Biochimica et Biophysica Acta, 449 (1976) 197–208

BBA 47188

AN ENRICHED REACTION CENTER PREPARATION FROM GREEN PHOTOSYNTHETIC BACTERIA

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(Received April 26th, 1976)

SUMMARY

Bacteriochlorophyll *a* reaction-center complex I from *Chlorobium limicola* f. *thiosulfatophilum* 6230 (Tassajara) was incubated in 2 M guanidine · HCl and then chromatographed on cross-linked dextran or agarose gel. Two principal components were separated: a larger component with photochemical activity (bacteriochlorophyll *a* reaction-center complex II) and a smaller component without activity (bacteriochlorophyll *a* protein). Complex II contains carotenoid, bacteriochlorophyll *a*, reaction center(s), and cytochromes *b* and *c*, but lacks the well characterized bacteriochlorophyll *a* protein contained in Complex I. Complex II carries out a light-induced reduction of cytochrome *b* along with an oxidation of cytochrome *c*.

INTRODUCTION

Bacteriochlorophyll *a* reaction-center Complex I (mol. wt. > 1.5 million) and bacteriochlorophyll *a* protein (mol. wt. ≃ 130 000) are both isolated from green photosynthetic bacteria [1, 2]. Complex I is photochemically active, as evidenced by light-induced oxidation of bacteriochlorophyll *a* (*P*-840) and cytochromes *b* and *c* [3], whereas bacteriochlorophyll *a* protein is photochemically inert [4]. Complex I also contains carotenoid in addition to bacteriochlorophyll *a* and cytochromes *b* and *c*; the bacteriochlorophyll *a* protein contains only bacteriochlorophyll *a* and protein [4]. Comparison of the absorption and CD spectra of bacteriochlorophyll *a* protein at 77 K to the corresponding spectra of Complex I suggested that Complex I might consist of a reaction-center complex combined with several macromolecules of bacteriochlorophyll *a* protein [5]. The presence of the reaction-center complex was proposed on the basis of absorption and CD bands at 833 and 832 nm respectively in the

Abbreviations: Brij 58, polyoxyethylene(20)-cetyl ether; CD, circular dichroism; DEAE, diethylaminoethyl; Triton X-100, polyoxyethylene(10)-*p*-octylphenyl ether.

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spectra of Complex I at 77 K. (At room temperature the absorption band appears as a shoulder at 835 nm.) Since these bands do not appear in the absorption and CD spectra of the bacteriochlorophyll *a* protein, they were ascribed to bacteriochlorophyll *a* molecules other than *P*-840 in the reaction-center complex.

MATERIALS AND METHODS

Preparation of Complex I

Chlorobium limicola f. *thiosulfatophilum*, strain 6230 (Tassajara), was grown anaerobically in 20-l bottles as previously described [2]. Cells were harvested 5–7 days after inoculation by centrifugation at 14 000 × *g* for 20 min. The cells (4–10 g) were resuspended in approx. 4 ml of 10 mM sodium phosphate (pH 7.4) per gram wet weight of cells. Solid L-ascorbic acid was added to give a final concentration of 10 mM. The cells were homogenized and then passed three times through a French pressure cell at 16 000 lb/inch^2. After the first pass, 0.5 μg DNAase per ml suspension was added, and the homogenate incubated for 30 min at room temperature. (After the cells were lysed, the material was handled only in green light.) The lysate was centrifuged at 48 000 × *g* for 90 min to sediment unbroken cells and intact vesicles. The floating carotenoid layer, the pellet and 2–4 ml of solution above the pellet were discarded. The remainder of the supernatant was applied to a sucrose gradient. Either continuous (20–50 %) or discontinuous (35, 45, 50 %) sucrose gradients were employed. These were centrifuged for 18 h at 110 000 or 260 000 × *g*. Fractions (approx. 2 ml) were examined in a Cary 14R spectrophotometer. Those fractions having the lowest ratios of A_{745}/A_{810} and A_{671}/A_{810} were combined and designated as "Complex I preparations".

Chromatography

Hydroxyapatite was equilibrated in 10 mM ascorbate and 0.2 M NaCl in 10 mM phosphate [6]. Each column was packed in a 10-ml syringe, and a peristaltic pump used to draw off eluate.

DEAE cellulose (Schleicher and Schuell Type 40) was washed with 10 mM phosphate (pH 7.4) and 10 mM ascorbate in the presence and absence of 7 M urea: Columns were packed in 20-ml syringes.

Cross-linked dextran (Sephadex G-100 or G-200) or agarose gel (Sepharose 4B or CL-4B) was equilibrated with buffer containing 10 mM sodium phosphate (pH 7.4), 10 mM ascorbate, and guanidine · HCl or urea and packed in a column (0.8 × 60 cm or 0.5 × 50 cm). Fractions (1–2 ml) were collected, and static absorbance readings made at 810, 835, and 900 nm.

Spectrophotometry

Absorption spectra were recorded on a Cary 14R spectrophotometer; CD spectra were recorded on a modified Cary 14 [7]. Bacteriochlorophyll *a* concentration was estimated at 810 nm (Complex I) or 813 nm (Complex II) on the assumption that $\Delta\varepsilon = 100\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ [2].

Reduced-minus-oxidized difference spectra were recorded on the 14R spectrophotometer equipped with a sensitive slidewire (full scale = 0.1 or 0.2). Minute amounts of solid L-ascorbic acid (Fisher Reagent Grade) or sodium dithionite

(Baker, purified) were used as reductants. Crystals of potassium ferricyanide (Baker Reagent Grade) were used as oxidant.

Absorbance changes due to far red actinic light [2] were recorded in the wavelength intervals 400–440 nm and 500–650 nm by a custom built scanning spectrometer [8] and by a Cary 14R spectrophotometer. Changes in cytochromes *b* and *c* were measured at 562 and 552 (or 418) nm respectively with the assumptions that $\Delta\varepsilon_{562} = \Delta\varepsilon_{552} = 20\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ [2, 9] and that $\Delta\varepsilon_{418} = 100\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$. Changes in bacteriochlorophyll *a* (*P*-840) were conveniently measured at 610 nm [10] with the assumption that $\Delta\varepsilon_{610} = 30\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$. (This value is assumed from the absorbance at 603 nm of bacteriochlorophyll *a* protein from *C. limicola* 2 K [4].)

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by the slab gel technique of Studier [11]. Samples were heated in a boiling water bath for 2 min in the presence of cracking buffer: 1 % sodium dodecyl sulfate, 1 % mercaptoethanol, 10 % glycerol, 5 mM Tris (pH 6.8) and 2 mM EDTA. Electrophoresis was carried out in the standard glycine buffer on 10 % acrylamide. Gels were stained with Coomassie Blue. Horse heart cytochrome *c* (13-kdalton polypeptide) and bacteriochlorophyll *a* protein (39-kdalton polypeptide) were used as markers. Cytochrome *c* migrated close to the front as expected [12].

RESULTS

Bacteriochlorophyll a reaction-center Complex I

The extent of light-induced cytochrome *c* (552 nm) oxidation in various preparations varied from 0.7 to 4.3 molecules oxidized per 100 molecules of bacteriochlorophyll *a* (see Table I). On the average 1.7 molecules cytochrome were oxidized per 100 molecules bacteriochlorophyll *a*. The total amount of cytochrome *c* in Complex I was considerably greater than the amount oxidized upon illumination; in the one preparation (21. 10. 1975 in Table I) there were 23 molecules of cytochrome *c* but less than six molecules of cytochrome *b* for every 100 molecules of bacteriochlorophyll *a*. No light-induced reactions of cytochrome *b* (562 nm) were observed under the conditions of our experiments (air +10 mM ascorbate) in which the redox potential of the environment is presumed to be about 0 V.

Bacteriochlorophyll *a* (*P*-840) often showed no light-induced steady-state changes at 610 nm, but sometimes the light-minus-dark difference spectrum showed a trough at 610 nm in addition to the trough at approx. 552 nm. The ratio of cytochrome *c* oxidized to *P*-840 oxidized appeared to be approximately 2; however, chemical difference spectra (Fig. 1) indicate that the total cytochrome *c* content is approximately eight times the *P*-840 content.

Dissociation of Complex I

Since attempts to fractionate Complex I on hydroxyapatite and DEAE cellulose (with and without 7 M urea) had failed, Complex I was treated with various dissociating agents in attempts to break up the complex without destroying the photochemical activity of the reaction center. The effects of Brij 58, LDAO, Triton

TABLE I

RELATIVE AMOUNTS OF BACTERIOCHLOROPHYLL *a*, CYTOCHROMES *b* AND *c*, AND *P*-840 IN COMPLEXES I AND II

Date	Complex I		Complex II			
	Cyt *c* / 100 Bchl *a*	Cyt *c* / *P*-840	Cyt *c* / 100 Bchl *a*		Cyt *c* / Cyt *b*	
	Light[a]	Light[a]	Light[a]	Total[b]	Light[a]	Total[b]
15.11.1974	0.8	—	1.7	—	—	—
	0.9[c]	—				
12.08.1975	—	—	—	5.8	—	—
8.09.1975	0.8	1.7	0.3	6.7	−1	2.7
17.09.1975	3.7	—				
25.09.1975	2.3	—	0.8[e]	—	—	—
	4.3[d]	—	0.3[d,e]	—	—	—
21.10.1975	0.7	—	0.8	7.6	−1	2.4
(9–13 days)	1.1	2.5	0.8	4.8	−2	approx. 4
20.11.1975	1.0	—				
26.03.1976	1.5	2.1				
Average	1.7	2.1	0.8	6.2	−1.3	3

[a] Light-induced difference spectrum.
[b] Chemical difference spectrum (reduced minus oxidized).
[c] Guanidine · HCl (2M) present.
[d] Brij 35 (0.2 %) present.
[e] Made with 1.5 M guanidine · HCl.
Cyt *c*, cytochrome *c*; Bchl *a*, bacteriochlorophyll *a*.

X-100, guanidine · HCl or urea in 10 mM phosphate (pH 7.4) and 10 mM ascorbate on the absorbance ratio A_{835}/A_{810} were tested versus concentration of each agent and also versus time of incubation. A decrease in the absorbance ratio was taken to indicate differential damage to the reaction center with respect to the "bulk" bacteriochlorophyll *a* in the complex.

Brij 58 (3.2, % w/w) showed no effect even after incubation for 4 h, but samples could not be chromatographed on cross-linked dextran (Sephadex) in the presence of Brij because of a reaction between Brij and the dextran which stopped the flow of the column. When lauryl dimethylamine oxide (0.8 %, v/v) and Triton X-100 (2.0 %, v/v) were tested, a substantial decrease in absorbance ratio was observed after 10 min incubation and they were eliminated from further consideration. When guanidine · HCl (2 M) and urea (7 M) were tested, the absorbance ratio was unaffected. These two agents appeared to be the most promising candidates for dissociating Complex I without destroying the reaction center. Complex I in 2 M guanidine · HCl retained the essential spectral and photochemical characteristics of the original complex in buffer. The shoulder at 835 nm remained in the absorption spectrum, and the CD spectrum (Fig. 5) was unchanged. In our original experiments photochemical activity, assayed as light-induced cytochrome *c* oxidation, remained essentially unchanged except for a doubling of the rate of reduction when illumination was stopped. However, in subsequent experiments we observed that the extent of cyto-

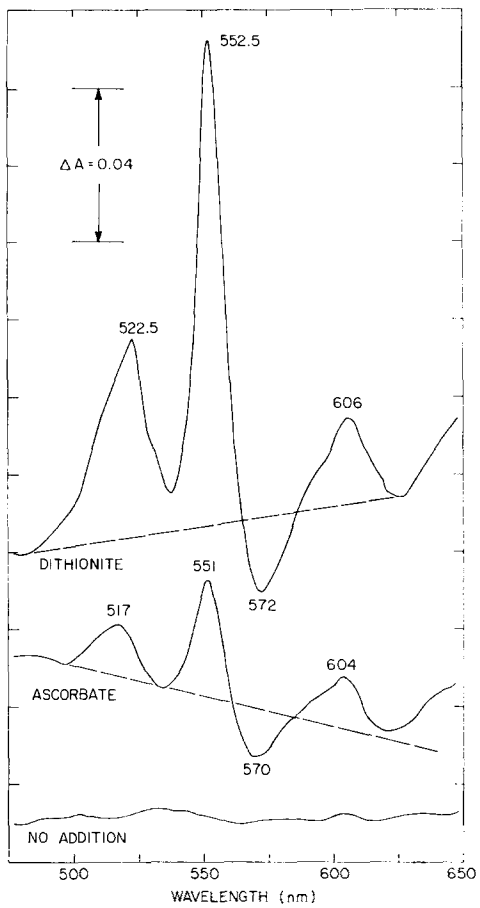

Fig. 1. Reduced minus-oxidized (no additions) difference spectra of Complex I ($A_{810} \simeq 2.7$, 21.10.1975).

chrome *c* oxidation in the presence of 2 M guanidine · HCl is usually considerably less than in its absence. We therefore tested the effect of guanidine · HCl as a function of both concentration and time of incubation and found that the extent of cytochrome *c* oxidation remaining after 1.2 h incubation in 2 M guanidine · HCl is only about 6 % of the control. The original observation, that guanidine · HCl did not greatly affect the extent of light-induced cytochrome *c* oxidation, has not been repeated.

Chromatography of Complex I treated with guanidine · HCl or urea

Originally Complex I was incubated for 30 min in separate experiments with 1 M guanidine · HCl and 7 or 8 M urea and then chromatographed on cross-linked dextran (Sephadex G-100). This procedure separated Complex I into at least two colored components. From starting material (Complex I) with $A_{835}/A_{810} = 0.21$, the first (A) and second (B) fractions off the column had absorbance ratios of 0.34 and 0.18, respectively. Improved separation was obtained by raising the guanidine · HCl to 2 M and by replacing Sephadex G-100 by agarose gel (Sepharose 4B, or

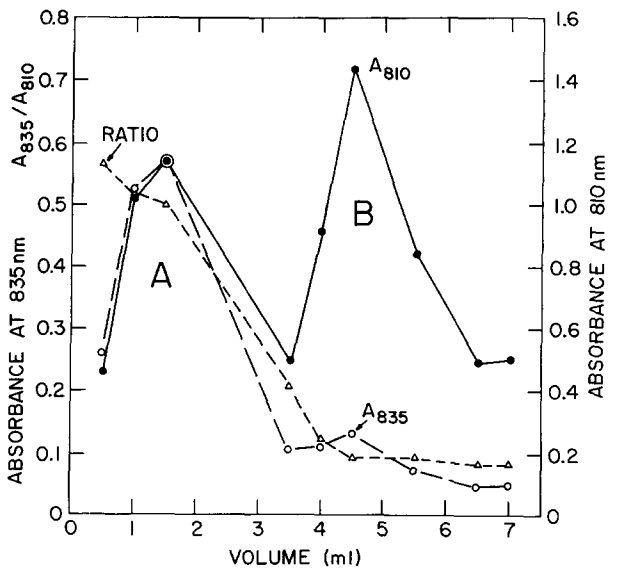

Fig. 2. Chromatography of Complex I in 2 M guanidine · HCl on Sepharose 4B. Sample (A_{810} = 4.7, A_{835} = 1.2, volume = 1.3 ml) was passed through a 0.5 × 50 cm column.

Sepharose CL-4B). Now fractions A and B routinely have absorbance ratios of approximately 0.45 and 0.09, respectively. Fig. 2 shows the separation of the two fractions. With 7 or 8 M urea and Sephadex G-100 the separation of fractions A and B was inferior to that obtained with guanidine · HCl.

Characterization of fractions A and B

The CD spectrum for Fraction A (Fig. 3) is distinctly different from that for Complex I; in particular the positive peak at 835 nm is substantially larger (based on equivalent bacteriochlorophyll content) in the spectrum for Fraction A. Furthermore the far red absorption peak (Fig. 4) is shifted from 810 nm in Complex I to approx. 813 nm in Fraction A, and the relative size of the 835 nm shoulder is doubled. The peaks at 414 and 674 in the absorption spectrum (Fig. 4) are attributed to chlorobium pheophytin (bacteriopheophytin *c*) and the peaks (or shoulders) at (320), 341 (approx. 375), 603, (approx. 800), 813 and (approx. 835 nm) are ascribed to bacteriochlorophyll *a*. Carotenoid gives rise to the peaks (or shoulders) at (approx. 440), 471 and 504 nm. We have named the large pigmented component in Fraction A "Bacteriochlorophyll *a* reaction-center Complex II". Table I shows that on the average there are approx. 6 cytochrome *c* molecules for every 100 bacteriochlorophyll *a* molecule in Complex II. Upon illumination approx. 2 cytochrome *c* molecules are oxidized for every reaction center (*P*-840) oxidized, consistent with the earlier finding that the reaction center in Complex I is associated with two functional cytochrome *c* molecules [10]. Therefore we estimate approx. 30–50 chlorophyll molecules per reaction center (active or inactive) in Complex II. Although Complex II contains less bacteriochlorophyll *a* per reaction center than does Complex I, more chlorophyll remains than can be assigned to reaction centers alone.

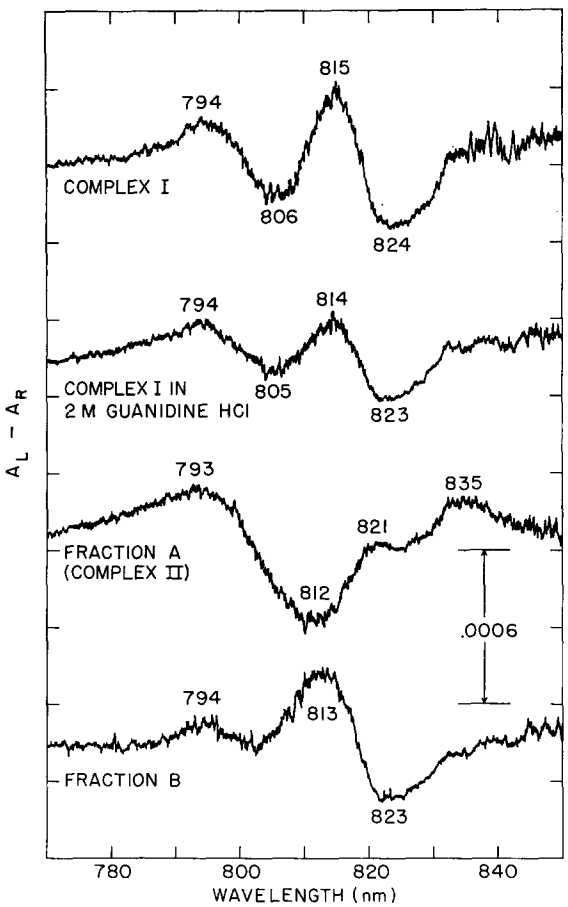

Fig. 3. CD spectra of Complex I preparations. (1) Complex I in 10 mM sodium phosphate and 10 mM sodium ascorbate. (2) Complex I in phosphate and ascorbate plus 2 M guanidine · HCl. (3) Fraction A (Complex II) from Sephadex column. (4) Fraction B.

As shown in Table I the extent of light-induced cytochrome *c* oxidation in Complex II varies from preparation to preparation. The most active preparation is comparable to the average of all preparations of Complex I based on equivalent chlorophyll content. However, on the average the extent of light-induced cytochrome *c* oxidation in Complex II is about half that observed in Complex I. Complex II contains about one third the total amount of cytochrome *c* as does Complex I, and, of this amount, only about 13% responds to light. The ratio of total cytochrome *c* to *P*-840 (both active and inactive) is estimated to be between 3 and 4, which is approx. half the ratio for Complex I. The ratio of cytochrome *c* oxidized in the light to *P*-840 oxidized in the light appears to be 2, the same as in Complex I.

As shown in Fig. 5 Complex II also exhibits a light-induced reduction of cytochrome *b* concomitant with the oxidation of cytochrome *c*. (The reduction was never observed in Complex I.) The ratio of total cytochrome *c* to cytochrome *b* appears to be 3 ± 1 (see Table I and Fig. 6).

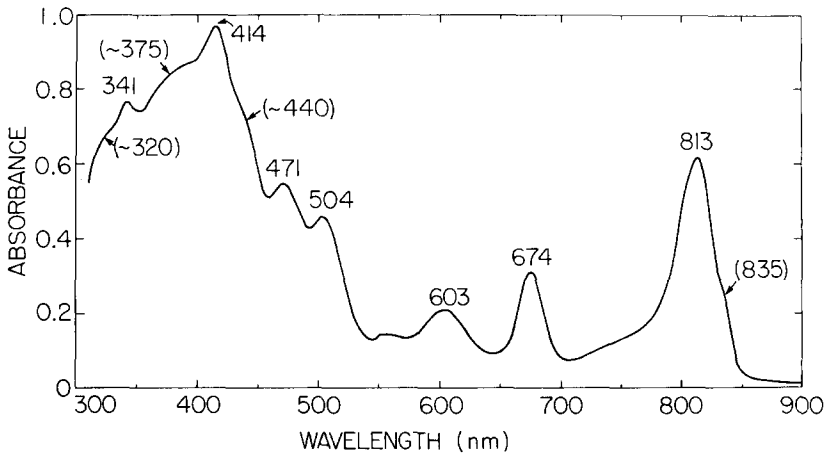

Fig. 4. Absorption spectrum of Fraction A (Complex II) in 2 M guanidine · HCl, 10 mM sodium phosphate (pH 7.4) and 10 mM sodium ascorbate (21.10.1975). $A_{674}/A_{813} = 0.50$. $A_{835}/A_{813} = 0.40$

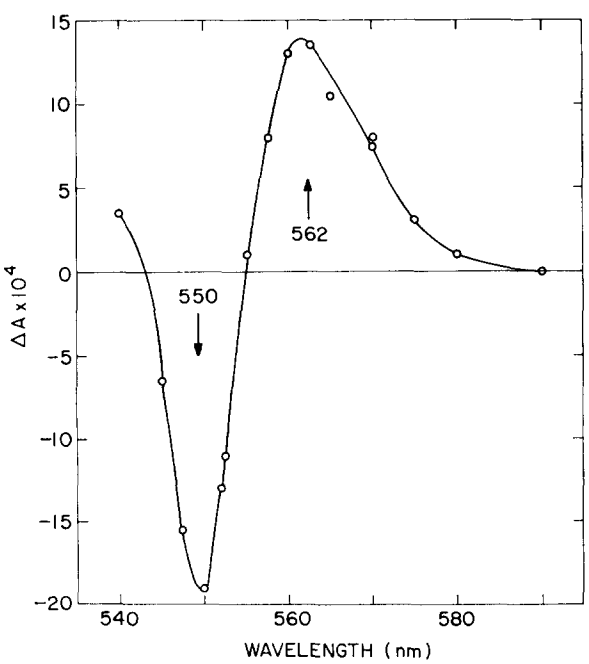

Fig. 5. Light-induced cytochrome reactions in Fraction A (Complex II, $A_{813} = 1.24$) 7 h after addition of 2 M guanidine · HCl to Complex I (21.10.1975).

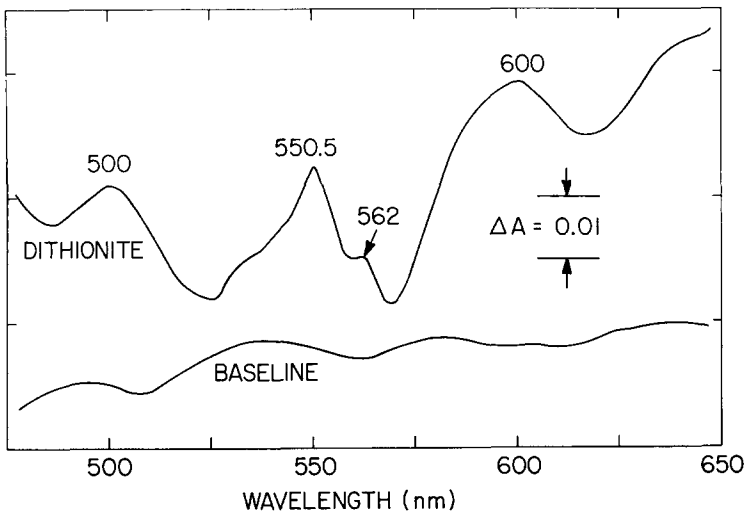

Fig. 6. Reduced(dithionite)-minus-oxidized(ascorbate) difference spectrum of Complex II (A_{813} = 1.58, 21.10.1975).

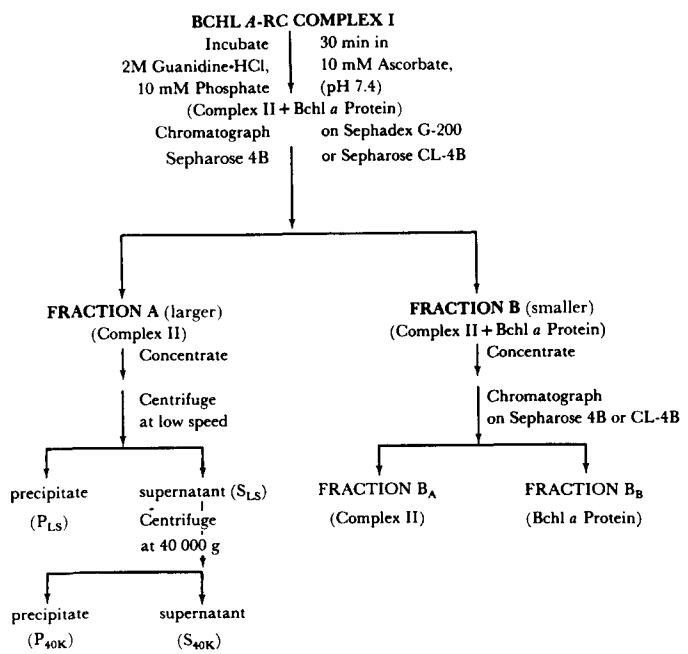

Fig. 7. Flow diagram for separation of various fractions after treatment of Complex I with 2 M guanidine · HCl. Bchl *a*, bacteriochlorophyll *a*; RC, reaction-centre.

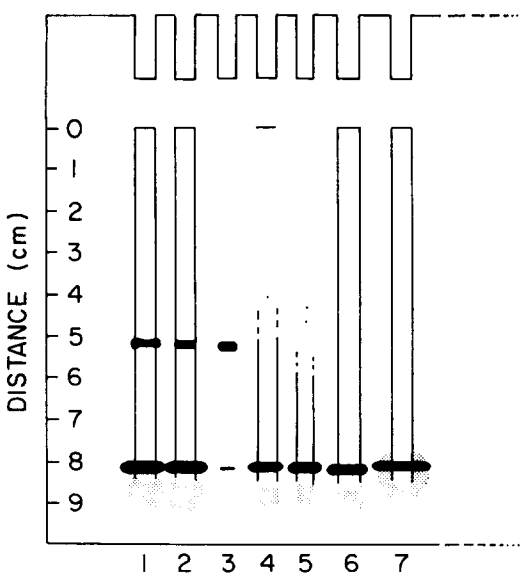

Fig. 8. Comparison of various fractions and purified bacteriochlorophyll *a* protein by gel electrophoresis. (1) Fraction B; (2) Fraction B_B; (3) bacteriochlorophyll *a* protein; (4) Fraction B_A; (5) Fraction A (P_{LS}); (6) Fraction A (P_{40K}); (7) Fraction A (S_{40K}). The figure was traced from a xerox image of a slab gel. The order of the samples has been rearranged for clarity.

When fraction A is concentrated, Complex II tends to precipitate. Various precipitate and supernatant subfractions (P_{LS}, P_{40K} and S_{40K}) can be obtained by centrifugation as shown in Fig. 7. When any of these subfractions is heated in cracking buffer and then electrophoresed, two bands appear as shown in Fig. 8: a compact (C) band and a diffuse (D) band. The C band migrates with the front and probably contains a variety of polypeptides with molecular weights below 15 000 [12]. The nature of the material in the D band is uncertain. When fraction B is denatured and electrophoresed three bands appear (Fig. 8). The top band (39 kdalton) is new; the middle C band and the lower D band appear to be the same bands observed for subfractions A (P_{LS}, P_{40K} and S_{40K}). When fraction B is rechromatographed on Sepharose, two fractions are obtained (Fig. 7). The first fraction (B_A) is similar to subfractions A (P_{LS}, P_{40K} and S_{40K}) and contains no 39-kdalton polypeptide. The second fraction (B_B) does contain the 39-kdalton polypeptide as well as other material in the C - and D - bands.

The CD spectrum of fraction B (Fig. 3) closely resembles that for bacteriochlorophyll *a* protein, especially in showing no peak at 835 nm [2]. Photochemical activity is low compared to that in fraction A or in Complex I. Fraction B_B has no detectable photochemical activity and closely resembles the bacteriochlorophyll *a* protein in absorption spectrum.

Interpretation

Complex I probably exists as a membranous fragment of molecular weight greater than 1.5 million [1]. In the presence of 1.5–2.0 M guanidine · HCl, some cytochrome *c* and all of the bacteriochlorophyll *a* proteins (mol. wt. = 140 000)

are dissociated from the complex. Complex II, which remains, contains bacteriochlorophyll *a* (*P*-840, *B*-800, *B*-813 and *B*-835), cytochrome *c*, cytochrome *b*, carotenoid, and chlorobium pheophytin (bacteriopheophytin *c*). (Chlorobium pheophytin is probably a contaminant, because its ratio to bacteriochlorophyll *a* varies from approx. 0.2 (Fig. 6) to 5 in different preparations.) Complex II is smaller than Complex I, but it is clearly much larger than the bacteriochlorophyll *a* protein. However the polypeptides present in Complex II (approx. 15 kdalton or less) are much smaller than the subunit polypeptide (39 kdalton) of the bacteriochlorophyll *a* protein. Cytochromes in Complex II probably account for some of the polypeptides in the C band, but further electrophoretic studies are required to identify these polypeptides.

Fraction B contains mostly bacteriochlorophyll *a* protein with a little Complex II plus impurities; Fraction B_B contains bacteriochlorophyll *a* protein plus low molecular weight impurities.

DISCUSSION

Although Complex II shows no evidence of polypeptides in the 20- to 30-kdalton range, such polypeptides still might be present in an amount too low to be detected by the electrophoretic technique used. Since the reaction centers of the non-sulfur purple bacteria, *Rhodopseudomonas sphaeroides* and *Rps. capsulata*, contain three polypeptides of 21, 24 and 28 kdalton respectively [13–15], we intend to find out unequivocally whether or not reaction centers from green bacteria contain similar polypeptides.

Fowler [3] showed that Complex I from *C. Limicola* 2 K contains approximately equal amounts of cytochrome *b* and cytochrome *c*. We have observed in both Complexes I and II from strain Tassajara that there is at least twice as much cytochrome *c* as cytochrome *b*. We also have evidence (see Fig. 1) in Complex I for an ascorbate-reducible cytochrome *c*-551 [9], which does not react to light. There may also be some dithionite-reducible cytochrome *c*-551 in Complex II (Fig. 6). Fowler [3] also demonstrated the light-induced oxidation of cytochrome *b* in Complex I when the redox potential of the environment was lowered to −0.25 V. We have shown the light-induced reduction of cytochrome *b* concurrent with the oxidation of cytochrome *c* in Complex II from strain Tassajara at a redox potential in the neighborhood of 0V. Similar observations in *Chlorobium* chromatophores have been made by Knaff and Buchanan [6]. From these observations we conclude that cytochromes *c* and *b* probably function in a cyclic electron transport chain in Complex I. In Complex II the linkage between the two cytochromes may be severely impaired.

ACKNOWLEDGEMENTS

Research was carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission and the U. S. Energy Research and Development Administration. Dr. J. Philip Thornber suggested the use of the guanidine · HCl to dissociate Complex I and provided constructive criticism of the manuscript.

NOTE ADDED IN PROOF (Received September 20th, 1976)

Polyacrylamide slab gel electrophoresis in 8 M urea (Downer, N. M., Robinson, N. C. and Capaldi, R. A. (1976) Biochemistry 15, 2930–2936) of dissociated Complexes I and II has shown the presence of at least six polypeptides in each complex (Table II).

TABLE II

MOLECULAR WEIGHT AND RELATIVE ABUNDANCE OF POLYPEPTIDES IN COMPLEXES I AND II

kdalton	Complex I	Complex II
42±4	+++++	+++
36±2	++	+
31±4	+	++++++
23±2	+++	++
21±2	+	+++
18±1	++	++

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