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Rapid communication

On the question of the identity of cytochrome *b*-560 in thylakoid stromal membranes

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Abstract

Stromal membranes enriched in PS I contain a low potential cytochrome with a reduced α-band peak close to 560 nm. The identity of this cytochrome component has been ascribed either to a low potential form of the Photosystem II cytochrome *b*-559 or to a different cytochrome with a reduced α-band of 560 nm. The half-bandwidth of the 560 nm component in stromal membranes is identical to that of purified cytochrome *b*-559. Western blots show that the stromal membranes contain an amount of PS II cytochrome *b*-559 α-subunit that is more than sufficient to account for the cytochrome *b*-560 detected spectrophotometrically in these membranes. These immunochemical data and the similarity of (i) the spectral peaks, and (ii) the redox properties of low potential PS II cytochrome *b*-559 and the *b*-560 component, suggest that the simplest inference is that the cytochrome *b*-560 protein in stromal membranes is identical to the PS II cytochrome *b*-559.

Abbreviations: A – absorbance; cyt – cytochrome; DCBQ – 2,5-dichloro-*p*-benzoquinone; E_{mx} – mid-point potential at pH x; hbw – half-bandwidth; LP – low potential; MD – menadiol; MES – 2-(N-morpholino)ethanesulfonic acid; MHQ – methylhydroquinone; PS I – PS II, photosystems I, II; SDS–PAGE – sodium dodecylsulfate polyacrylamide gel electrophoresis

Introduction

Chloroplast thylakoid membranes contain two known cytochrome *b* proteins, cytochromes *b*-559 and b_6 (Cramer and Whitmarsh 1977), that are encoded, respectively, by the chloroplast *psb*E-*psb*F and *pet*B genes. The cyt *b*-559 α and β polypeptides are known to be an intrinsic part of the Photosystem II reaction center (Nanba and Satoh 1987), and the presence of the cytochrome in the PS II reaction center contrasts with the photosynthetic bacterial reaction center (Deisenhofer and Michel 1989). The cyt b_6f complex is present to a significant extent in both granal and stromal membrane fractions (Whitmarsh 1986).

The function and significance of lower potential forms of cyt *b*-559 is a problem with a long history (Cramer and Whitmarsh 1977), as is the question of whether thylakoid membranes contain an additional *b* cytochrome with redox and spectral properties somewhat similar to those of cytochrome *b*-559 (Rolfe et al. 1987). The reduced α-band maximum of cyt *b*-559 associated with Photosystem II in thylakoid membranes has generally been reported to be 559 nm, but also at 560 nm (Cramer and Butler 1967, Buser et al. 1992) and at 559.8 nm for the purified cyto-

chrome (Widger et al. 1984). Part of the interest in this cytochrome arises from the unusually positive midpoint potential of the cytochrome in native thylakoid membranes (E_m = +390–400 mV) (Horton et al. 1976). However, some of the enigmatic aspects of this cytochrome arise from (i) the lability of this high potential redox state (Cramer and Whitmarsh 1977), and (ii) the heterogeneity of its redox and spectral properties, including the presence in thylakoid membranes of 1–2 low potential heme equivalents in excess of the equivalents associated with the reaction center (Heber et al. 1976, Vallon et al. 1987). 'Low potential' PS II cyt *b*-559 is defined as the cytochrome species reducible by (a) dithionite or (b) ascorbate or menadiol, but not by the weak reductants ferrocyanide or hydroquinone. The measured E_m of low potential PS II cyt *b*-559 in thylakoid membranes ranges from +32 mV (Peters et al. 1983) to +110 mV (Rich and Bendall 1980).

Materials and methods

1. *PS I-enriched stromal membrane vesicles*

Stromal membranes prepared from lettuce or pea thylakoids according to Rich et al. (1987) had a Chl *a*/*b* ratio of 4.5:1, determined according to Porra et al. (1989). Membranes prepared by digitonin treatment of spinach thylakoids (Peters et al. 1983), provided for these studies by I. Baroli and Dr A.R. Crofts, had a Chl *a*/*b* ratio = 7.3, were devoid of PS II O_2 evolution activity (H_2O to DCBQ), and contained a *b*-cytochrome component with a reduced α-band peak at 560 nm with E_{m7} = + 50 mV (Baroli et al. 1991).

2. *Purified and crude cytochrome b_6f complexes*

Purified cyt b_6f complex was prepared for spectrophotometric experiments from pea by a modified (Rich et al. 1987) method of Hurt and Hauska (1981). A crude digitonin cyt b_6f complex was obtained from pea or lettuce by protamine sulfate fractionation of a PS I-enriched digitonin extract of chloroplasts (Nelson and Neumann 1972).

3. *Redox difference spectra*

These were measured as in Rich et al. (1987), using a measuring beam hbw = 0.25 nm. Protein complexes in residual detergent and membranes were resuspended in 50 mM K-MES buffer, pH 6.0.

4. *Immunochemical assays*

Membrane fractions were separated on a 15–20% acrylamide gradient SDS–PAGE including 6 M urea, and transferred (130 mA, constant current, 45 min) on nitrocellulose paper (0.45 μ pore diameter).

Results and discussion

The presence of a cytochrome component with a reduced α-band at 560 nm can be seen in stromal membrane vesicles (Fig. 1A, menadiol-methylhydroquinone spectrum), but not in the cyt b_6f complex (Fig. 1B) purified from lettuce chloroplasts. The methylhydroquinone – fully oxidized and dithionite-menadiol spectra show the presence of apparently pure (by the criterion of peak absorbance wavelength and symmetric absorbance band) cytochrome *f* (λ_{max} = 554 nm) and b_6 (λ_{max} = 564 nm) components in the approximate ratio of 1:2, as expected for the cyt b_6f complex (Hurt and Hauska 1982). The *b*-560 component in Fig. 1A appears to have a slight asymmetry on the long wavelength side. However, when this spectrum is deramped and deconvoluted, it is fit very well with one major and one minor gaussian component (Fig. 1C) having maxima at 560.2 nm and 549.8 nm, with hbw = 12.3 and 6.0 nm, respectively. The 12.3 nm hbw is identical to that of the difference spectrum of purified PS II cyt *b*-559 (Widger et al. 1984). If an additional component with a 563 nm peak is added, the fit is worse (data not shown). It was calculated that 0.84 cyt *b*-560 hemes/2 cyt b_6 hemes were present in these membranes.

The existence of this *b*-560 component in thylakoid membrane fractions enriched in PS I

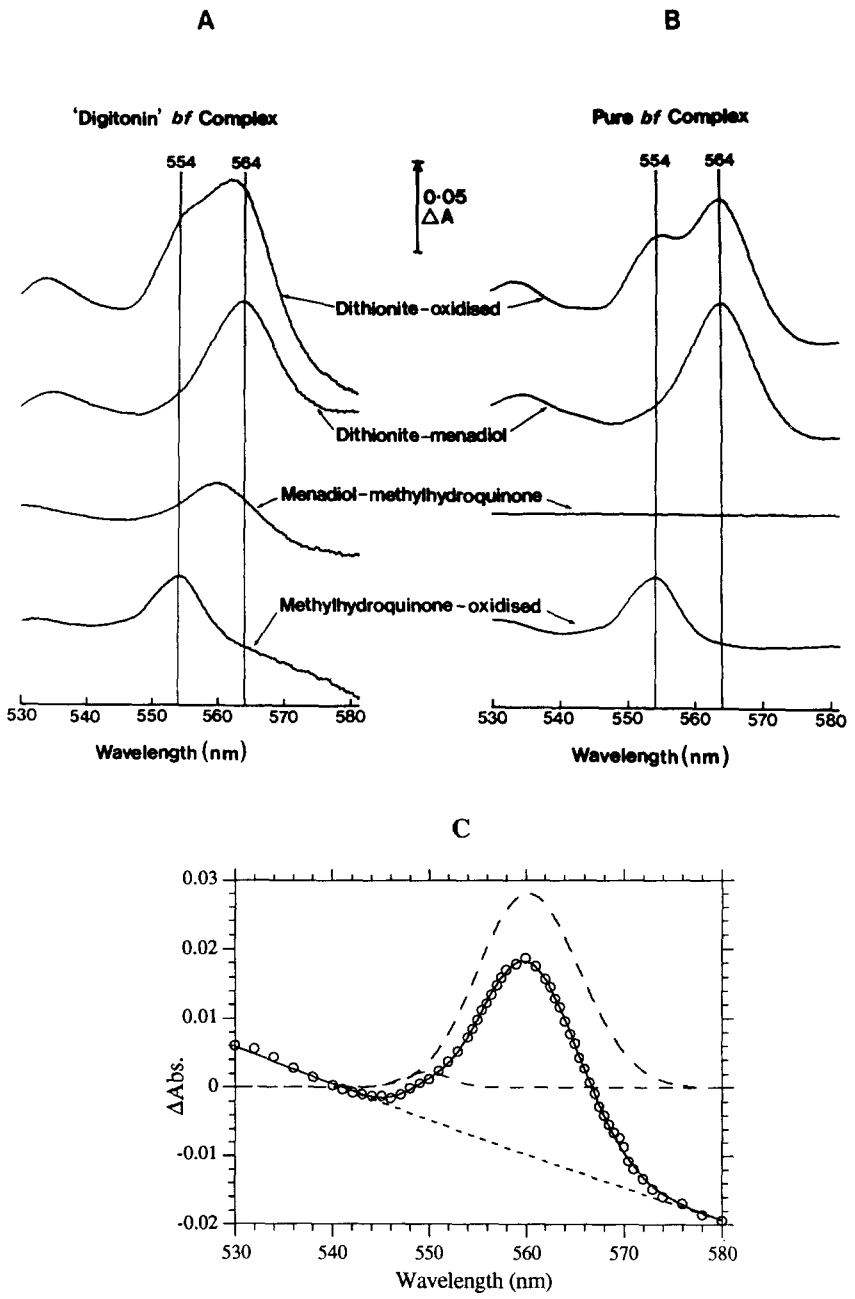

Fig. 1. Redox difference spectra of (A) crude cyt b_6f complex from lettuce (150 μg Chl/ml), and (B) purified lettuce cyt b_6f complex, both at a b_6f concentration of 1.5 μM. From top, the spectra are (i) dithionite reduced-oxidized, (ii) dithionite-MD, (iii) MD-MHQ, and (iv) MHQ-oxidized. (C) Deconvoluted spectrum of (A-iii) obtained using 'Peak Fit' (Jandel), with two gaussian components having maxima at 560.2 and 549.8 nm and hbw = 12.3 and 6.0 nm, respectively. (○) subset of spectral data with 1 nm spacing (2 nm on wings); (– –) gaussian components and ramp used for deconvolution. Ferricyanide, oxidant; (a) MHQ (1 mM), (b) MD (1 mM), or (c) dithionite used as reductant of (a) cyt *f* and high potential PS II cyt *b*-559, (b) PS II cyt *b*-559_{LP} and/or cyt *b*-560, and (c) cyt b_6. For calculation of stoichiometry of cyt components with peaks at 560 and 564 nm (cyt b_6), the following ΔA ratios were assumed: for '*b*-560,' $\Delta A_{564}/\Delta A_{560} = 0.75$; for b_6, $\Delta A_{560}/\Delta A_{564} = 0.72$; then, ΔA of '*b*-560' alone = $[\Delta A_{560} - (0.72)\ (\Delta A_{564})]/[1 - (0.75)\ (0.72)]$, and ΔA of b_6 alone = $[\Delta A_{564} - (0.75)\ (\Delta A_{560})]/[1 - (0.72)\ (0.75)]$

has been noted previously, and has been attributed to the presence of (i) low potential PS II *b*-559 (cyt b-559_{LP}) (Anderson and Boardman 1973, Peters et al. 1983); (ii) alternatively, the *b*-560 component in plant (Rich and Bendall 1980, Vallon et al. 1987, Baroli 1991) or *Chlamydomonas reinhardtii* thylakoid membranes (Rolfe et al. 1987) has been attributed to a different cytochrome with an $E_{m7} = +50$ mV (Baroli et al. 1991). Either hypothesis would account for the presence of significant amounts of LP *b*-559-like cytochrome in thylakoids (Heber et al. 1976).

These arguments for a distinct cyt *b*-560 are apparently consistent with the difference in α-band peaks in the *C. reinhardtii* F18 cyt b_6f^- mutant between the PS II cyt *b*-559 and a lower potential cyt *b*-560 that was also PS II-linked (Rolfe et al. 1987). However, a difficulty with spectrophotometric analysis of cytochromes in thylakoid membranes of *Chlamydomonas* is the difficulty of separating thylakoid and mitochondrial membranes (Atteia et al. 1992). This might be a particularly severe problem when analyzing the origin of cytochromes present at a low level.

The possible presence of the PS II cyt *b*-559 α-polypeptide in stromal membranes from pea and spinach was tested immunochemically with Western blots, using antibody to the COOH-terminal tridecapeptide of this cyt *b*-559 α-subunit that has been described previously (Tae et al. 1988). PS I membranes were used from pea instead of lettuce because they had better defined bands on SDS–PAGE, while possessing a very similar cytochrome content. The main conclusion from these Western blots is that the stromal membranes have a relatively high content of the cyt *b*-559 α- and D2 polypeptides characteristic of the PS II reaction center (Fig. 2). Moreover, using as a reference the antibody reactivity of the cytochromes in thylakoids (lanes B and G), where the content of each cytochrome is equal within a factor of two, the amount of cyt *b*-559 α-subunit in these membranes (lanes C, D, Fig. 2) exceeds the amount of cyt b_6 polypeptide (lanes H, I, Fig. 2). This comparison can be made relatively simply, in spite of the qualitative nature of Western blots, because the thylakoid membrane reactivity with the antibodies to cyt *b*-559 α-subunit and cyt b_6 (lanes B and G, respectively), are qualitatively comparable. The intensity

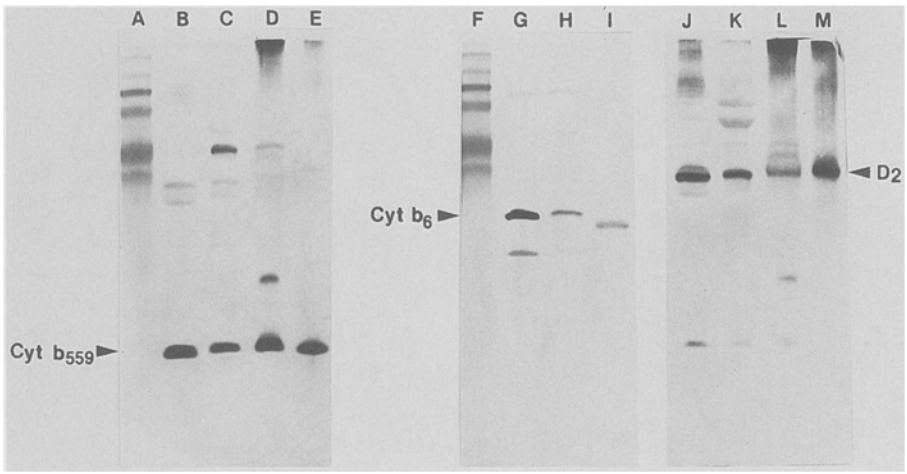

Fig. 2. Western blot analysis of spinach thylakoid membranes (lanes B, G and J) and stromal membranes from: (i) spinach (Peters et al. 1983) [lanes C, H and K]; (ii) crude b_6f complex from pea (Nelson and Neumann 1972) with a *b*-560:b_6 heme ratio = 0.79:2.0 (lanes D, I and L). Antibodies to cyt *b*-559 (lanes B–E), cyt b_6 (lanes G–I), and the PS II D2 polypeptide (lanes J–M). Lanes A, F, molecular weight (kDa) standards: triose phosphate isomerase, 26.6; lactate dehydrogenase, 36.5; fumarase, 48.5; pyruvate kinase, 58; fructose-6-phosphate kinase, 84; β-galactosidase, 116; α_2-macroglobulin, 180. Purified D1-D2-cyt *b*-559-CP 47 PS II preparation (5 μg, lanes E, M) from spinach was used as a size marker for cyt *b*-559 and D2. Cyt b_6f complex from spinach was used as a control (not shown) to confirm M_r values of cyt b_6 from spinach thylakoids and stromal membranes (lanes G, H) compared to pea stromal membranes (lane I); membrane equivalent of 10 μg chlorophyll added to each lane.

of the D2 polypeptide band in the spinach stromal membranes (lane K, Fig. 2) is also relatively high. The smaller staining intensity at the position of the D2 polypeptide in crude b_6f complex from pea is presumably related to a significant amount of protein not entering the gel (lane L).

Abundance of cytochrome b-559 α-subunit in stromal membranes

The Western blot data (Fig. 2) indicate that there is more than enough PS II cyt *b*-559 α-subunit present in the stromal membranes to account for the *b*-560 component. The measured cyt *b*-560 heme:cyt b_6 heme ratio is 0.42:1. If cyt *b*-560 is really PS II cyt *b*-559, then the polypeptide ratios of the PS II cyt *b*-559 α:cyt *b*-559 β:cyt b_6 would be: 0.84:0.84:1 because there are two *b* hemes in the cyt b_6 polypeptide. Assuming the *b*-560 spectral component is associated with the PS II cyt *b*-559 protein, the spectrophotometric data indicate that the content of cyt *b*-559 α and β polypeptides that is present in the PS I membranes is smaller than that of the cyt b_6 polypeptide (Hurt and Hauska 1982, Cramer et al. 1986). However, the ratio of PS II cyt *b*-559 α-subunit to cyt b_6 polypeptide, normalized to their relative content in thylakoids, as shown in the Western blots (Fig. 2), is >1. That is, there is enough cyt *b*-559 α-subunit to account for the *b*-560, the excess polypeptide perhaps belonging to nascent centers (see below). The properties of the 560 nm α-band in the stromal membranes are, therefore, not sufficiently different from that of the PS II cyt *b*-559 to warrant a conclusion that the stromal cyt *b*-560 corresponds to a distinct cytochrome.

Concerning the Western blot analysis, it is important to note that similar experiments were carried out with stromal membranes isolated by French Press treatment by Vallon et al. (1987) who arrived at a different conclusion. The fraction of cyt *b*-559 in stromal membranes detected immunochemically and by chemical difference spectra in those studies was 8.8% and 19%, respectively, of the total cyt *b*-559, which suggested the existence of a cyt *b*-559_{LP} or *b*-560 antigenically distinct from the PS II high potential form.

PS II components in PS I membrane; repair, assembly

The presence of a significant concentration of the D1, D2 and cyt *b*-559 PS II reaction center polypeptides in stromal membranes has been noted previously (Mattoo and Edelman 1987, Callahan et al. 1989). The D1 protein found in centers defective in $Q_A \rightarrow Q_B$ electron transfer in the stromal membrane fraction has been proposed to be newly synthesized protein that is assembled in the stroma as part of a repair cycle to replace damaged and rapidly turning over D1 protein in the appressed membrane (Melis 1991). Less is known about damage, turnover and new assembly of the D2 and cyt *b*-559 polypeptides, although light-induced degradation of D1 protein caused migration of PS II polypeptides (D2, cyt *b*-559, 33 kDa and CP 43) from the appressed to non-appressed membrane regions (Hundal et al. 1990, Aro et al. 1990).

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