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SOME PHOTOREACTIONS OF ISOLATED CYTOCHROME *b*-559

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SUMMARY

Cytochrome *b*-559 was isolated from spinach and the alga *Bumilleriopsis filiformis* (Xanthophyceae) and characterized by functional properties:

(a) It was active as electron acceptor in a diaphorase system using NADPH as donor and ferredoxin and ferredoxin-NADP reductase as redox proteins.

(b) It exhibited photooxidation with Photosystem-I particles, when illuminated with 707 nm light.

(c) It was photooxidized by Photosystem-II particles and 652 nm light at room temperature. Light > 702 nm was ineffective.

The data corroborate previous reports on redox reactions of bound cytochrome *b*-559.

INTRODUCTION

Localization and function of cytochrome *b*-559 in the photosynthetic electron transport is a controversial question, some aspects of which were compiled recently [1–4].

Up to now, the possible role of cytochrome *b*-559 was concluded by photoreactions which can be measured with isolated chloroplasts or subchloroplast particles retaining the protein bound to the thylakoid membrane. Several problems arise: light-induced absorption changes of cytochrome *b*-559 can be misinterpreted when they are accompanied by corresponding changes of cytochrome *f* or *b*-563 of the Photosystem-I region. Absorption changes of cytochrome *b*-559 are not detectable in isolated untreated chloroplasts or intact algal cells [5–8]. In order to measure them, part of the photosynthetic electron transport has to be either inhibited by low temperatures [6, 9, 10], by carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone [11] or the oxygen-evolving system has to be impaired, e.g. by Tris washing [6]. In addition, cytochrome *b*-559 occurs in at least two different redox potential forms [11–13], which have been

Abbreviations: HP, LP, "high" or "low" midpoint potential form of cytochrome *b*-559, compare refs. 12, 13. DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)-methylglycine; Tris, *N*-(tris-hydroxymethyl)-aminomethane.

ascribed to different reactions and localization. It was claimed that part of cytochrome *b*-559LP should interact with Photosystem I, cytochrome *b*-559HP with Photosystem II [14], although it was demonstrated conclusively that cytochrome *b*-559 is not closely associated with Photosystem I [15].

Experiments with isolated cytochrome *b*-559 are few, since only recently it has been possible to isolate it in a homogeneous form [13, 16, 17]. This paper deals with reactive isolated cytochrome *b*-559 in reconstitution experiments with sub-chloroplast particles and tries to add some data on the function of this cytochrome within the photosynthetic electron transport. Some of these findings have been reported elsewhere [18].

MATERIALS AND METHODS

Isolation of cytochrome *b*-559LP, from *Bumilleriopsis* and *Spinacia* has been described recently [13, 17]. The cytochrome is kept in solution by 2 % Triton X-100, which was lowered to approx. 0.2 % in the assays by dilution. Ferredoxin and ferredoxin-NADP reductase were prepared from the alga *Bumilleriopsis filiformis* as described [19], plastocyanin from spinach [20].

The enzymic activity of isolated cytochrome *b*-559 from both specimens was measured in a diaphorase assay with NADPH as electron donor, including ferredoxin-NADP reductase and ferredoxin [21]. Since the *b*-type cytochrome could be prepared in a pure form only in very low concentrations (see below), the assay method had to be refined: all rates were determined in an absorbance range of 0–0.005 using the dual-wavelength mode with 559 nm as measuring and 540 nm as reference wavelength (Aminco-Chance spectrophotometer; model DW-2; Aminco, Silver Spring, Md.). The test was done in an open cuvette (3 ml) at room temperature, slit width 3 nm, recorder response 300 ms. It contained in a 1 ml reaction volume: Tris/Cl, pH 7.0*, 27 μmol/$NADP^+$, 0.7 μmol/Na-isocitrate, 1 μmol/isocitrate dehydrogenase, 63 μg/$MgCl_2$ 0.7 μmol/cytochrome *b*-559 (from spinach) 0.22 nmol or cytochrome *b*-559 (from *Bumilleriopsis*), 0.16 nmol. The cytochrome preparation had to be free of urea, which was achieved by a 6×1 h dialysis vs. 10 mM Tricine, pH 7.9, including 2 mM dithiothreitol, thereby preserving 80 % of its activity. Triton X-100, forming a micelle of about 70 000 molecular weight, remained with the cytochrome after this procedure and could be removed by prolonged dialysis (2 weeks) which led, however, to an inactive cytochrome. (Determination of urea and Triton according to the methods in refs. 22 and 23, respectively.) Since the cytochrome was kept reduced by dithiothreitol, it had to be titrated back with 1 mM potassium ferricyanide before starting the assay described above, while the sample was mixed in a cuvette with a stirring device. The resulting ferrocyanide did not inhibit the reaction in these small concentrations [21]. Although the reduced low-potential *b*-type cytochromes are autoxidizable, this reaction can be neglected during the course of the assay.

Photosystem-I particles from spinach were prepared according to Boardman and Anderson [24] using digitonin. No photoreaction could be detected, neither due to

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* Tris · Cl is used at pH 7.0 although its pK_a value is unfavourable. With other buffers (morpholinopropane sulfonic acid or piperazine-*N*,*N*'-bis 2-ethane sulfonic acid) the rates were substantially lower.

cytochrome *b*-559 nor to cytochrome *f* (with 1 μM plastocyanin added to the assay). The molar ratio of chlorophyll to P700 was 97 : 1 (determined after Marsho and Kok [25]).

Photosystem-II particles were prepared according to Vernon et al. [26]. However, bioglass was substituted for CPG-10-2.000 (controlled pore glass with pores of 2108 Å). Particles with 100 to 200 μg of chlorophyll were obtained from isolated chloroplasts equivalent to 100 mg of chlorophyll. The photosynthetic activity of these preparations was assayed in a medium as follows: 20 mM Tricine · NaOH, pH 7.9/ 0.25 M sucrose/0.1 mM DCIP/0.5 mM 1,5-diphenylcarbazide. Chlorophyll content was 2–5 μg/ml. Rates were found to be variable depending on the season in which the spinach was grown (*Spinacia oleracea*, strain Atlanta, grown in the University garden on thoroughly fertilized soil). With spinach harvested during fall, rates were about 1000 μmol DCIP reduced per mg chlorophyll per h, otherwise figures of 300–400 were obtained. The reaction was DCMU-sensitive (40 % inhibition with 10^{-6} M, 81 % inhibition with 10^{-5} M; a similar inhibition was reported previously [26]). Difference absorption spectra exhibited small amounts of cytochrome *f* or cytochrome *b*-563 present (approx. 1 molecule per 2–3000 chlorophylls which, however, did not show any photoreactions). The ratio of chlorophyll to cytochrome *b*-559 in the particles varied between 68 : 1 and 130 : 1. A residual Photosystem-I activity was low: in the presence of 0.5–1 μM plastocyanin, sucrose and buffer as mentioned above, the 2,3,5, 6-tetramethyl-*p*-phenylenediamine/ascorbate → methylviologen system yielded an O_2 uptake of 30 and 40 μmol only per mg chlorophyll per h, whereas the untreated chloroplast material gave rates of about 1500. Photosynthetic NADP reduction exhibited 10–17 μmol NADPH/mg chlorophyll · h. The assay for this reaction consisted of: 30 mM phosphate buffer, pH 6.7/0.4 mM NADP/7.5 mM Na-ascorbate/ 50 μM DCIP/1 μM plastocyanin/2.5 μM ferredoxin/0.2 μM ferredoxin-NADP reductase.

Photoreactions again were measured by the dual-wavelength mode (559–540 nm) with the Aminco spectrophotometer in a 3 ml cuvette equipped with a small magnetic stirrer, consistently at room temperature. Actinic light intensity in the empty cuvette was $0.8–1 \times 10^5$ ergs/cm^2 × s, defined by a red cut-off filter (Schott RG 630, 2 mm) and additional interference filters from Balzers, as mentioned in the legends (compare Fig. 4). A particular narrow-band filter (built on special order by Seavom, Franconville, France) was used additionally for Photosystem-I reactions. It only had a half-bandwidth of 7 Å with a transmittance of 54 % and a peak wavelength of 702 nm. In this case, no cut-off filter was used to obtain the same light intensities as mentioned above. The photomultiplier was shielded against stray light by a Balzers Filtraflex green DT filter and a Corning filter, No. 9788.

Chemicals. Buffer and other chemicals were purchased from Merck, Darmstadt, enzymes from Boehringer, Mannheim. Controlled pore glass was from Serva-Heidelberg.

RESULTS

Fig. 1 demonstrates the enzymatic reduction of spinach cytochrome *b*-559 dependent on ferredoxin. Without ferredoxin, the reaction was almost zero. Due to the Triton X-100 content of our cytochrome preparations, we could only use 0.1–0.2

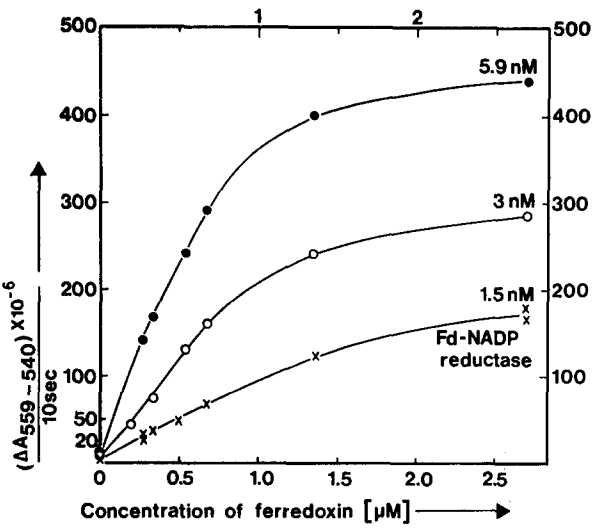

Fig. 1. Reduction of isolated cytochrome *b*-559 from spinach with NADPH, ferredoxin and ferredoxin-NADP reductase. A differential extinction coefficient (559–540 nm) of 20 $mM^{-1} \times cm^{-1}$ was used [34].

μM cytochrome *b*-559, which did not allow maximum rates. This assay was taken as a means to indicate reactivity of the isolated cytochrome, since it was observed that a denatured cytochrome preparation, although it might still be reduced by ascorbate, did not react in this enzymatic assay or in photoreactions with particles. It should be mentioned that sodium dithionite had never to be applied in order to achieve com-

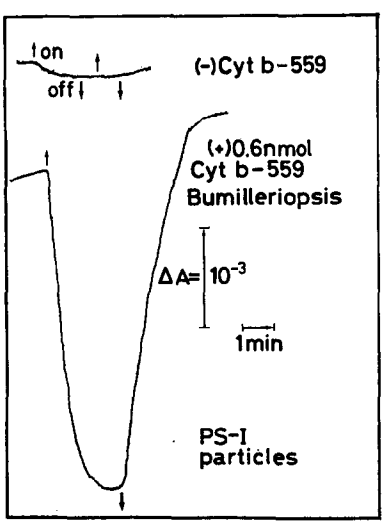

Fig. 2. Photooxidation of cytochrome *b*-559 from *Bumilleriopsis* with Photosystem-I particles from spinach. Actinic light: 713 nm. Besides the cytochrome, the assay included: 20 mM Tris/Cl, pH 8.0/ 0.25 M sucrose/9.5 μg/ml of chlorophyll. Cyt, cytochrome; PS, Photosystem.

plete reduction of the isolated cytochrome. Ferredoxin concentrations higher than indicated in Fig. 1 did not exhibit a decrease in activity. So, ferredoxin does not compete with the cytochrome at the reductase. The K_m can be estimated to be in the range of 0.8–1 μM ferredoxin. The rates were about 50 times less than those obtained with, e.g. purified algal cytochrome *c*-553 under saturating conditions [21]. This is due to the low concentration of the cytochrome *b*-559 used (see above), which does not yield the maximum velocity and due to the Triton content, which affects the reductase activity.

Cytochrome *b*-563 could be isolated in higher concentrations and was also reduced in this assay system. The K_m for ferredoxin was only 0.64 μM [18]. Isolated cytochrome *b*-559 from *Bumilleriopsis* is reduced in the same manner as that from spinach.

Fig. 2 shows the photoreaction of cytochrome *b*-559 from *Bumilleriopsis* with Photosystem-I particles. Half-time of oxidation was about 25 s. This oxidation in the light was followed by reduction in the dark, which was due to dithiothreitol present, and, as expected, neither DBMIB nor DCMU inhibited this reaction (up to 20 μM). Apparently, the (added) cytochrome is electron donor to Photosystem I, by-passing the corresponding inhibition sites. The observed absorbance change cannot be due to cytochrome *b*-563 present in these particles, since at the most it would possibly contribute less than 10 % of the absorbance change of this experiment.

Preparations of Photosystem-II particles from spinach [8] harvested in the fall exhibited photoreduction by Photosystem-II light of 5 % of the bound cytochrome *b*-559LP, (Fig. 3). The reaction was not sensitive to 10^{-6} M DCMU (10 μM inhibited to 20–30 % only), nor did DBMIB (3–30 μM) inhibit the reduction. This photoreduction could not be increased, on the contrary, it was not observed with particles prepared from spinach grown during other seasons of the year, although those preparations had the same amount of bound cytochrome *b*-559. After addition of soluble cytochrome *b*-559 from spinach or *Bumilleriopsis* (with 5 mM dithiothreitol present),

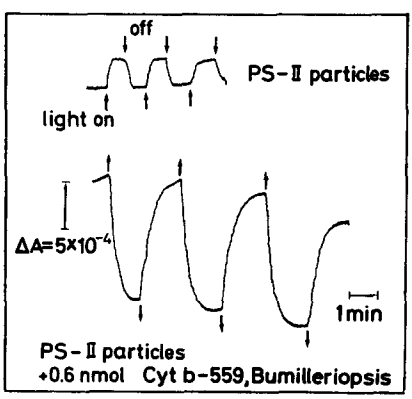

Fig. 3. Photoreaction of cytochrome *b*-559 from *Bumilleriopsis* with Photosystem-II particles from spinach. Actinic light: 652 nm. For the assay including 0.3 mM 1,5 diphenylcarbazide see legend of Fig. 2; the particles were equivalent to 20.4 μg of chlorophyll per ml. Note that the extent of photooxidation depends on the preparation of cytochrome *b*-559 itself and on the Photosystem-II particles (compare Fig. 4). Downward deflection indicates absorbance decrease. Cyt, cytochrome; PS, Photosystem.

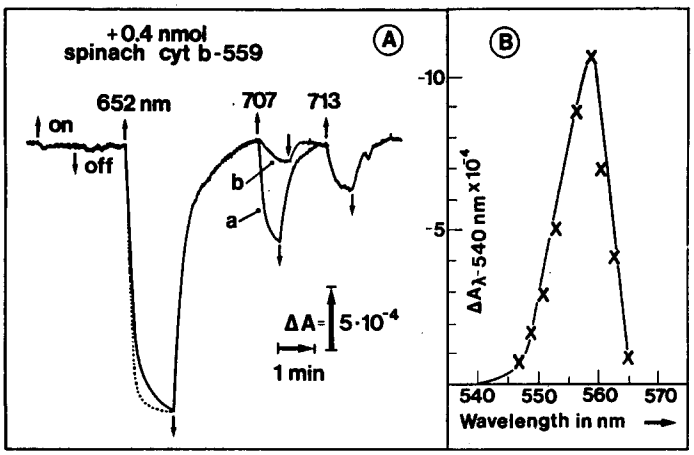

Fig. 4. A. Photooxidation of cytochrome *b*-559 by Photosystem-II particles: dependence on wavelength of actinic light. Both cytochrome and particles were prepared from spinach, for composition of the reaction mixture see legend of Fig. 2. Dotted line, experiment without 1,5-diphenylcarbazide; solid line, with 0.3 mM 1,5 diphenylcarbazide; chlorophyll content was 38 μg/ml. Actinic light was defined by the following interference filters: 652 nm, 707 nm (tracing a) and 713 nm, the half-bandwidths were 10, 11.5 and 11 nm, respectively. In tracing b an ultra-narrow band filter with 702 nm peak wavelength was applied (see Materials and Methods). B. Difference absorption spectrum of cytochrome *b*-559 photooxidation by 652 nm light. Photosystem II particles were equivalent to 24 μg of chlorophyll per ml; cytochrome *b*-559 0.1 μM.

Photosystem-II particles exhibited a photooxidation of about 40 % of the cytochrome added when illuminated with 652 nm light. This deflection was superimposed upon the small accompanying photoreduction of the bound cytochrome *b*-559 mentioned above. Half-time of photooxidation was about the same as in Fig. 2.

When the reaction was performed with particles having no active membrane-bound cytochrome *b*-559, a photooxidation of added cytochrome *b*-559 up to 90 % could be observed. With 1,5 diphenylcarbazide as electron donor for Photosystem II the rate of the reaction decreased (Fig. 4), but the extent of photooxidation remained the same. Photooxidation sometimes was not complete and varied with different preparations of the Photosystem-II particles used. When it was less than 90 % (as in Fig. 3), addition of DCMU (3–30 μM) increased the deflection by 20–40 %. A similar observation was made in the presence of DBMIB. Cytochrome *b*-559 oxidized by potassium ferricyanide is not photoreduced although it could be enzymatically reduced (diaphorase assay of Fig. 1). The small absorbance decrease consistently observed could not be interpreted as photoreduction of cytochrome *b*-559. Photosystem-II particles prepared with 4 M urea and 2 % Triton X-100 [27] in order to remove (part of) the cytochrome *b*-559 did not exhibit photoreaction activity.

To exclude a participation of Photosystem I in this reaction with Photosystem-II particles, the oxidation was measured with light of 652, 707 and 713 nm all of the same intensity (Fig. 4A). As demonstrated, the photooxidation of cytochrome *b*-559 decreased by 72 % when the reaction was induced with 707 nm light and 84 % when 713 nm light was applied. Although there is considerable oxidation even in the latter case, it can be explained by the comparatively wide half-bandwidth of the

filter (11 nm). The filter having a peak wavelength of 713 nm allows about 2 % of 682 nm light intensity to pass, which activates also Photosystem II. The experiment emphasizes that under high light intensities the normally used interference filters are not satisfactorily selective for Photosystem-I excitation. The ultra-narrow band filter with 702 nm peak wavelength decreased the photooxidation of cytochrome *b*-559 by 91 % vs. illumination with 652 nm light. The residual deflection of 9 % is in accordance with the content of Photosystem-I system contaminations as can be calculated from the photosynthetic electron transport activity as given under Materials and Methods.

DISCUSSION

The assay on enzymatic activity of isolated cytochrome *b*-559 using NADPH, ferredoxin-NADP reductase and ferredoxin should not indicate a possible functional role within the terminal part of the photosynthetic electron transport chain. As pointed out, this assay appears to be a sensitive indicator of whether the isolated cytochrome *b*-559 is denatured or not. It should be noted that without ferredoxin no reduction of cytochrome *b*-559 is possible, which is in contrast to cytochrome *c*-553 (i.e. the cytochrome *f* from the alga *Bumilleriopsis*) which can be reduced in this system without ferredoxin, the rate being approximately 10 % of the maximum reaction velocity. Also with mammalian cytochrome *c* reaction was not observed without ferredoxin [21].

For bound cytochrome *b*-559HP it has been suggested that oxidation by System I involves System I components with an electron transfer at equal potential [28, 30, 31]. Another suggestion was that the transfer of electrons from Photosystem II to System I requires cytochrome *b*-559 in its low-potential form and is mediated by plastoquinone [32, 33].

The photooxidation of cytochrome *b*-559 by Photosystem-I particles did proceed without adding plastocyanin. Although removal of plastocyanin from the Photosystem-I particles is probable, we cannot rule out that some was left to allow reaction with cytochrome *b*-559. At any rate, lack of DBMIB inhibition indicates that cytochrome *b*-559 does not donate its electrons to Photosystem-I via plastoquinone.

The oxidation of cytochrome *b*-559 by Photosystem-II particles has been shown to be a typical Photosystem II-dependent reaction (Fig. 4). It can be observed at room temperature, since the water-splitting system is destroyed. In contrast to reports on bound cytochrome *b*-559 [6, 9, 10] (see also ref. 4 for review), this oxidation induced by 652 nm light proceeds at room temperature with the added redox protein being in the low-potential form, occasionally superimposed by a reduction of part of the cytochrome *b*-559 still bound to the particle. This reduction was found not to be inhibited by DCMU as reported by others [8]. Photoreduction of added cytochrome *b*-559 could not be demonstrated. Without 1,5 diphenylcarbazide present, the (reduced) cytochrome *b*-559 itself, seemingly, is the electron donor instead of the water system, similar to the data measured by low-temperature photooxidation and Tris washing [9, 10]. The stimulation of the photooxidation by DCMU or DBMIB can tentatively be interpreted as an inhibition of the back-reaction of the reduced cytochrome *b*-559. This back-reaction may proceed either via plastoquinone (as proposed by Maroc and Garnier [28], or via the primary acceptor Q (compare ref. 29). It should be noted that cytochrome *b*-559 prepared from either spinach or from the alga *Bumilleriopsis* gave comparable data.

Light-induced absorbance changes of significant amplitude of cytochrome *b*-559 can only be observed after certain perturbations of the isolated chloroplasts (see Introduction). This experimental problem brings about the difficulty of interpretation and is the reason why so many hypotheses have been put forward. A unifying concept for a functional physiological role of this cytochrome has still to be developed.

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