Isolation and Comparison of Molecular Properties of Cytochrome *b*-559 from Both Spinach Thylakoids and PS II Particles

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Z. Naturforsch. **44c**, 415–422 (1989); received November 28, 1988

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Cytochrome *b*-559, Spinach Thylakoids, PS II Particles, High and Low Potential, Interconvertible Forms

Cytochrome *b*-559 has been purified from both spinach (*Spinacia oleracea* L.) thylakoids and photosystem II particles in order to compare some of its more polemic molecular properties. In the two cases, cytochrome *b*-559 (which is initially present in its reduced high-potential form and in its oxidized low-potential form) is purified as a single species by preparative disc electrophoresis in Triton-containing polyacrylamide gel, the isolated protein exhibiting similar molecular mass and almost identical low midpoint redox potential and absorption spectra. Moreover, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified cytochrome *b*-559 reveals, again in both cases, only one polypeptide band of similar molecular mass. Integration of the isolated low-potential form into liposomes partly restores the high-potential form. These data corroborate our previous results and indicate that there exists in thylakoids only a cytochrome *b*-559 molecular species tightly bound to PS II and that the two naturally occurring low- and high-potential couples are physiological and correspond to interconvertible states of the genuine heme protein.

A prolonged and yet unsolved discussion exists in the literature as to the number, midpoint potentials, pH-dependence, molecular weights and other properties of the enigmatic, membrane-bound *b*-559 cytochrome which is present in chloroplasts [1–7]. To begin with, cytochrome *b*-559 exhibits *in situ* different behaviour [1–5] and is exceptionally difficult to bring into solution, its extraction requiring sonication in the presence of detergents [2, 8, 9].

It has been generally admitted [1–4, 7, 10, 11] that the labile high-potential (HP) form of cytochrome *b*-559, tightly bound to photosystem II (PS II) and apparently located solely in the appressed regions of plant chloroplasts which constitute the grana, is the physiological relevant species and that the stable low-potential (LP) form is a non-physiological HP altered form. Some authors [1–3, 12–15] have even claimed that chloroplasts normally contain another physiological LP cytochrome *b*-559 form which is found in photosystem I (PS I) particles and exhibits a significantly lower potential than the altered HP form. According to these authors, when chloroplasts are fractionated with digitonin, HP cytochrome *b*-559 remains associated with PS II, while the physiological LP cytochrome *b*-559 species, which is located in the unappressed stromal membranes, either follows PS I [3, 12–14] or is obtained in a distinct cytochrome *bf* particle [12, 16–18]. The question is further complicated by the fact that a range of intermediate redox forms may exist [1, 2, 4, 6–8]. Our group [19, 20] has recently characterized the redox and acid-base states of cytochrome *b*-559 in both spinach thylakoids and PS II particles and has besides presented evidence that its LP and HP forms are, respectively, non-protonated and protonated interconvertible molecular pairs of the same heme protein.

In 1971 Garewal and Wasserman [21, 22] reported the extraction and purification of cytochrome *b*-559 in its middle potential (MP) form from spinach thylakoids. Key steps in the procedure included removal of chlorophyll from the particles with ethanol, solubilization of the cytochrome (stabilized with dithiothreitol) at pH 8 with 2% Triton X-100/2–4 M urea, removal of impurities through a DEAE-cellulose column and, as a final step, polyacrylamide gel electrophoresis with non-ionic detergent. It was concluded that cytochrome *b*-559 is a "mosaic lipoprotein" of molecular weight (MW) 111,000 with about eight small polypeptide chains of three kinds but only one size (MW around 6000). The method was slightly modified in 1982 by Zielinsky and Price [9], who

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0341–0382/89/0500–0415 $ 01.30/0

used it to show that cytochrome *b*-559 is translated and assembled in isolated chloroplasts [23] and corroborated that it has a subunit MW of 6000. More recently, Matsuda and Butler [24] purified, following Garewal and Wasserman [21, 22] and Zielinski and Price [9] methods, a single lipoprotein (MW 110,000) as a LP form; chromatography on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gave a single band corresponding to the 6 kDa polypeptide monomer.

Lach and Böger solubilized cytochrome *b*-559 in its HP form [25] and prepared the heme protein in a homogenous form by chromatography on a Biogel A column [26], thus eliminating the requirement for the lengthy electrophoretic step of previous methods [8, 9]. Analytical SDS-PAGE revealed [26] a subunit MW of 17,000 for the protein from the alga *Bumilleriopsis* and 37,000 for the protein of spinach. A heme protein with a subunit MW of 14,000, which appeared to be related to cytochrome *b*-559, was observed by Maroc and Garnier [27] in the alga *Chlamydomonas reinhardtii*. Koenig and Moller [28] isolated LP cytochrome *b*-559 from chloroplasts of barley using a DEAE-Sepharose column and found that the heme protein of 120 kDa could be dissociated, depending on the electrophoretic conditions used, into components of 31, 30, 18 and 9 kDa.

In 1983 Metz *et al.* [29] presented a rapid and simple procedure for the purification of LP cytochrome *b*-559, using oxygen-evolving PS II preparations as the starting material and DEAE-cellulose column as a chromatographic step. Lithium dodecyl sulfate polyacrylamide gel electrophoresis of the purified cytochrome *b*-559 from both spinach and maize revealed then a major polypeptide band (9 kDa) and two minor bands (10 kDa and 6 kDa). Starting in 1984 Widger *et al.* [30] purified cytochrome *b*-559 from spinach thylakoids using three chromatographic steps (DEAE-cellulose, hydroxylapatite and DEAE-Sephacel) and found that it had (besides a small polypeptide of 6 kDa) a dominant polypeptide of 10 kDa, which was further purified, analyzed and sequenced in the first 27 residues. This sequence, together with the antibody generated to the purified cytochrome polypeptide, allowed [4] location of the corresponding gene on the plastid chromosome of spinach and determination of its complete nucleotide sequence and thereby the amino acid polypeptide sequence of an 82 amino acid polypeptide of a molecular mass of 9.16 kDa containing a single histidine residue. Biochemical, biophysical and molecular biological data obtained by the same group [4] have later suggested that spinach cytochrome *b*-559 contains a second 38 amino acid polypeptide of 4.27 kDa, also presenting a single histidine residue. Thus, assuming a heterodimer structure ($\alpha\beta$, one heme), the molecular mass of the apoprotein of spinach chloroplast cytochrome *b*-559 can be calculated to be 13.43 kDa.

The present investigation deals with the purification of cytochrome *b*-559 from both spinach thylakoids and PS II particles. Electrophoretical analysis of the purified LP heme protein revealed in the two cases only a polypeptide subunit of similar molecular mass. The isolated LP form can be partially reconverted into the HP form by integration into liposomes.

Materials and Methods

Purification of cytochrome b-559

Purification to apparent electrophoretic homogeneity of native cytochrome *b*-559 from both spinach thylakoids [19] and PS II particles [20] was carried out by the method of Garewal and Wasserman [8, 21, 22], but with certain modifications that, according to the results obtained, may have been quite significant.

Solubilization of the cytochrome was accomplished, after chlorophyll removal with ethanol and Tris washing, by dispersing the pellet in 2% Triton X-100 (w/v), 4 M urea, 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol and sonicating the dispersion for 2 min on ice (10-ml aliquots and eight 15-s periods with 1-min cooling intervals), using a Branson sonifier, model B-12, selected to a sonic power of 90 W. The sonified suspension was clarified by centrifugation ($60{,}000 \times g$, 30 min), and the supernatant was loaded on to a DEAE-cellulose column (Whatman DE-52). The 5×25 cm column (bed volume about 450 ml) was equilibrated with 2% Triton X-100 (w/v), 2 M urea, 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol. The sample volume was kept to less than 10% of the bed volume by concentration with dry Sephadex G-25, and the column was developed with the equilibration solution at a flux of 6 ml h^{-1}. Fractions of 2 ml were taken and spectrophotometrically (559 nm) analyzed, and those containing cytochrome *b*-559 were pooled, concentrated and made to 10% glycerol prior to electrophoresis.

Electrophoresis was performed in a Shandom System with tubes of 15 mm internal diameter, using a 1-cm long, 4% polyacrylamide stacking gel and a 7-cm long, 10% polyacrylamide resolving gel. The composition of the electrode buffer was 0.5% Triton X-100, 5 mM Tris-HCl, 39 mM glycine (pH 8.3), 4 mM Tris-thioglycolate, 1 mM dithiothreitol. The process was conducted at 4 °C for 18 h by applying a constant current of 1.5 mA. On completion, the cytochrome *b*-559 band was cut, homogenized by hand in the same electrophoresis buffer (no detergent) and stirred under argon for 2 h to facilitate diffusion. The gel was then removed by centrifugation ($30{,}000 \times g$, 1 h), and aliquots of the resulting solution were used for further analysis and characterization of the purified cytochrome.

When PS II particles were used as starting material for cytochrome *b*-559 purification, a 3×19 cm DEAE-cellulose column (bed volume about 100 ml) was used for binding of impurities, and tubes of 6 mm internal diameter were employed in the electrophoretic step.

Molecular weight determination by gel filtration

Calibration of the MW of the native heme protein purified by electrophoresis was attained in a 40 cm × 26 mm Sephadex G-150 Fine column equilibrated with 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.1 M NaCl. The standard proteins (2 mg each) were localized in the eluted fractions (1.5 ml) by absorbance at 230 nm, and cytochrome *b*-559 was monitored by absorbance of the Soret band (415 nm) of its oxidized form. The following MW protein markers were used: catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and α-chymotrypsinogen (25 kDa).

Molecular weight determination by SDS-PAGE

Analytical disc polyacrylamide gel electrophoresis of the purified enzyme was performed under denaturing conditions, in the presence of 0.1% sodium dodecyl sulfate, by using tubes of 4 mm internal diameter and a 1.5-cm (4%) stacking gel and a 7-cm (10%) running gel. Prior to its deposit in the gel, the cytochrome *b*-559 and standard proteins samples were heated for 5 min at 100 °C in the presence of 1% SDS and 1% 2-mercaptoethanol. Electrophoresis was run first for 1 h at 2.5 mA/gel and then for 3–4 h at 3–4 mA/gel. The gels were stained with Coomassie Brilliant blue, and the mobility of the blue band was measured relatively to the electrophoretic front (bromphenol blue). The following MW protein markers were used: phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin soybean inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa) and cytochrome *c* (12.4 kDa).

Alternatively, analytical polyacrylamide electrophoresis was carried out in a slab gel apparatus (Protean II from Bio-Rad) as described by Schägger and von Jagow [31]. A separating gel of 16.5% acrylamide and $16 \times 17 \times 0.07$ cm was used; the gel was overlaid by a 10% spacer gel (1–2 cm) that in turn was overlaid by a 4% stacking gel (1–2 cm). The treatment of the sample (30 min at 40 °C in 4% SDS and 2% 2-mercaptoethanol), as well as the composition of anode and cathode buffers, were as described by the authors. About 5 μg protein was applied per band. Electrophoresis was started at a constant voltage of 30 V for about 1 h and, when the sample had completely left the sample pocket, the current was raised to a constant value of 30 mA (voltage at start, 100 V; at end, 350 V). Fixing, staining and destaining of proteins were also made as in [31]. The MW-SDS-17 protein kit from Sigma Chemical Co. was used as marker. It contained: myoglobin, 16.95 kDa; myoglobin fragment 1 + 2, 14.4 kDa; fragment 1 + 3, 10.7 kDa; fragment 1, 8.16 kDa; fragment 2, 6.21 kDa, and fragment 3, 2.51 kDa.

Absorbance spectra

Spectra of the native cytochrome *b*-559 preparations eluted from the electrophoretic gels were carried out *versus* appropriate blanks in a Beckman DU-7 spectrophotometer. Due to the presence of dithiothreitol in the elution medium, the sample cuvette was bubble for a few seconds with oxygen in order to oxidize completely the cytochrome. On the other hand, to ensure total reduction of the cytochrome, a few crystals of dithionite were added prior to recording its reduced spectrum.

Redox titrations

Potentiometric redox titrations of the native cytochrome *b*-559 preparations eluted from the electrophoretic gels were carried out under argon in 3-ml cells, thermostated at 20 °C, using 0.4 μM cytochrome suspensions in 50 mM Tricine buffer (pH 7.5)

in the presence of suitable redox mediators (20 μM 1,2-naphthoquinone, 20 μM diaminodurol, 20 μM duroquinone, 10 μM phenazineethosulphate, 2.5 μM phenazinemethosulphate). Absorbance changes were followed in an Aminco DW-2a dual wave-length spectrophotometer at 559–570 nm after oxidation with ferricyanide and controlled reduction with 50 mM dithionite. Redox potentials were simultaneously determined with a Beckman-4500 potentiometer provided with a combined Pt-Ag/AgCl Ingold electrode previously calibrated against a saturated solution of quinhidrone (E'_m, pH 7, +280 mV at 25 °C).

Restoration of the high-potential form

The isolated native cytochrome *b*-559 purified to apparent electrophoretic homogeneity and exhibiting the midpoint redox potential proper to the low-potential form was incorporated into liposomes prepared as in [6] but with some modifications. A 400 μl sample of purified cytochrome *b*-559 (1 μM) was added to a mixture of 20 μl of 100 mM phosphatidylethanolamine and 5 μl of 100 mM phosphatidylcholine, previously flushed with argon, and gently stirred for 2 h at 4 °C under argon. The final liposome preparation was finally diluted with 400 μl of 10 mM Tricine-KOH buffer, pH 7.5, containing 15% glycerol.

Results

Fresh spinach thylakoids as well as oxygen-evolving PS II particles (which are devoid of cytochromes *f* and *b*-563) present, as shown previously [19, 20], cytochrome *b*-559 in both the reduced form of the high-potential pair (about 2/3) and the oxidized form of the low-potential pair (about 1/3). When the heme protein was purified by the procedure described under Materials and Methods, using either thylakoids or PS II particles as the starting material, reduced cytochrome *b*-559 appeared in the Triton-containing polyacrylamide gel after the disc electrophoresis step as a pink-orange sharp band. As shown in Fig. 1, the gel which corresponded to the thylakoid preparation, but not that of the PS II preparation, exhibited besides a slower-moving band of reduced cytochrome *f*. After elution of cytochrome *b*-559 from its gel slice, its MW determination was performed by gel filtration on a Sephadex G-150 column (see Materials and Methods) and yielded a value of about 200,000 (data not shown).

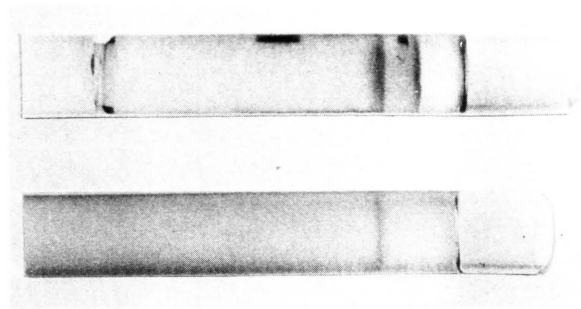
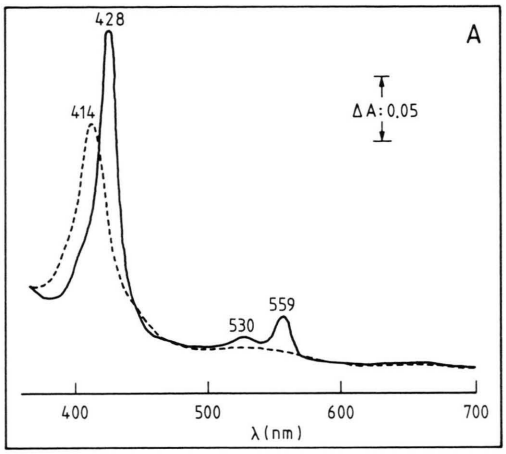


Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of spinach cytochrome *b*-559. Top: Protein purified from thylakoids. Bottom: Protein purified from PS II particles (the cytochrome *f* band is lacking). The purification procedure is described in Materials and Methods.

The absolute absorption spectra in the visible region of reduced and oxidized cytochrome *b*-559 purified to apparent homogeneity by gel electrophoresis from both thylakoids and PS II preparations are shown in Fig. 2. It can be seen that they are identical whatever the origin of the preparation. The low temperature (77 K) spectrum of the reduced cytochrome *b*-559 form shows the appearance of shoulders at 512, 535 and 575 nm, but no split in the α peak, which is displaced towards 556 nm.

When the isolated cytochrome from either source was potentiometrically titrated, it exhibited only the midpoint potential proper to the low-potential couple (E'_m, pH 7.5, +115 and +158 ±10 mV for the heme protein coming from thylakoids and PS II particles, respectively), with a *n* value close to 1 (Fig. 3). Moreover, although the HP form was completely absent of the purified cytochrome, reconstitution of the isolated LP cytochrome *b*-559 (dithionite-reducible) into phosphatidylcholine-phosphatidylethanolamine vesicles promoted the conversion of 1/3 into the hydroquinone-reducible HP form (Fig. 4). It should be indicated that this conversion is rather tricky and was achieved only in 25% of the experimental trials.

Analytical disc SDS-PAGE of denatured cytochrome *b*-559 isolated from either thylakoids or PS II particles showed only one polypeptide band of 14.6 and 14.0 kDa, respectively, when carried out as described in Materials and Methods (Fig. 5). However, when SDS-PAGE of the same purified cytochrome preparations was alternatively performed in



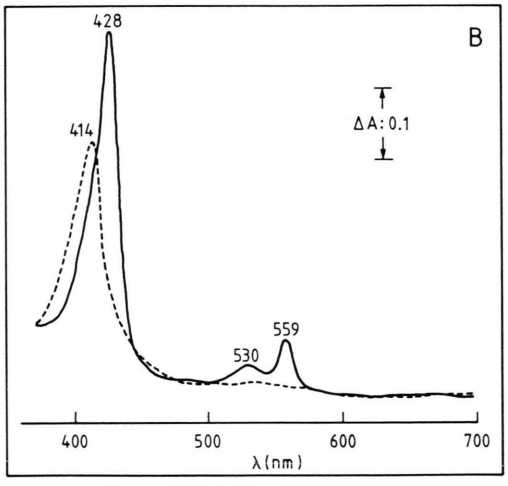


Fig. 2. Absolute absorption spectra of cytochrome *b*-559 purified from spinach thylakoids (A) and PS II particles (B). Dotted line: Oxidized cytochrome. Solid line: Dithionite-reduced cytochrome. The absorption maxima in the alpha, beta and Soret bands are indicated over the corresponding peaks. For other details see Materials and Methods.

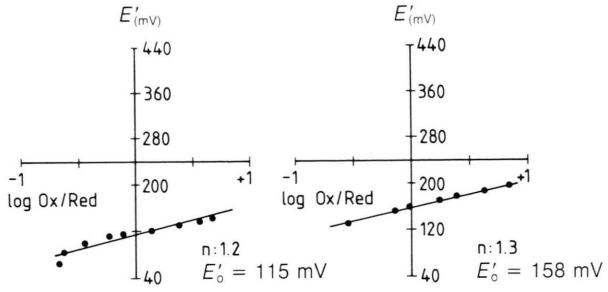


Fig. 3. Potentiometric redox titration of cytochrome *b*-559 purified from spinach thylakoids (left) and PS II particles (right). Experimental conditions are given in Materials and Methods. The serial redox potential values belong to an experiment among a set of ten, the corresponding midpoint potentials values varying ±10 mV.

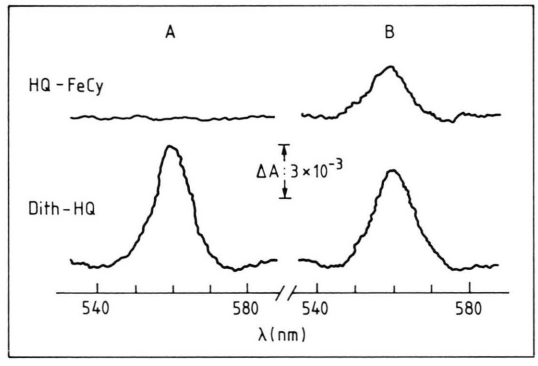


Fig. 4. Redox difference spectra of isolated cytochrome *b*-559 (A) and after its integration into liposomes (B). Single-beam absorption spectra were measured after sequential additions of 0.5 mM ferricyanide (FeCy), 20 mM hydroquinone (HQ) and a few grains of dithionite (Dith.). Cytochrome *b*-559 was isolated and incorporated into liposomes as described in Materials and Methods.

a slab gel apparatus (see also Materials and Methods) as described by Schägger and von Jagow [31] only one polypeptide band was again revealed, but of 8.6 and 8.5 kDa, respectively (Fig. 6).

Discussion

The elusive character of cytochrome *b*-559 function implies either that its mechanism of action is very complicated or perhaps and more likely that the question is not being asked in the proper way. In either case, elucidation of the until now very controversial molecular properties of native cytochrome *b*-559 must be considered essential to the interpretation of its function. The present investigation, carried out in parallel, for comparative purposes, with spinach thylakoids and PS II particles indicates that chloroplasts contain only a molecular species of cytochrome *b*-559 which is tightly bound to PS II and can be isolated as a pure heme protein in its low-poten-

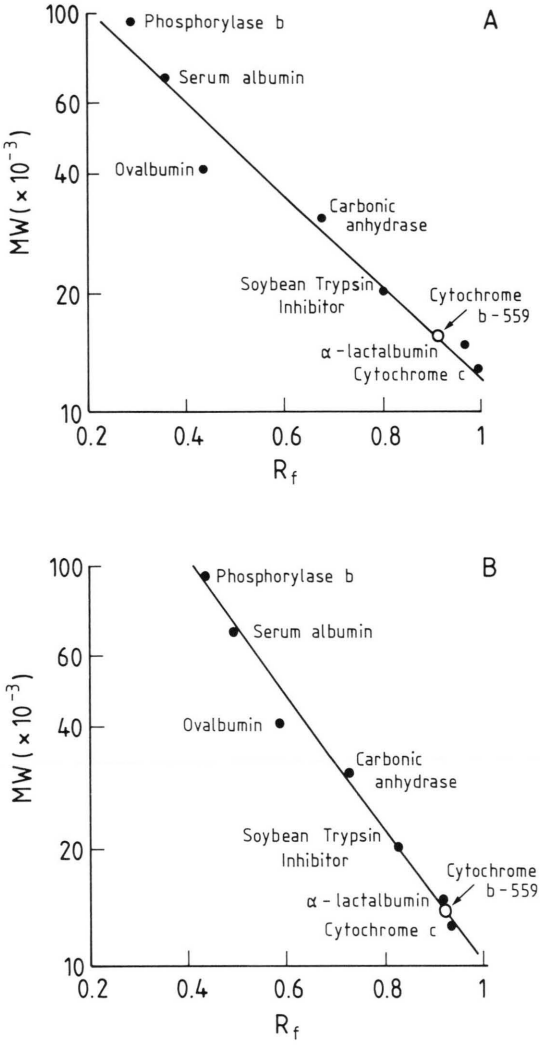


Fig. 5. Molecular weight determination by disc SDS-PAGE of cytochrome *b*-559 purified from spinach thylakoids (A) and PS II particles (B). Experimental conditions are given in Materials and Methods. The represented values are averages of five independent experiments.

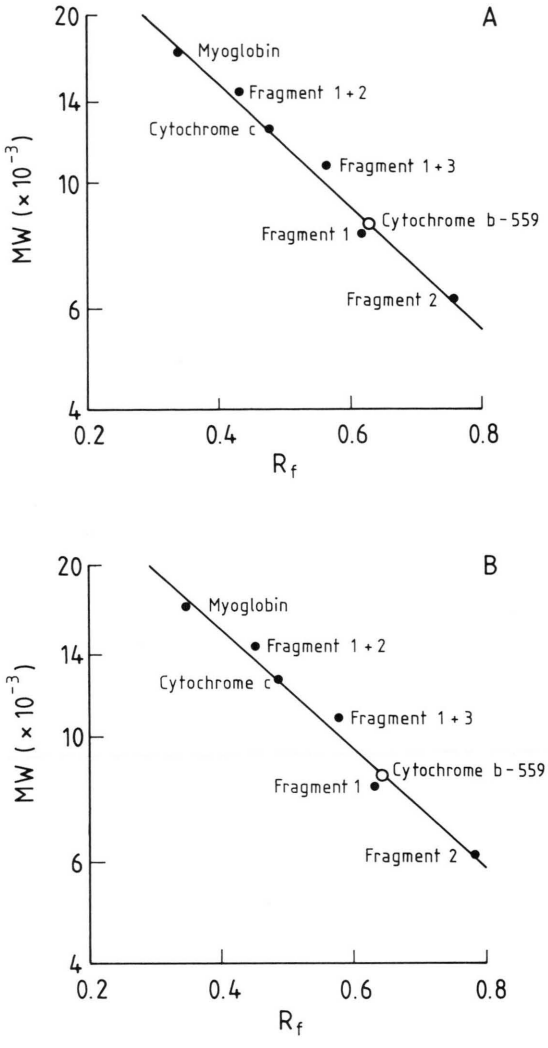


Fig. 6. Molecular weight determination by slab SDS-PAGE of cytochrome *b*-559 purified from spinach thylakoids (A) and PS II particles (B). Experimental conditions are given in Materials and Methods. The represented values are averages of six independent experiments.

tial form. The reported results corroborate our previous research showing that in fresh thylakoids [5, 19] and PS II particles [5, 20] cytochrome *b*-559 is present both as the reduced form of the protonated HP pair (about 2/3) and as the oxidized form of the non-protonated LP pair (about 1/3). The two other forms of the LP and HP couples are absent in the initial preparations because the oxidized HP form is electronically energized and unstable and the reduced LP form is autooxidizable.

It is already well established [1–5] that the HP form of cytochrome *b*-559 is labile towards treatments, such as sonication or incubation in detergents, that may alter or disrupt membrane structure and that cause its conversion to the LP form. As a matter of fact, during the extraction of cytochrome

b-559 carried out by sonication in the presence of the detergent Triton X-100, the reduced HP form initially present in chloroplasts becomes transformed in the oxidized LP form (*cf.* [5, 19, 20]). That this transformation may be physiologically reversible is at the present time against general opinion. In this respect, our group [20] has recently demonstrated, using spinach PS II particles, the endergonic conversion *in situ* of the non-protonated and oxidized LP form of cytochrome *b*-559 into its corresponding protonated and oxidized HP form. Furthermore, as shown first by Matsuda and Butler [6, 24] and confirmed by us in this work, incorporation of purified LP cytochrome *b*-559 into liposomes can convert it partially back to the HP form. We have also very recently shown [32] by integration of purified complex III from baker's yeast into liposomes that this relevant property of chloroplast cytochrome *b*-559 is shared by the energy-transducing cytochrome *b*-564 of mitochondria. In the latter case, it was proven moreover [32] that restoration of the HP form of cytochrome *b*-564 is concomitant with redox-linked proton translocation.

Garewal and Wasserman [8, 21, 22], as well as Matsuda and Butler [24], achieved an homogeneous preparation of spinach cytochrome *b*-559 as a lipoprotein complex of MW about 110,000, and Koenig and Moller [28] isolated from barley the same heme protein, but having an apparent MW of 120,000. According to Cramer *et al.* [4] the MW of the spinach lipoprotein in aqueous solution indicates that it is heterodisperse over a range between 100,000 to 300,000. The MW value reported in this paper for the native spinach heme protein from either spinach thylakoids or PS II particles is 200,000. As pointed out by Widger *et al.* [30], it appears possible that MW measurements of such a non-polar protein as cytochrome *b*-559 in aqueous solution may not readily distinguish a true oligomeric lipoprotein from an artificial aggregate. Actually, when Triton X-100 is depleted, a soluble undenatured aggregate is formed which cannot penetrate a 10% polyacrylamide gel [8].

Sodium dodecyl sulfate apparently denatures and completely dissociates the heme protein molecule to its polypeptide chains. Notwithstanding, a subunit MW of about 6000 for an octameric cytochrome *b*-559 lipoprotein from spinach was found by Wasserman [8, 22], Price [9, 23] and Butler [6] groups, whereas the minimum polypeptide MW was found to be 37,000 for the cytochrome *b*-559 from spinach and 17,000 for that from the alga *Bumilleriopsis* in Böger's laboratory [26]. More recently, a 9–10 kDa polypeptide associated with cytochrome *b*-559 has been purified from barley [28], spinach and maize [29, 30] chloroplasts, and a second smaller polypeptide (MW about 6000) was also found to be associated with this heme protein [4]. According to Cramer *et al.* [4] cytochrome *b*-559 seems to be a heme-cross-linked hetero-oligomer constituted by the polypeptide products (apparent molecular masses of 9.16 and 4.27 kDa) of two plastid chromosome genes (*psbE* and *psbF*). Our results indicate that the purified cytochrome *b*-559 that we have isolated from either spinach thylakoids or PS II particles revealed only one kind of electrophoretical subunit. The peculiar hydrophobic character of cytochrome *b*-559, proper to its tight association with chloroplast membranes, may explain why, in contrast with hydrophilic horse heart cytochrome *c* – which was always used as a reliable marker –, yielded in analytical SDS-PAGE electrophoresis a value of either 14 kDa or 9 kDa, depending on the method and conditions employed (Fig. 5 and 6). In this respect, it is also of interest to mention that a cytochrome *b*-559 polypeptide of 14 kDa has been observed in SDS-PAGE gels of chloroplast fragments of mutants of *Chlamydomonas reinhardtii* [27].

An oxygen-evolving PS II reaction center complex containing LP cytochrome *b*-559 has been lately purified from spinach [33]. SDS-PAGE of this complex disclosed two small subunits of cytochrome *b*-559 (9 and 4.5 kDa), although the smaller subunit was not resolved. Satoh's group has also succeeded in isolating from spinach thylakoids a PS II reaction center complex, consisting of D1 and D2 polypeptides and cytochrome *b*-559 polypeptides (9 and 4.5 kDa), that is capable of reversible photochemical accumulation of reduced pheophytin [34]. More recently, Barber *et al.* [3] have been able to remove the cytochrome from the D1/D2 complex and have suggested that the minimal unit for the PS II reaction center is the D1/D2 heterodimer binding the reaction center chlorophyll and pheophytin molecules.

In conclusion, there seems to be little doubt according to present evidence that cytochrome *b*-559 is only a molecular species that can exist in two interconvertible redox pairs and is closely linked to PS II. However, its function remains still a matter of conjecture and the different groups seem to diverge more and more as time goes on. The following pro-

posals are at present being considered: a redox carrier between the two photosystems which binds protons on the oxidizing side of PS II and leads to proton-release in the splitting of water [6, 24]; a redox carrier in a cyclic, proton-conducting pathway in PS II [35, 36]; a mediator in the (re)assembly of the water-splitting enzyme in chloroplast development and in response to stress [4]; a charge balancer [37]. Our own findings [5, 19, 20] are consistent with the operation of cytochrome *b*-559 as a transducer of redox energy into acid-base energy between the reducing side of PS II and the oxidizing side of PS I on the one hand, and between the extra- and intrathylakoid spaces, on the other hand. The most recent evidence pertaining to its location at the coupling site between plastoquinone and cytochrome *f* will be reported elsewhere [38].

Acknowledgements

Research supported by grant SEUI-PBO401 from Dirección General de Investigación Científica y Técnica, Spain. J. M. O. is the recipient of a predoctoral fellowship from the Ministerio de Educación y Ciencia, Spain. The authors wish to thank Mrs. A. Friend and Mrs. M. J. Pérez de León for helpful secretarial assistance.

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