Variable Composition of Cytochrome b_6-f Particles

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Cytochrome b_6-f Particles, Solubilization, Structure of Detergents

Cytochrome b_6-f particles were prepared from chloroplasts of spinach and the heterokont alga *Bumilleriopsis filiformis* using digitonin in comparison with non-ionic detergents. The cytochrome b_6-f particles are solubilized in different amounts according to chemical structure and HLB value of the detergent (see Griffin, J. Soc. Cosmet. Chem. **1**, 311 [1949]). Also the ratio of the two cytochromes in the isolated particle depends on the detergent used. In case of spinach, it varies from Cyt b_6: f = 1.5:1 to 4.9:1. These investigations do not strengthen the idea that a cytochrome b_6-f complex with a stoichiometric composition is present in the photosynthetic membrane. Application of different detergents is a means for selective solubilization of thylakoid membrane components.

Introduction

Nelson and Neumann [1] reported the preparation of a cytochrome b_6-f particle by digitonin treatment of lettuce chloroplasts, with a stoichiometric composition of cytochrome b_6: f = 2:1. The particle appeared to be a tightly bound entity, since it could not be separated by DEAE chromatography or ammonium sulfate precipitation, suggesting a close connection of both cytochromes, which led to the speculation that cytochrome f might be reduced by cytochrome b_6 *in vivo*. Furthermore, Anderson and Boardman [2] found a cytochrome b-559LP present in their cytochrome b_6-f preparation. This paper shows that the type of detergent * used leads to different solubilization of cytochromes and, thereby, to cytochrome b_6-f particles of different composition.

Investigations indicating a correlation between non-ionic detergent structure [3–5] and their ability to solubilize biological membranes were carried out with mitochondria [6], microsomes [7] or virus and bacterial membranes [8, 9]. No comparative data have been reported for thylakoids of chloroplasts, and very few non-ionic detergents have been introduced in this field. In this investigation, digitonin is compared with several non-ionic detergents of the BRIJ, TWEEN and TRITON series by preparation of the cytochrome b_6-f particle.

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Abbreviations: Cyt b_6, cytochrome b_6 (= cytochrome b-563); Cyt b-559LP, cytochrome b-559 with low midpoint potential (ref. [12]); Cyt f, cytochrome f; Chl, chlorophyll; Tricine, N-[tris-(hydroxymethyl)-methyl)-glycine.

* Griffin [3] denotes as detergents only those surfactants which have HLB values of 13–15. This strict definition has not been generally accepted. Therefore, this paper uses surfactant and detergent as synonyms.

Materials and Methods

Cyt b_6-f particle from spinach

800 g of spinach (*Spinacia oleracea* L., strain Atlanta, grown in the open from April to June) was washed and deribbed, and homogenized with 500 ml containing 0.4 M sorbitol, 15 mM NaCl, 50 mM Tricine/NaOH, pH 8.0, in a Waring Blendor for some seconds (at 4 °C). The homogenate was pressed through nylon cloth (100 μm mesh) [10] and centrifuged for 1 min at 1000 × *g*, the supernatant centrifuged again for 7 min at 4000 × *g*. The pellet contained the chloroplasts which were osmotically shocked by resuspending them in 60–80 ml or 5 mM Tricine/NaOH, pH 8.0. After homogenization in a Potter-Elvehjem homogenizer, they were centrifuged for 10 min at 48000 × *g*. Thereafter, the chloroplast fragments were resuspended in a medium containing 0.4 M sorbitol, 15 mM NaCl and 50 mM Tricine/NaOH, pH 8.0, and adjusted to a chlorophyll content of 2.0 mg/ml. Chlorophyll determination was done according to ref [11].

The subsequent procedure was taken from Nelson and Neumann [1]. The chloroplast fragments were mixed with an aliquot of a 2.5% detergent solution (see Table I). A 14-h incubation was followed by a protamine sulfate precipitation step (1 ml protamine sulfate, 2 mg/ml, was given to 10 ml). After centrifugation, the cytochromes were determined in the supernatant by difference absorption spectra according to Hind and Nakatani [12]. Instrument: split beam spectrophotometer (Perkin-Elmer, model 124),

Table I. Detergents checked for preparation of cyt b_6-f particles from spinach chloroplasts.

No.	Trade Name	Chemical Name	HLB value	Solubilization of Cyt b_6-f
1	TRITON X-15	POE * (1) p-t-octylphenol	3.6	—
2	TRITON X-35	POE (3) p-t-octylphenol	7.8	—
3	TRITON N-57	POE (5) nonyl phenol	10.0	—
4	TRITON X-45	POE (5) p-t-octylphenol	10.4	—
5	TRITON X-207	alkylaryl polyether alcohol	10.7	—
6	TWEEN 85	POE (20) sorbitan trioleate	11.0	—
7	BRIJ 96	POE (10) oleyl alcohol	12.4	+
8	TRITON X-114	POE (7-8) p-t-octylphenol	12.4	+
9	TRITON X-155	alkylaryl polyether alcohol	12.5	—
10	BRIJ 56	POE (10) cetyl alcohol	12.9	+
11	TRITON N-101	POE (9-10) nonyl phenol	13.4	+
12	TRITON X-100	POE (9-10) p-t-octylphenol	13.5	+
13	TRITON CF-54	modified polyethoxy adduct	13.6	—
14	TRITON CF-10	alkylaryl polyether	14.0	—
15	TRITON CF-21	alkylaryl polyether	14.0	+
16	TRITON X-102	POE (12-13) p-t-octylphenol	14.6	+
17	TWEEN 60	POE (20) sorbitan monostearate	14.9	—
18	TWEEN 80	POE (20) sorbitan monooleate	15.0	—
19	TWEEN 40	POE (20) sorbitan monopalmitate	15.6	—
20	BRIJ 58	POE (20) cetyl alcohol	15.7	+
21	TRITON X-165	POE (16) p-t-octylphenol	15.8	+
22	TWEEN 20	POE (20) sorbitan monolaurate	16.7	+
23	TRITON X-67	alkylpolyether alcohol	16.8	+
24	BRIJ 35	POE (23) lauryl alcohol	16.9	+
25	TRITON X-305	POE (30) p-t-octylphenol	17.3	—
26	TRITON X-405	POE (40) p-t-octylphenol	17.9	—
27	TRITON B-1956	modified phthalic glycerol alkyl resin	—	—
28	TRITON CF-32	amine polyglycol condensate	—	—
29	TRITON DF-12	modified polyethoxylated alcohol	—	—
30	DIGITONIN	*Digitalis* saponin	—	+
31	TRITON QS-15	sodium salt of amphoteric surfactant	—	—

Nos.: 1–5, 8, 9, 11–14, 16, 21, 23, 25, 26: ref. 13; Nos. 6, 17–19, 22, 24: ref. 4; Nos. 7, 10, 20: ref. 14; No. 15: ref. 9.

* POE, polyoxyethylene; the figure indicates the number of POE groups in the molecule.

(+), Cyt b_6-f particle is solubilized. (−), No solubilization observable (Cyt b_6-f below 0.05 μM).

The HLB value (= *h*ydrophile-*l*ipophile *b*alance, see refs. 3, 4) is used for non-ionic detergents and represents the percentage (by weight) of hydrophilic groups in the detergent molecule divided by 5. Those groups are oxyethylene in the TRITON series with additional glycerol, sorbitol in the TWEEN types. The HLB values vary between 0 (completely hydrophobic) to 20 (completely hydrophilic). — All detergents of the table are non-ionic except for TRITON QS-15 (anionic).

absorbance display 0 – 0.1 extinction for 25 cm paper width; see ref. [10] for further details.

Deviations from this method are given in the text. The particle preparations done with digitonin were purified further after the protamine sulfate step according to Nelson and Neumann [1].

Besides digitonin the detergents noted in Table I were used. Both the trade name and the "chemical name" are given there, since different trade names often denote the same product.

Cyt b_6-f particles from Bumilleriopsis filiformis Vischer

The alga was cultivated according to ref. 16 with a nutrient medium given in ref. 17. 19 g of a wet algal paste were suspended in 19 ml of a 0.4 M sorbitol, 15 mM NaCl, 50 mM Tricine/NaOH, medium (yielding 2.0 to 2.2 mg chlorophyll per ml) and homogenized with 41 g of glass beads (0.5 mm ϕ) for 1 min in a cell homogenizer of Braun, Melsungen/Germany, under cooling with CO_2 snow according to ref. 16. After separation of the glass beads, the homogenate was diluted (11 ml of 5 mM Tricine/NaOH, pH 8.0, per 1 g algal paste). After removal of unbroken cells by centrifugation for 5 min at $10000 \times g$, the chloroplast particles were spun down at $26000 \times g$ for 30 min. The pellet was homogenized in the sorbitol medium (see above) and adjusted to a chlorophyll content of 2.0 mg/ml. The subsequent procedure was the same as described for spinach. With *Bumilleriopsis* chloroplasts only those detergents were checked which led to a substantial solubilization of Cyt b_6-f from the spinach thylakoid as noted in Table I (marked with +).

Results

1. Cyt b_6-f particles from spinach

As shown in Table II, the cytochrome b_6-f particle obtained from spinach has different b_6-f ratios depending on the detergents used. Both cytochrome contents vary considerably with different detergents (comp. cols. 2 and 3).

Even stronger is the variation of the chlorophyll content of the particles (454 μg/ml with TRITON

Table II. Solubilization of the Cyt b_6-f particle from spinach chloroplasts by non-ionic detergents.

(1) Detergent	(2) μM concentration of: Cyt f	(3) Cyt b_6	(4) Molar ratio Cyt b_6:Cyt f	(5) Chl / Cyt b_6	(6) Chl / Cyt f	(7) Cyt b-559 LP
BRIJ 35	0.55	1.32	2.4	19	45	<0.05
BRIJ 56	0.43	1.01	2.4	48	112	<0.05
BRIJ 58	0.22	0.76	3.5	34	118	<0.05
BRIJ 96	0.23	0.64	2.8	100	278	<0.05
TWEEN 20	0.17	0.50	2.9	58	171	<0.05
TRITON CF-21	0.13	0.63	4.9	188	915	0.06
TRITON N-101	0.45	1.10	2.4	245	598	0.06
TRITON X-67	0.16	0.36	2.3	106	238	0.07
TRITON X-100	1.00	2.40	2.4	87	208	0.06
TRITON X-102	0.64	1.64	2.6	20	50	0.1
TRITON X-114	1.05	1.60	1.5	284	432	<0.05
TRITON X-165	0.35	1.16	3.3	10	34	0.1
Digitonin	0.76	1.52	2.0	38	76	
Digitonin *	1.05	2.24	2.1	29	63	

Concentrations were determined by difference absorption spectra after the protamine sulfate step according to refs. 12, 10.

* Data from ref. 1.

X-100, 12 μg/ml with TRITON X-165). A low chlorophyll content of the Cyt b_6-f particle has to be achieved for further investigation of the particle. In this respect, digitonin is surpassed by some detergents, which still have the same solubulizing activity (comp. cols. 3 and 5).

Table III demonstrates that an incubation time of 14 h (with 1.25% detergent) is not the optimum

Table III. Solubilization of the Cyt b_6-f particle from spinach thylakoids influenced by incubation time and concentration of detergent.

Detergent	Incubation time [h]	Concentration [%]	Ratio Cyt b_6/f	Chl content [μg/ml]
BRIJ 35	22	1	1.7	28
	1.5	0.5	2.0	12
	14	0.5	1.9	17
	22	0.5	1.6	20
TRITON X-102	22	1	3.1	51
	1.5	0.5	2.5	19
	14	0.5	1.9	18
	22	0.5	1.8	34

treatment with all detergents. With BRIJ 35, higher concentration and longer incubation time increase chlorophyll, but not the amount of the solubilized Cyt b_6-f particle. TRITON X-102 is unfavourable for preparative procedure. Here, the concentration of solubilized particle increases parallel to the chlorophyll content of the preparation.

2. *Cyt b_6-f particles from Bumilleriopsis*

Table IV demonstrates the composition of a Cyt b_6-f particle from *Bumilleriopsis* prepared with

Table IV. Solubilization of the Cyt b_6-f particle from *Bumilleriopsis* chloroplasts.

Detergent	μM concentration of: Cyt f	Cyt b_6	Molar ratio Cyt b_6/f	Chl / Cyt b_6	Chl / Cyt f
TRITON CF-21	1.86	0.20	0.11	670	72
TRITON X-100	2.60	1.75	0.67	138	93
TRITON X-102	2.14	1.40	0.65	15	10
TRITON X-114	2.03	1.50	0.75	381	282
Digitonin	3.95	4.40	1.10	93	104

For determination procedure see Table II.

several detergents. The situation is different from spinach chloroplasts, obviously due to the fact that algal cytochrome f is a peripheral protein, which is easily solubilized. Therefore, this protein can be removed by passing the particle through a Biogel A-1.5 m column. Subsequently, after protamine sulfate precipitation, the concentration of Cyt f in the supernatant is always greater than of Cyt b_6, except when digitonin is used. With spinach, the Cyt b_6/f-ratio is highest with 4.9 using TRITON CF-21, it has its lowest value (= 0.11) with *Bumilleriopsis*. The smallest Cyt b_6:Cyt f ratio of 1.5 is obtained from spinach with TRITON X-114, from *Bumilleriopsis* it is 0.74 with this detergent, the highest figure obtained besides digitonin. The chlorophyll contents of the preparations were about the same except for the digitonin preparation.

Cytochrome f is eluted first during purification of the Cyt b_6-f particle on a DE 52 column, followed by a fraction which contains Cyt b_6 and Cyt f together (see Fig. 1). The shoulder at 430 nm in the absorp-

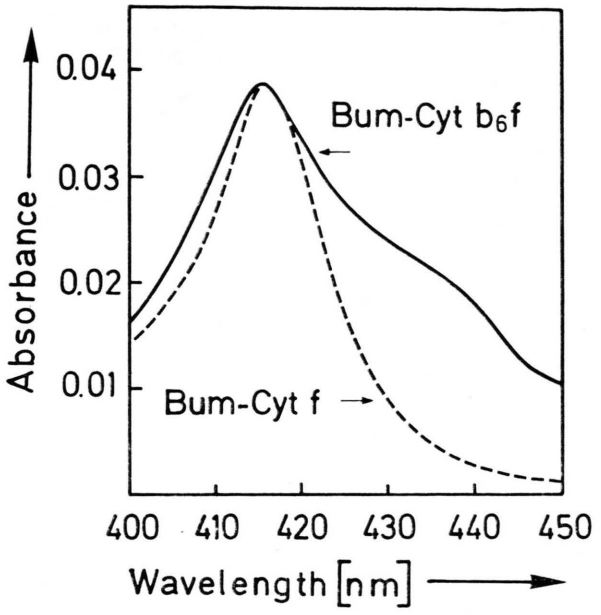

Fig. 1. *Bumilleriopsis:* Comparison of absorption spectra of chlorophyll-free cytochrome b_6-f particle (after elution on DE 52) and cytochrome 553 (= cytochrome f). Scan speed 0.5 nm min; slit width 0.5 nm.

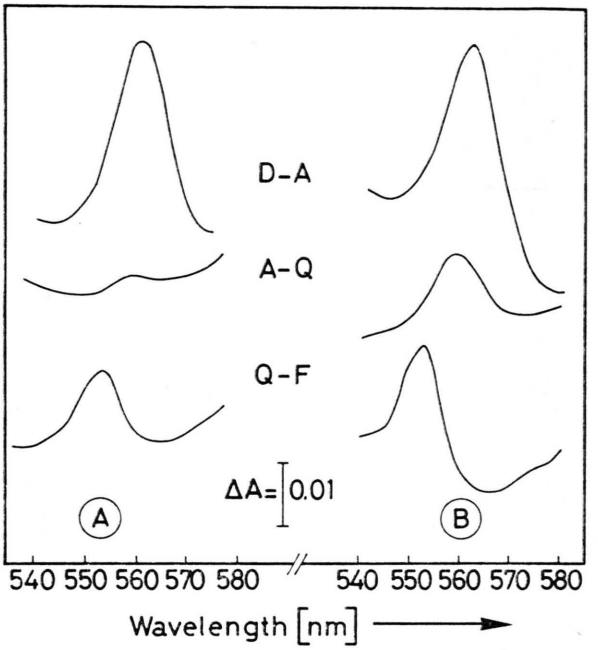

Fig. 2. Difference absorption spectra of cytochrome b_6-f particles from spinach chloroplasts. A, B: Two preparations after the protamine sulfate step. Q−F: Hydroquinone minus K-hexacyano-(III)-ferrat. A−Q: Na-ascorbate minus hydroquinone. D−A: Na-dithionite minus Na-ascorbate. Reduction of Cyt b_6 is a slow process [18], which is prolonged in the presence of digitonin. Therefore, complete reduction is assumed in the spectrum D−A, when no further increase of the maximum is detectable. Scan speed 0.5 nm/min; slit width 1 nm.

tion spectrum indicates that Cyt b_6 is present. Its content, however, has substantially decreased after the protamine sulfate precipitation.

3. *Presence of Cyt b-559LP in the Cyt b_6-f particle*

The question whether this cytochrome is present in our preparations can be decided already after the protamine sulfate precipitation. After this step, the chlorophyll content with most detergents used is so much decreased that the optical density is sufficiently low to allow determination of the necessary difference absorption spectra. From spinach, Cyt b_6-f particles are obtained which may contain Cyt b-559LP or not. It is noteworthy that in preparations with a high Cyt b_6:f ratio there is no or only very little Cyt b-559. Decreasing Cyt b_6/Cyt f ratios increase the content of Cyt b-559. Fig. 2 A demonstrates a preparation with a ratio of Cyt b_6:Cyt f = 2.55 which contains 0.1 μM Cyt b-559, while Fig. 2 B shows a ratio of 1.65, the particle containing the comparatively high concentration of 0.55 μM Cyt b-559.

Fig. 3 shows a dithionite minus ascorbate difference absorption spectrum of a chlorophyll-free Cyt b_6-f particle. The maxima observed under these conditions are the same as from homogeneously purified cytochrome b_6 [21]. When this sample is reduced by ascorbate and measured against a hydroquinone-treated reference, no absorbance due to Cyt b-559 is observed.

4. *Further purification of the Cyt b_6-f particle*

For determination of the three cytochromes dealt with in this paper, a further purification is not recommended, since cytochrome b_6 and cytochrome f are sensitive to further treatments to a different extent. The ratio of Cyt b_6: Cyt f of a digitonin preparation (comp. also ref. 1) is decreased, probably due to the loss on non-covalently bound heme in cytochrome b_6. Furthermore, peak shifts may be observed. The α-peak at 563 nm of Cyt b_6 can be placed to 561 nm, and the maximum of the γ-band thens hifts to 429 − 428 nm. This is true for the Cyt b_6-f particle itself as for a preparation of Cyt b_6 purified to homogeneity.

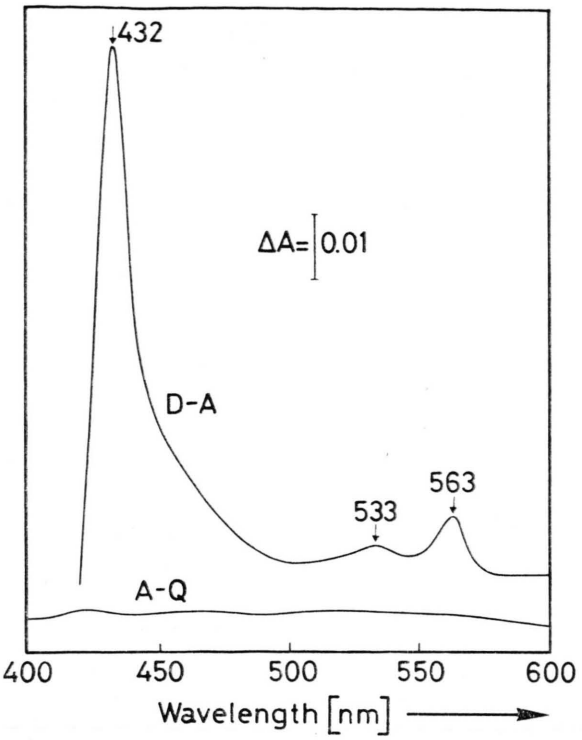

Fig. 3. Difference absorption spectrum of a chlorophyll-free cytochrome b_6-f particle from spinach chloroplasts after elution from a DE 52 column. Scan speed 0.5 nm/min; slit width 0.5 nm.

Discussion

The widely used digitonin solubilizes a Cyt b_6-f particle from spinach thylakoids, which contains the cytochromes in a molar ratio of 2:1, thereby confirming the data of Nelson and Neumann[1]. Although this ratio corresponds approximately to the amount of these cytochromes in spinal thylakoids[10], this stoichiometry is not obtained with other non-ionic detergents, yielding preparations with a cytochrome ratio of 1.5 – 4.9, their values being obviously characteristic for the detergent applied. The solubilization of cytochrome b_6 – *i. e.* incorporation of this protein into the detergent micelle – is highest with TRITON X-100. It is 100% referred to the cytochrome content *in vivo*. Using detergents of the TRITON X-series (having basically the same chemical composition), their solubilizing ability decreases with lower and higher HLB values (*i. e.* increase and decrease of the hydrophilic part in the molecule, see Table I). Detergents which lead to substantial solubilization of thylakoid cytochromes are in the HLB range of 12.4 to 16.9. Most detergents used for solubilization of membrane proteins from other organelles also exhibit an HLB range of 12.5 to 14.5. The often applied TRITON X-100 has an HLB value in between.

A definite comparison is difficult between detergents of different chemical structures (BRIJ, TWEEN, TRITON), since besides the HLB value their solubilizing ability is due to the nature of the lypophilic group in the molecule and to the fact whether or not there is an additional hydrophilic part present besides the polyoxyethylene (*e. g.* TWEEN). This explains why, for example, TRITON CF-54 with its HLB value of 13.6 does not solubilize the cytochrome b_6-f particle.

Different extent of solubilization by the detergents checked was also noted with the non-polar chlorophyll. As expected, it is solubilized best with detergents of low HLB values (*e.g.* TRITON X-114), whereas those with high HLB data (15.7 – 16.9) solubilize very little.

The different cytochrome content of the cytochrome b_6-f particles from spinach is due to different affinity of detergents to the respective cytochromes and not due to their possible denaturation. In case of the latter, a ratio of Cyt b_6 : Cyt f lower than 2 is expected, since cytochrome b_6 is more sensitive against denaturation. The changing composition of the Cyt b_6-f particle obtained with different detergents can be explained by varying extent of solubilization of Cyt b_6 and f, respectively, during treatment of the thylakoid. Preparative procedures using detergents therefore do neither indicate nor prove a strong binding of both cytochromes *in vivo*[1]. If this were the case, solubilization should generally lead to a particle containing these proteins in a constant stoichiometric relationship.

The results presented here demonstrate that neither the cytochrome b_6-f particle from spinach nor *Bumilleriopsis* contain cytochrome b-559LP. After digitonin treatment of thylakoids and centrifugation, photosystem II activity is mostly found in the pellet[15]. Apparently, this cytochrome is a contamination of the Cyt b_6-f particle, or it is a denatured cytochrome b_6, since denaturation shifts the maximum of the α-band to shorter wavelengths. Absence of Cyt b-559 in the Cyt b_6-f particle corroborates the idea that cytochromes b_6 and f have their functional localization within the photosystem-I region[19, 20].

In case of a redox function of cytochrome b-559 within photosystem I, one should expect its amount at least in the range of cytochrome f, which is not found.

In this paper, investigations on selective solubilization activity of various detergents [8, 9] are extended to photosynthetic membranes. Detergents with different HLB values and chemical composition appear to be a suitable means for selective solubilization of certain thylakoid membrane-bound components.

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