this difference is not clear, but may reflect a difference in the stability of the PS II reaction centres used (also see below).

It is interesting to note that, in a very careful and detailed study, Thompson et al. [25] observed a loss of the high potential form of cytochrome *b*-559 in PS II samples depleted of the 17, 23 and 33-kDa polypeptides and the manganese complex. In fact, Thompson et al. [25] observed that the high potential form only contributes 44% to the total reducible cytochrome *b*-559 content in untreated PS II particles and that this is reduced to 18% upon the removal of the 17 and 23-kDa polypeptides. Our data indicate that it is possible for the high-potential form of cytochrome *b*-559 to exist even in the most simple PS II particle, namely the D1/D2/cytochrome *b*-559 complex, and that it can contribute up to approx. 30% of the total reducible cytochrome *b*-559 content.

We observe that high detergent concentrations do not destroy cytochrome *b*-559, but cause a negative shift in the redox potentials which are observed (Fig. 2). Under these conditions the high potential form is lost and more negative redox forms of cytochrome *b*-559 are observed. In Fig. 2, curve b, only two redox forms appear to be present, but at other detergent levels more than two redox forms were observed (data not shown). This suggests that a wide distribution of redox forms might exist for cytochrome *b*-559 under certain conditions. We have no simple explanation for

why the redox waves observed under high detergent conditions (e.g., Fig. 2, curve b) appear steeper than those observed in Fig. 1. It should be noted that high detergent concentrations cause the PS II reaction centre to be unstable [17].

The unusually-low-potential form of cytochrome *b*-559 (−500 mV) observed by Shuvalov et al. [11] could be due to the effect of detergent reported here. However, the concentration of detergent used by these authors (0.05% Triton X-100) is considered to be 'low' in a study by Newell et al. [26], in which the effect of the detergent Triton X-100, on the conformation of the isolated PS II reaction centre complex, was studied.

We have shown here that if the reaction centre complex is handled appropriately then cytochrome *b*-559 can have redox potentials quite close to those found in intact membrane systems. The preparation and solubilisation conditions used here are the same as those used for our other PS II reaction centre studies [27–29]. We therefore conclude that the previous data reported for the redox states of cytochrome *b*-559 within the isolated PS II reaction centre (see Ref. 11) are misleading and do not reflect the cytochrome *b*-559 potentials for the most active state of this complex.

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