Isolation and Some Molecular Properties of Plastidic Algal Cytochrome b-559

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(Z. Naturforsch. **32 c**, 75–77 [1977]; received November 18, 1976)

Cytochrome b-559, Algae, Spinach

Cytochrome b-559, an integral protein of chloroplast thylakoids, was prepared in a homogeneous form from the alga *Bumilleriopsis filiformis*. The protein is easily denatured and can be solubilized by treatment of isolated thylakoids with high concentrations of urea and detergents in addition to weak sonification. The reduced form exhibits absorption maxima at 559, 530 and 429 nm. By comparative determination, a molecular weight of 17 000 was found for the protein from *Bumilleriopsis*, whereas that for spinach has 37 000 daltons. Both proteins have a low, but variable lipid content which is not a necessary part of the enzymatically active cytochrome b-559. The purified cytochrome exhibits a low midpoint redox potential (reducible by ascorbate); during the preparation a transient "high-potential" form (reducible by hydroquinone) was also solubilized.

Introduction

Cytochrome b-559 is an integral membrane protein of chloroplast thylakoids although its function in photosynthetic electron transport has not yet been elucidated. It cannot be solubilized and isolated in a native form by conventional means, since it is hydrophobic and labile against strong sonification and oxygen. Its isolation from spinach chloroplasts employing urea and detergents was reported by Wasserman and collaborators [1, 2]. Their method had to be substantially modified in order to achieve preparation of this cytochrome from the alga *Bumilleriopsis filiformis* VISCHER (Xanthophyceae). In this paper, a modified procedure will be presented, which has also been successful to solubilize cytochrome b-559 of high midpoint redox potential from spinach chloroplasts [3].

Materials and Methods

The alga was grown in sterile liquid culture, according to Böger [4], with an improved nutrient medium [5] but the iron content increased 5-fold. Isolation of chloroplasts was performed as reported [6], but their osmotic shocking was omitted: The washed algae paste was suspended in 20 mM Tris, pH 8.0, including 0.15 M NaCl. After cell homogenization, centrifugation steps were done in the same medium. The chloroplast material was then extracted with ethanol as described for spinach material [3]. By this treatment, cytochrome c-553 (= algal cytochrome f) is modified to a low redox potential form which can only be reduced by sodium dithionite. During isolation of Cyt b-559, care had to be taken to avoid contamination of the preparations with this peripheral soluble c-type protein. Alcohols of longer chain lengths (*e. g. n*-butanol) did not change this situation, on the contrary, aggregation of the chloroplast particles was increased and, thereby, the inclusion of cytochrome c-553 rendering it more difficult to separate it from the b-cytochrome.

After the ethanol extractions and Tris washing [3], the grey particles were suspended in a mixture of 4 M urea, 2% Triton X-100, 2 mM DTT and 50 mM Tris, pH 8.0. The particle concentration is important at this point. Best solubilization is obtained with about 25 mg of protein/ml, which was achieved by suspending ethanol-extracted chloroplast material that had 100 mg of chlorophyll in 12 ml medium at start.

Sonification and further centrifugation steps were done as described for the preparation of spinach Cyt b-559 [3]. The resulting supernatant was chromatographed on a Biogel A-1.5 m column (200 to 400 mesh; 5 × 84 cm; elution velocity 30 to 40 ml/h).

For the analytical polyacrylamide disc gel electrophoresis see legend of Fig. 1. On gels according to Weber and Osborn [8], Cyt b-559 did not exhibit sharp bands even in the absence of urea.

Pyridine hemochromogenes were determined according to Appleby [9] (comp. Lach *et al.* [10]); cleavage

Abbreviations: Cyt, cytochrome; Tris, N-tris(hydroxymethyl)-aminomethane (buffer, adjusted with HCl); DTT, dithiothreitol; TEMED, N,N,N′,N′-tetramethyl-ethylenediamine; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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of the heme group was performed after Teale [11]. In order to check CO binding, the cytochrome was gassed for 10 min with pure carbon monoxide (99.997 vol.%, 10 vpm O_2) and its absorption spectra compared *vs.* that of the control.

For determination, the lipids of the cytochrome preparation were extracted according to Bligh and Dyer [12] and measured by the method of Zöllner and Kirsch [13]. Beforehand, the Triton X-100 was decreased to 0.02% by a 3 week dialysis. (Complete removal of the detergent by a 72 h dialysis as reported [2] could not be achieved due to the low critical micelle concentration of Triton). The extinction in the lipid assay caused by Triton was substracted.

SDS disc gel electrophoresis of spinach Cyt b-559 was performed by the method of Weber and Osborn [8] (7.5% gel). A 10% gel for PAGE without SDS of Cyt b-559 from spinach [1, 15] cannot be used since an R_F of 0.19 only was achieved. Therefore, the gel concentration was decreased and riboflavin/TEMED were used as catalysts for polymerization. Then, the R_F value was 0.64 in a 3.75% gel for spinach Cyt b-559, whereas it was 0.31 when the gel was polymerized with ammonium peroxodisulfate.

Protein in the crude extracts was determined according to Lowry *et al.* [16]. Due to Triton X-100 present and low protein concentrations (below 0.1 mg/ml), protein determinations of pure Cyt b-559 were measured as reported by Sims and Carnegie [17].

Results and Discussion

Isolation of Cyt b-559 from *Bumilleriopsis* was possible only with freshly harvested algae, since from frozen cells the yield was low. Furthermore, strong sonification could not be used for solubilization of algal Cyt b-559 due to denaturation. So, simple homogenization by glass beads was the method of choice before treatment of chloroplasts with urea and Triton X-100.

Increase of iron in the nutrient medium increased algal growth (approximately 26 g of wet algal paste per liter culture suspension after a 7 day growth period) and raised the content of all the cytochromes when referred to chlorophyll or protein. By difference absorption spectra of isolated chloroplast material (as it was used for preparation of the cytochrome) a molar ratio of 300 chlorophylls to 1 cytochrome b-559 was determined. The ratio of the high-potential to the low-potential form was found as 2 : 1 (comp. [3]). In contrast to spinach, the c-type cytochrome 553 is present in *Bumilleriopsis* in the same concentration as cytochrome b-559.

Cytochrome b-559 from *Bumilleriopsis* exhibits similar spectral properties as that from spinach, however, it is more sensitive to denaturation. Consequently the yield is low: with *Bumilleriopsis* 24 nmol per 100 μmol of chlorophyll were obtained (which is a 8% yield). Fig. 1 * demonstrates an absorption spectrum of *Bumilleriopsis* Cyt b-559 in the oxidized and reduced form. Homogeneity was proved by analytical PAGE (Fig. 2), the R_F values of *Bumilleriopsis* and spinach Cyt b-559 are given in Table I. The most striking difference between the two cytochromes is in the molecular weights of the protein moiety, which was reproducible with 3 preparations from both *Bumilleriopsis* and spinach, regardless of different lipid content. The purified algal cytochrome has a low redox potential (reducible by ascorbate), which we also found with the spinach cytochrome. However, after sonification of the chloroplasts, also a high-potential form (reducible by hydroquinone) was solubilized, which disappeared during further purification. Presumably, it was transformed to the more stable low-potential form.

Table I. Comparison of some molecular properties of cytochrome b-559 from *Bumilleriopsis* and spinach.

	Bumilleriopsis	Spinach
Molecular weight (PAGE, +SDS)	17,000 (±1000)	37,000 (±1000)
Lipid content %	5–25	5–18
R_F (PAGE, −SDS; 10% gel)	0.90	0.19 *
Acid-dissociable heme	+	+
CO binding	−	−
Chlorophyll / Cyt b-559	>0.2	>0.1

* Purification test was performed on 3.75% polyacrylamide gel polymerized with riboflavin (see Methods).

Figs 3 a, b demonstrate the SDS gel electrophoresis of Cyt b-559 from the alga. Electrophoresis of integral membrane proteins is difficult [19]; the cytochrome could be run in the gels only with Triton present. On the other hand, not all of the marker proteins gave definite bands under this condition. This led to slight deviations of the regression lines

* Figs 1–3 see Plate on page 76 a.

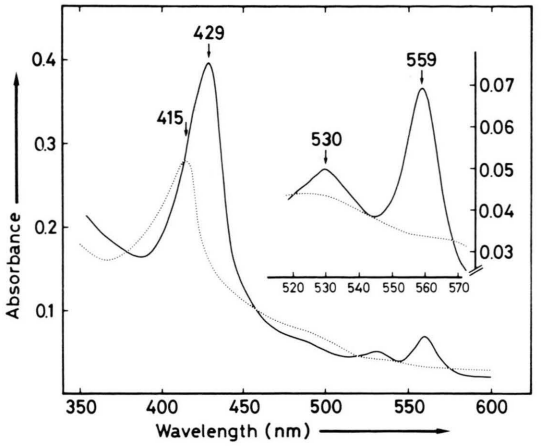

Fig. 1. Absorption spectra of cytochrome b-559 from *Bumilleriopsis* measured in a mixture of 4 M urea, 2% Triton X-100, 2 mM DTT, 50 mM Tris, pH 8.0. Solid line, reduced; dotted line, oxidized. Oxidation was performed with ammonium peroxodisulfate instead of K-hexacyano-III-ferrat in order to scan the region of the protein without dialysis. A partial denaturation by this oxidizing agent cannot be excluded. — Instrument: Perkin-Elmer spectrophotometer, model 124.

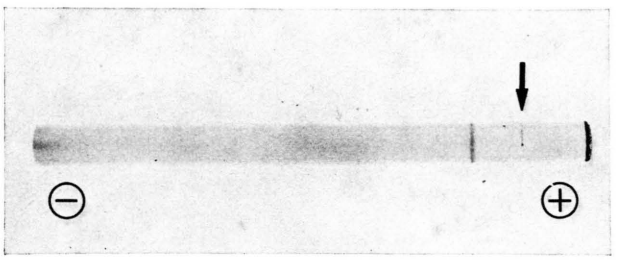

Fig. 2. Analytical polyacrylamide disc gel electrophoresis of *Bumilleriopsis* cytochrome b-559. Gels had 10% acrylamide containing 0.5% Triton X-100, 10% glycerol and 4 mM DTT. Protein, 38 μg per gel; running time 8 h with 4 mA per gel tube. Electrophoresis buffer: 10 mM Tris, pH 8.3, with 78 mM glycine, and 0.5% Triton X-100.

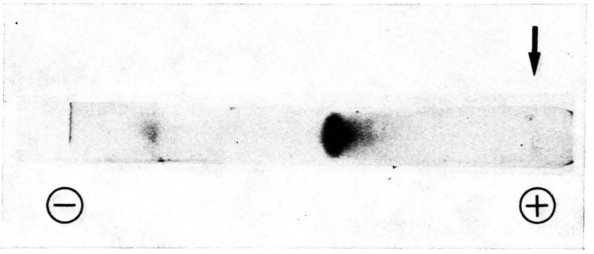

Fig. 3 a. SDS polyacrylamide electrophoresis of *Bumilleriopsis* cytochrome b-559. The protein was boilt for 5 min in the presence of 1% SDS and 20 mM DTT and subsequently incubated at 37 °C for 12 h in the same medium. Gel: 5% polyacrylamide with 0.1% SDS, 0.5% Triton X-100 and 4 mM DTT. Protein, 45 μg per gel, running time 4 h with 8 mA per gel tube. Electrophoresis buffer 0.1 M Tris, pH 8.3, with 0.5% Triton X-100 and 0.1% SDS.

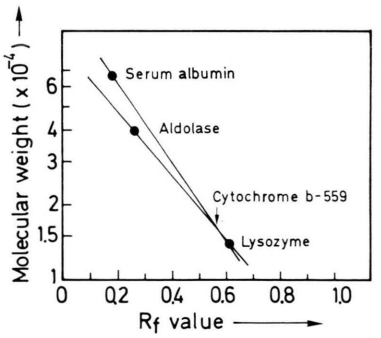

Fig. 3 b. Molecular weight determination of cytochrome b-559 by SDS gel electrophoresis according to Fig. 3 a. Location of the bands was determined by densitometry on a Beckman spectrophotometer Acta MVI, extinction range 0—1, scan rate 1.5 cm/min.

with the three marker proteins used as shown in Fig. 3 b, which could be tolerated since the value of 17000 daltons for molecular weight was obtained repeatedly regardless of some uncertainty of band location. This molecular weight was determined with or without DTT (20 mM) present in the gels, mercaptoethanol (20 mM) or urea (8 M).

The preparations from both spinach and the alga had a variable lipid content (Table I), which was much lower than published by the Wasserman group (they reported a constant lipid content of 56% for spinach Cyt b-559). The same is true for the chlorophyll content. These authors reported 4 chlorophylls per molecule Cyt b-559, which may be explained by improper separation of the pigment on the Biogel column. Chromatography conditions as mentioned in Methods have to be carefully observed since the K_{av} values for chlorophyll and Cyt b-559 are 0.58 and 0.52, respectively. It is important to start the preparation with ethanol-extracted particles which do not anymore exhibit a greenish tint.

Our preparations from both *Bumilleriopsis* and spinach can be reduced enzymatically by NADPH *via* (plastidic) ferredoxin-NADP reductase and are photoreactive in reconstitution experiments with subchloroplast particles [20].

This work was supported in part by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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