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Relationship between O_2 evolution capacity and cytochrome *b*-559 high-potential form in Photosystem II particles *

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As previously shown for inside-out vesicles by Larsson et al. (Larsson, C., Jansson, C., Ljungberg, U.L., Åkerlund, H.E. and Anderson, B. (1984) in Advances in Photosynthesis Research, Vol. I, pp. 363–366 (Sybesma C., ed.), Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands), we observed that NaCl 1 M washing of Photosystem II particles prepared by Triton X-100 treatment of spinach thylakoids induces both an inactivation of oxygen evolution and transformation of cytochrome *b*-559 from its high-potential to its low-potential form. A partial reactivation of water oxidation by 24 kDa polypeptide refixation is accompanied by a partial restoration of the cytochrome *b*-559 high-potential (HP) form. In contrast, reconstitution of water splitting by Ca^{2+} addition is not associated to a reestablishment of the cytochrome (HP) form. We conclude that cytochrome *b*-559 HP plays no role in water oxidation.

The washing of PS II particles by high concentrations (1–2 M) of NaCl releases from the membrane two extrinsic proteins of apparent 18 and 24 kDa molecular weight, and, in parallel, leads to an inhibition of oxygen evolution [1–3]. The amplitude of the inhibition varies, and ionic conditions in the assay, especially Ca^{2+} and Cl^- concentrations, seem to be critical [4,5]. It has been shown that centers unable to evolve oxygen after the salt treatment can still perform partial positive charge accumulation on the donor side of PS II [3,6]. From the luminescence measurements, Boussac et al. [7] concluded that the NaCl washing blocks the S_3 to S_0 transitions.

Reactivation of oxygen evolution can be obtained either by refixation of 24 kDa polypeptide [2,8] or by addition of millimolar concentration of Ca^{2+} [4,9]. In the former case micromolar concentrations of Ca^{2+} are required [10].

The 18 kDa protein seems not necessary if 10 mM Cl^- are present in the resuspending medium [11].

These two proteins (18 and 24 kDa) may only play a conformational role in concentrating and maintaining Ca^{2+} and Cl^- required for water oxidation [10,11].

PS II particles contains cytochrome *b*-599, but the role of this component is still obscure. The cytochrome occurs in two forms, the high-potential form and the low-potential form. The high-potential form is labile and converted in the low-

* Dedicated to the memory of W.L. Butler.

Abbreviations: Chl, Chlorophyll; Cyt, cytochrome; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HP and LP Cyt *b*-559, High Potential and Low Potential cytochrome *b*-559; PS II, Photosystem II; Mes, 4-morpholineethanesulphonic acid.

potential form by many of the same treatments that also cause a loss of O_2-evolution capacity [12–14]. The conversion to the low-potential form is also caused by the treatment of inside-out vesicles with NaCl [15]. Because the inactivation of O_2 evolution by NaCl treatment is partially reversible, it seemed interesting to examine if restoration of oxygen evolution was associated with the transformation of low-potential cytochrome back to the high-potential form.

Oxygen-evolving Photosystem II particles were prepared by Triton X-100 treatment of spinach thylakoids according to Ref. 16. Before use, the concentrated particles (1.2 mg Chl/ml) were stored at 77 K in a medium containing 30% (v/v) ethylene glycol, 300 mM sucrose, 10 mM NaCl, 25 mM Mes (pH 6.6). The treatment with NaCl (1 M) was performed in the light as in Ref. 8. Isolated 24 kDa protein was prepared as in Ref. 8.

Hill activity was determined by measuring the initial rate of oxygen evolution under saturating white light with phenyl-benzoquinone (0.45 mM) as the Hill oxidant. The particles were used at a concentration of 20 μg Chl/ml in a medium containing Mes (25 mM), NaCl (10 mM) and EGTA (10 μM). The cytochrome *b*-559 concentration was determined in the same medium, plus 10% v/v ficoll 400, at 40 μg Chl/ml with an Aminco DW 2A spectrophotometer. We measured absorption changes at 559 nm versus 570 nm as in Ref. 7 induced by the sequential addition of ferricyanide (0.25 mM), ferrocyanide (2 mM), hydroquinone (2 mM), ascorbate (4 mM) and dithionite (3 mM) (final concentrations). As shown in Fig. 1 for two cases, we also scanned the spectra between 530 and 590 nm once in the various redox conditions, and we observed that dithionite reduction does not induce detectable shift of the absorption maximum indicating that cytochrome *b*-559 is the major cytochrome present in these particles. The concentration in cytochrome *b*-559 was calculated according to Ref. 12, i.e., with $\epsilon_{559-570nm} = 15 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The content in cytochrome *b*-559 HP corresponding to the hydroquinone reducible forms was about equal to the content of cytochrome *b*-559 found in the reduced state before the addition of ferricyanide.

In Table I are reported Hill activities and contents in high-potential forms of cytochrome *b*-559

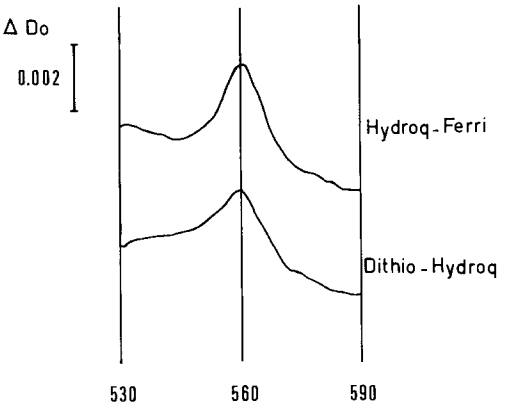

Fig. 1. Absorption spectra of reduced cytochrome *b*-559. The sample was first oxidized by ferricyanide (Ferri) then reduced successively by ferrocyanide, hydroquinone (Hydroq.), ascorbate and finally dithionite (Dithio). For detailed conditions, see text.

in PS II particles before and after NaCl 1 M treatment and in the high-salt-treated particles after subsequent addition of Ca^{2+} 10 mM or refixation of the 24 kDa protein. We determined both the hydroquinone and ferrocyanide-reducible amounts of cytochrome *b*-559 because Horton and Croze [18] demonstrated that the redox potential of the high-potential form can vary depending upon the conditions.

NaCl washing does not affect the cytochrome *b*-559 total content which is 2 cytochromes per 280 chlorophylls; Miyao and Murata [19] found 2 cytochromes per 220 chlorophylls. The difference can be due to a larger antenna size of PS II, in our case. It is known that the antenna size can vary upon growth conditions [20].

In untreated particles about half of the cytochrome is in the high-potential forms. An addition of $CaCl_2$ (10 mM) slightly stimulated the Hill reaction activity, but did not affect the potential of the cytochrome.

NaCl 1 M treatment induced together with the decrease of O_2 evolution a large transition from high- to low-potential forms as reported previously in inside-out vesicles by Larsson et al. [15]. Notice that the ferrocyanide reducible form is the most affected. A $CaCl_2$ addition considerably restored oxygen evolution, but did not restore cytochrome *b*-559 to its high potential form either in dark- or in light-adapted samples. We included the light

TABLE I

EFFECTS OF NaCl 1 M WASHING, $CaCl_2$ 10 mM ADDITION, AND 24 kDa POLYPEPTIDE REFIXATION ON THE CYT *b*-559 HP CONTENT AND THE HILL ACTIVITY OF PS II PARTICLES

Ca^{2+}, when added, was incubated with the sample for 5 min in the dark. The 24 kDa protein, when added, was used at 0.2 mg protein/mg chlorophyll and incubated for 1 h at 0°C. The values in parenthesis were obtained after a 30 s illumination of the sample. For further experimental details, see the text. Chl/Cyt *b*-559 ratio are 140 ± 6 and 144 ± 7, respectively in control and NaCl 1 M washed particles.

Particles	Additions	Cyt *b*-559 (% of total Cyt *b*)		O_2 evolution (%)
		Hydroquinone-reducible	Ferrocyanide-reducible	
Control	None	59 ± 4	34 ± 4	80 ± 2
	Ca^{2+}	60 ± 4	30 ± 4	100 ± 2 [a]
NaCl 1 M washed	None	34 ± 2.5	17 ± 1	26 ± 5
	Ca^{2+}	28 (27) ± 5	12 ± 4	75 ± 5
	24 kDa	39 ± 2	24 ± 1	45 ± 5
	24 kDa + Ca^{2+}	37 (23) ± 2	18 ± 2	71 ± 5

[a] 100% Oxygen evolution rate corresponds to 330 μmol O_2/mg Chl per h, temperature 20°C.

adaptation because in this way the conditions of the cytochrome measurements were better comparable with the conditions of the Hill activity measurement. Another reason for the illumination was that Butler and Matsuda [21,22] have suggested that protonation associated to water splitting may be required to build the high-potential form. Refixation of 24 kDa protein alone induces a partial restoration only of the high-potential form reducible by ferrocyanide together with partial regeneration of oxygen evolution activity. This result is consistent with data obtained by Larsson et al. [15]; $CaCl_2$ addition after 24 kDa refixation did not increase the amount of the high-potential form, whereas it stimulated the oxygen-evolution rate.

Butler and Matsuda [22] suggested that cytochrome *b*-559 HP is not absolutely required for oxygen evolution, but rather helps to stabilize the high S states. Measurements performed by Boussac et al. [7] on the same preparations as utilized here, did not support this hypothesis. Indeed they observed that S_2 deactivation in NaCl-washed particles reactivated by Ca^{2+} addition is the same as in untreated particles (34 and 35 s, respectively). S_3 deactivation is only slightly accelerated (28 and 40 s, respectively). The data thus suggest, in line with earlier indications [12] that cytochrome *b*-559 has no role whatsoever in water oxidation.

Butler and Matsuda [22] also proposed that the high-potential form necessitates a hydrophobic environment; thus refixation of 24 kDa polypeptide may create this hydrophobicity at the level of cytochrome *b*-559. Moreover, Ljungberg et al. [23] showed by immunology that cytochrome *b*-559 is structurally associated to 24 kDa protein. However, a strict correlation between the presence of 24 kDa protein and cytochrome *b*-559 HP is not supported by data from Ono et al. [24]. They showed that oxygen-evolving ImL plastids (from plants grown in intermittent light) contain the 24 kDa protein but almost no cytochrome *b*-559 HP form.

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