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A CYTOCHROME *f*-b_6 COMPLEX WITH PLASTOQUINOL-CYTOCHROME *c* OXIDOREDUCTASE ACTIVITY FROM *ANABAENA VARIABILIS*

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A cytochrome *f*-b_6 complex has been isolated from thylakoid membranes of *Anabaena variabilis*. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed the presence of four major polypeptides – cytochrome *f* (molecular mass 31 or 38 kDa, depending on sample preparation for the electrophoresis), cytochrome b_6 (molecular mass 22.5 kDa), a band with slightly lower mobility than cytochrome b_6 resolved only in gels containing urea, and a 16.5 kDa polypeptide, – plus two small polypeptides below 10 kDa. The complex is devoid of chlorophyll and carotenoids, contains 2 cytochromes b_6 per cytochrome *f*, but no cytochrome *b*-559. The Rieske Fe-S center is present in substoichiometric amounts of cytochrome *f*. The complex is active in plastoquinol-cytochrome *c* oxidoreductase activity, which is specific for electron-accepting proteins with positive net charge at physiological pH. Cytochrome *c* from horse heart, cytochrome *c*-553 and plastocyanin, both from *A. variabilis*, are equally well reduced. The specificity for the electron-donating quinol is less stringent. The sensitivity of the oxidoreductase to various inhibitors is retained during the isolation procedure, 2,5-dibromo-3-methyl-isopropyl-*p*-benzoquinone being the most efficient. The complex exhibits oxidant-induced reduction of cytochrome b_6, and photooxidation of cytochrome *f* as well as transient photoreduction of cytochrome b_6 when illuminated in the presence of plastoquinol, plastocyanin and Photosystem I reaction centers.

Introduction

A cytochrome *f*-b_6 complex retaining plastoquinol-plastocyanin-oxidoreductase activity has been isolated by us from spinach chloroplasts and characterized in detail [1]. Its polypeptide composition was found to be simpler than that known for cytochrome *b*-c_1 complexes from mitochrondria, although the redox components comprising the complex were the same: two cytochromes *b*, one cytochrome *c* and the Rieske Fe-S center [2–5]. In our search for the minimum polypeptide composition of polyprenyl quinol-cytochrome *c* oxidoreductases we extended our studies to photosynthetic bacteria, where these complexes may be involved in both photosynthetic and respiratory electron transport [6,7]. Here we report on the isolation and characteristics of an active cytochrome *f*-b_6 complex from the cyanobacterium *Anabaena variabilis*.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)glycine; Chl, chlorophyll; kDa, kilodalton.

Methods

The oxidoreductase was isolated from thylakoids of *A. variabilis* Kütz. (ATCC 29413) by a modification of the method developed for spinach chloroplasts.

The cyanobacterium was grown and harvested as described previously [8] and was stored frozen

at −20°C. All subsequent steps of isolation were performed at 0–4°C. Membranes were isolated as before [8] except that flushing with N_2 was omitted and the time of homogenization was extended to 1 min. The final membrane pellet was suspended in 0.4 M sucrose, 2 M NaBr, 10 mM Tricine-Tris (pH 8.1) to a chlorophyll concentration of 1.0 mg/ml. After 30 min, the suspension was diluted with an equal volume of water and centrifuged for 1 h at 48000 × *g*. The membrane pellet was washed once in 0.2 M mannitol, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 15 mM Tricine-Tris (pH 8.1) (centrifugation as before) and was resuspended in 0.4 M $(NH_4)_2SO_4$, 0.2 M sucrose, 1.5 mM KCl, 1.5 mM $MgCl_2$, 0.5% (w/v) cholate, 30 mM octylglucoside, 20 mM Tricine-Tris (pH 8.1) to a chlorophyll concentration of 1.5 mg/ml. After incubation for 30 min it was centrifuged for 1 h at 300000 × *g*. A saturated solution of $(NH_4)_2SO_4$ (pH 7.5) was added to the supernatant to give 45, 55 and 70% saturation. After each step, the solution was stirred for 20 min and centrifuged for 10 min at 27000 × *g*. The material precipitating between 55 and 70% saturation of $(NH_4)_2SO_4$ was dissolved in a minimal volume of 20 mM Tricine-Tris (pH 8.1) containing 0.2% (v/v) Triton X-100 and dialyzed for 1 h against 20 mM Tris-HCl (pH 8.1), 0.2% (v/v) Triton X-100. The material was loaded on linear sucrose density gradients (7–30%, w/v) containing 20 mM Tris-HCl (pH 8.1), 0.2% (w/v) cholate, 10 mM octylglucoside, and was centrifuged for 14 h in a Beckman SW-60 rotor at 330000 × g_{max}. The lower brown band containing the oxidoreductase was removed with a syringe. It could be stored in liquid N_2 without loss of activity. An upper greenish-brown band observed in the gradients contained chlorophyll and cytochrome *c*-549 (see Ref. 9).

The isolation procedures for the plastocyanins and cytochromes used as electron acceptors for the oxidoreductase have been listed before [1,8]. Cytochrome *c* from horse heart (type III) was purchased from Sigma (Munich, F.R.G.). Quinols were prepared as described previously [1]. Tetramethyl-*p*-benzoquinone (Sigma) was reduced according to the method of Izawa and Pan [10]. Sources of other chemicals were as listed in Ref. 1.

Chl *a* was determined in methanolic extracts according to the method of Mackinney [11] and protein as described by Bensadoun and Weinstein [12]. SDS-polyacrylamide gel electrophoresis was performed in slab gels in either the buffer system of Laemmli [13] or, in the presence of urea, in the buffer system of Harms et al. [14], as specified in the legend to Fig. 2. Gels were stained for heme-linked peroxidase activity [15] and/or for protein [16].

Oxidoreductase activity was measured at 20°C in an Aminco DW-2 spectrophotometer in the dual-wavelength mode. For reduction of cytochromes, the measuring beam was set to the wavelength of the α-peak (cytochrome *c* from horse heart, 550 nm; cytochromes c_2 from *Rhodopseudomonas capsulata* and *c*-552 from *Euglena gracilis*, 552 nm; cytochrome *c*-553 from *A. variabilis* 553 nm). The reference wavelength was 540 nm. Reduction of plastocyanins was followed at 597 minus 500 nm. The optical band pass was 1 nm throughout. First, the uncatalyzed reduction was measured by addition of quinol, then the catalyzed reaction was started by addition of the cytochrome *f*-b_6 complex.

The cytochromes of thylakoids and of the isolated oxidoreductase were quantified by redox difference spectra (ascorbate minus ferricyanide, dithionite minus ascorbate) as described by Almon and Böhme [17]. Acid-labile sulfur was estimated according to the method of Golbeck and San Pietro [18].

Results and Discussion

Table I shows the course of purification. The final cytochrome *f*-b_6 preparation is enriched about 20-fold on a protein basis and over 300-fold on a chlorophyll basis with a yield of 15%, all with respect to cytochrome b_6. From the ratio of cytochrome b_6 to protein, a molecular mass of 222 kDa can be derived for the protein moiety, assuming 2 cytochromes b_6 per complex. This is probably an upper limit because of some contaminating proteins (see Fig. 2). The complex contains 2 cytochromes b_6 per cytochrome *f* (Fig. 1). Estimations of this ratio in the membranes and during early steps of the purification are complicated by the closeness of the α-band of cytochromes *f* and *b*-559 [17]. Cytochrome *b*-559 is absent from the

TABLE I

PURIFICATION AND COMPOSITION OF PLASTOQUINOL-CYTOCHROME *c* OXIDOREDUCTASE

The preparation and assays are described in Methods. Oxidoreductase activity (μmol cytochrome *c* reduced/nmol cytochrome b_6 per h) was measured in the presence of 20 mM Mes-Tris (pH 6.1), 25 μM cytochrome *c*, 50 μM plastoquinol-1 and 50 nM cytochrome b_6 in the form of the cytochrome *f*-b_6 complex.

Purification step	Cytochrome b_6 (μM)	Chl/ cytochrome b_6 (mol/mol)	Cytochrome b_6/ protein (nmol/mg)	Cytochrome b_6/ cytochrome *f* (mol/mol)	Oxido-reductase activity	Yield of cytochrome b_6 (%)
Extract	5.3	317	0.54	–	68	100
300000 × *g* supernatant	3.7	162	3.1	–	50	70
$(NH_4)_2SO_4$	32.3	85	4.2	1.98	28	48
Sucrose gradient	10.0	0.9	9.0	2.07	19	15

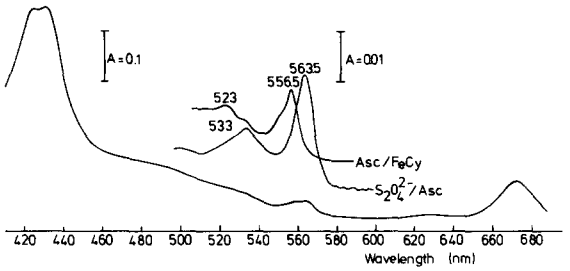

Fig. 1. Absorption spectra of the isolated cytochrome *f*-b_6 complex from *A. variabilis*. For the absolute spectrum, the cytochromes were reduced with $Na_2S_2O_4$. (Center) Difference spectra (ascorbate-reduced minus ferricyanide-oxidized (Asc/FeCy) and dithionite-reduced minus ascorbate-reduced ($S_2O_4^{2-}$/Asc)) showing the presence of cytochromes *f* and b_6, respectively. The concentration of the cytochrome *f*-b_6 complex was 0.9 μM in cytochrome b_6.

purified complex, as is evident from the difference spectra in Fig. 1. Spectra at low temperature show the characteristic split α-peak for cytochrome *f*, and an asymmetric α peak for cytochrome b_6 [17].

The polypeptide composition of the complex is shown in Fig. 2. Electrophoresis using the method of Laemmli [13] gives three major bands of 31, 22.5 and 16.5 kDa, and two small polypeptides below 10 kDa (Fig. 2A and B). The larger two bands react in the test for heme (Fig. 2B). The largest polypeptide can be shifted to about 38 kDa by boiling the sample prior to electrophoresis, a phenomenon described before for cytochrome *f* of *Anacystis* [19]. Based on molecular mass, the largest subunit is identified as cytochrome *f* [19–28], whereas the 22.5 kDa protein should represent cytochrome b_6. Fig. 2 also shows the polypeptide pattern resolved by polyacrylamide gel electrophoresis in the presence of urea [14], which is more complex. The two larger polypeptides, which both probably represent cytochrome *f*, give heme stain and can be shifted to the largest species by boiling. The 22.5 kDa polypeptide found in the absence of urea is resolved into two bands, the faster migrating, diffuse one giving heme stain (Fig. 2C). Also, the 16.5 kDa subunit seen in the absence of urea is resolved into a major and a minor component, migrating very close, in the presence of urea. Since this splitting was only observed with one preparation out of a total of eight examined on urea gels, the subunit is assumed to consist of one polypeptide. The two small components below 10 kDa are also visible on the pattern obtained in the presence of urea.

Measurement of the EPR spectra revealed a signal at 1.9 *g* characteristic of the Rieske Fe-S center. It was about 20% in amplitude compared to the analogous signal obtained with the cytochrome *f*-b_6 complex from spinach or with the cytochrome *b*-c_1 complex from beef heart mitochondria, with respect to cytochrome *f* or c_1 (Malkin, R. and Prince, R.C., unpublished results). Estimation of acid-labile sulfur [18] gave 0.4–0.6 S per cytochrome *f*, which corresponds to 0.2–0.3 Rieske centers. The presence of the Rieske Fe-S protein is also indicated by the cross-reaction of the cytochrome *f*-b_6 complex from *A. variabilis* with an antiserum obtained against the Rieske Fe-S protein purified from spinach chloroplasts [5]. The Ouchterlony double diffusion test indicates partial identity of the two Fe-S proteins (not shown). From all this we conclude that the Rieske Fe-S protein is present in substoichiometric amounts in the preparation from *A. variabilis*. It possibly corresponds to the weakly stained polypeptide which has a somewhat lower mobility than cytochrome b_6 in gels containing urea (Fig. 2C).

The loss of oxidoreductase activity during purification (Table I) might be explained by a loss of

TABLE II

ELECTRON DONORS FOR THE QUINOL-CYTOCHROME *c* OXIDOREDUCTASE OF *A. VARIABILIS*

The assay is described in Methods. The mixture contained 20 mM Mes-Tris (pH 6.1), 75 μM quinol (added from 10 mM stock solutions), 9 μM cytochrome *c* from horse heart and 23 nM cytochrome b_6 in the form of the cytochrome *f*-b_6 complex. Assays contained 0.75% (v/v) ethanol except in the cases of duroquinol (0.4% ethanol plus 0.4% ethylene glycol) and of plastoquinol-9 and ubiquinol-9 (0.15% l(v/v) Triton X-100).

Electron donor	Oxidoreductase activity (μmol/nmol cytochrome b_6 per h)
Plastoquinol-1	16
Ubiquinol-1	15
Trimethyl-*p*-benzoquinol	0.8
Duroquinol	12
Plastoquinol-9	20
Ubiquinol-9	5

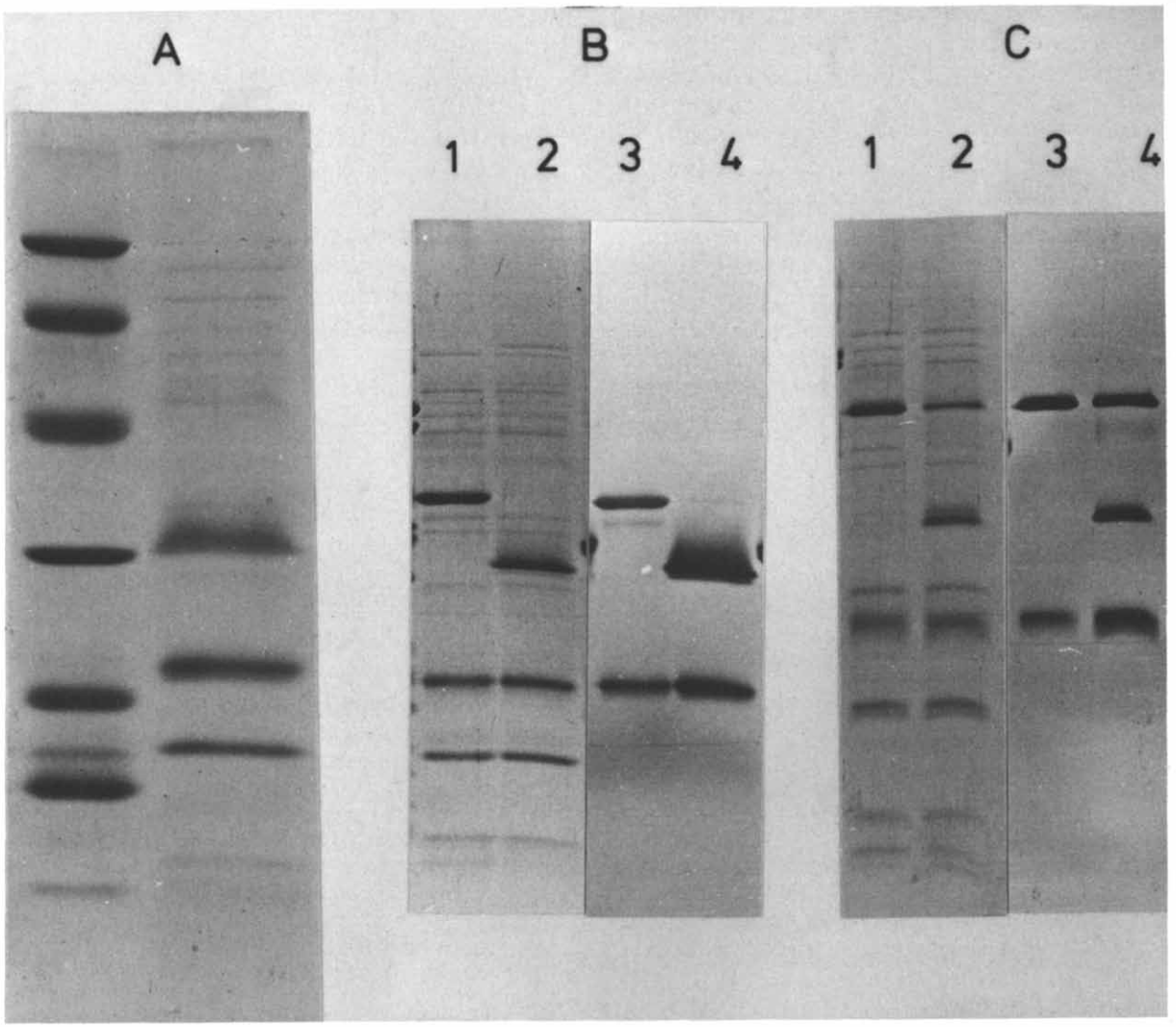

Fig. 2. Polypeptide composition of the cytochrome *f*-b_6 complex. (A) SDS-polyacrylamide gel according to the method of Laemmli [13], polyacrylamide gradient from 12 to 18%. The sample was dissolved in sample buffer at room temperature. Left lane: molecular mass standards (92.5, 66.5, 45, 31, 21.5, 14.3 kDa). Right lane: cytochrome *f*-b_6 complex with 0.45 nmol cytochrome b_6 (B) Gel system as in A, but another preparation of the complex. Lane 1, complex heated in sample buffer for 5 min at 100°C before application to the gel; lane 2, heating omitted. Lanes 3 and 4 are identical to lanes 1 and 2 but are stained for heme-linked peroxidase activity. Complex containing 0.1 nmol cytochrome b_6 was applied to each lane. (C) Separation on an SDS-polyacrylamide gel containing urea [14]. The separating gel contained 15% acrylamide. Arrangement of lanes and all other details as in B.

the Rieske Fe-S protein. This component can be removed from cytochrome *b*-*c* complexes rather easily [3,5,29]. However, the highest oxidoreductase activity observed with purified preparations from *A. variabilis* (35 μmol cytochrome *c* reduced/nmol cytochrome *b* per h) is higher than that reported for the preparation from spinach (20 μmol cytochrome *c* reduced/nmol cytochrome *b* per h), which contains the Fe-S protein in close to equimolar amounts to cytochrome *f* [1,5]. Oxidoreductase activity is highest with plastoquinols, although ubiquinol-1 and duroquinol are almost as efficient electron donors (Table II). Trimethyl-*p*-benzoquinol is only poorly oxidized, as was also

shown for the oxidoreductase from spinach [1]. The concentrations of plastoquinol-1 and -9 for half-maximal activity were 20 and 40 μM, respectively. Plastoquinol-9 has the advantage over plastoquinol-1 that its uncatalyzed reaction with cytochrome *c* becomes prominent above pH 8.5 only [1], but has the disadvantage that it has to be added in the presence of Triton X-100, which inhibits oxidoreductase activity by micellar 'surface dilution' [3]. The pH optimum for the oxidoreductase from *A. variabilis* with both plastoquinols is about pH 7.5, similar to the finding with spinach [1]. For plastoquinol-9 the activity at pH 8.1 is 1.7-fold of that measured at pH 6.1 (cf. Tables II and III). The maximal rate measured with the purified complex with plastoquinol-9 at the pH optimum was approx. 35 μmol cytochrome *c* reduced/nmol cytochrome b_6 per h. This amounts to about 40% of the electron-transport rate from Photosystem II to Photosystem I measured with isolated thylakoids [30], if one assumes 2 cytochromes b_6/cytochrome *f* and about 500 Chl/cytochrome *f*. To obtain high activity it is important to isolate the complex as quickly as possible. The presence of Triton X-100 during density gradient centrifugation, successfully used for the isolation of the complex from spinach [1], inactivated the oxidoreductase activity almost completely.

Table III shows the specificity of the isolated oxidoreductase for electron-accepting cytochromes *c* and plastocyanins, which is more expressed than for its counterpart from spinach [1]. Highest rates are obtained with cytochrome *c* from horse heart and the candidates for the physiological electron acceptor, cytochrome *c*-553 and plastocyanin from *A. variabilis*. All three are basic proteins [31,32]. The concentration of cytochrome *c* from horse heart and of cytochrome *c*-553 giving half-maximal activity was 2 and less than 1 μM, respectively. Because of the low extinction coefficient, such a value could not be estimated for plastocyanin from *A. variabilis* which gave maximal activity at 10 μM. It is worth noting in this context that cytochrome *c*-553 and plastocyanin are also known to replace each other functionally as electron donors for Photosystem I [32,33] as well as for cytochrome oxidase [8].

In comparison to spinach [1] the oxidoreductase from *A. variabilis* is much less sensitive to inhibitors (Table IV). This holds for the isolated cytochrome complex as well as for thylakoid membranes. The most efficient is 2,5-dibromomethylisopropyl-*p*-benzoquinone.

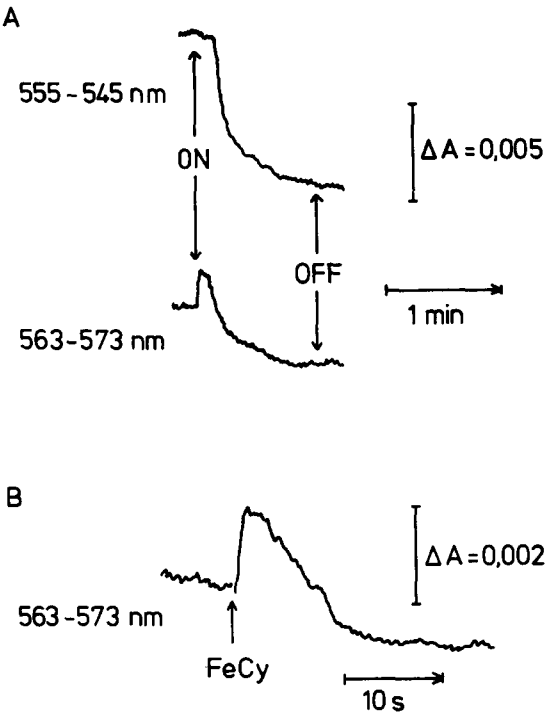

Fig. 3. (A) Photoreduction of cytochrome b_6 and photooxidation of cytochrome *f* by Photosystem I reaction centers. The reaction was measured in an Aminco DW-2 spectrophotometer in the dual-wavelength mode. The reaction mixture contained 20 mM Tricine-Tris (pH 8.1), 60 mM KCl, 5 mM $MgCl_2$, 0.05% (v/v) Triton X-100, 20 μM plastoquinol-1, 25 μM spinach plastocyanin, 0.7 μM Photosystem I reaction centers (as P-700), and 2.4 μM cytochrome b_6 in the form of the cytochrome f-b_6 complex. The reaction centers were isolated from spinach chloroplasts as described in Ref. 38. The wavelength pairs 555−545 and 563−573 nm were chosen for cytochromes *f* and b_6, respectively, to minimize spectral overlap. Actinic light from a slide projector was filtered through a Schott RG 665 red glass, the photomultiplier being protected by a Schott BG 18 blue glass plus $CuSO_4$ solution. ON, actinic light on; OFF, actinic light off. No absorption changes were observed in the absence of the cytochrome complex. (B) Ferricyanide-induced reduction of cytochrome b_6. The reaction conditions were as before, but plastocyanin and Photosystem I reaction centers were omitted, the concentration of plastoquinol-1 was 60 μM and that of cytochrome b_6 in the form of the complex was 1.2 μM. The reaction was performed in the dark and started by addition of ferricyanide (FeCy) to 0.125 mM.

TABLE III

ELECTRON ACCEPTORS FOR THE QUINOL-CYTOCHROME *c* OXIDOREDUCTASE OF *A. VARIABILIS*

The assay mixture contained 20 mM Tricine-Tris (pH 8.1), 10 μM electron acceptor, 75 μM plastoquinol-9, 0.15% (v/v) Triton X-100 and 23 nM cytochrome b_6 in the form of the cytochrome f-b_6 complex.

Electron acceptor	Oxidoreductase activity (μmol/nmol cytochrome b_6 per h)
Cytochrome *c*-553 from *A. variabilis*	32
Plastocyanin from *A. variabilis*	31
Cytochrome *c* from horse heart	34
Cytochrome *c* from *Rhodopseudomonas capsulata*	9
Cytochrome *c*-552 from *Euglena gracilis*	1
Plastocyanin from spinach	9

The cytochrome f-b_6 complex exhibits oxidant-induced reduction of cytochrome b_6, in the presence of plastoquinol-1, upon addition of ferricyanide (Fig. 3B), a reaction which is characteristic of cytochrome *b-c* complexes [34,35]. The transient reduction is about 7% of cytochrome b_6 present, which is substantially smaller than that observed with the preparation from spinach (Hurt, E. and Hauska, G., unpublished results), which again could reflect the low content in the Rieske Fe-S center. Transient reduction of cytochrome b_6 can also be observed when a mixture of the complex from *A. variabilis*, plastocyanin and Photosystem I reaction center, both from spinach, is illuminated (Fig. 3A). A delayed photooxidation of cytochrome f is also seen in this system. About 5% reduction of cytochrome b_6 is obtained at the peak of the transient, then an oxidation level is reached which is higher than that before illumination. Plastoquinol probably keeps part of cytochrome b_6 reduced in the dark and becomes oxidized in the light. The extent of cytochrome f photooxidation is about 40%, and its delay is probably caused by plastoquinol. The transient photoreduction of cytochrome b_6 could either be caused by the same

TABLE IV

INHIBITORS OF THE OXIDOREDUCTASE ACTIVITY

Activity was measured in 20 mM Mes-Tris (pH 6.1), 25 μM cytochrome *c*, 40 μM plastoquinol-1, 50 nM cytochrome b_6 in the form of the cytochrome complex and the inhibitors. Ethanol never exceeded 1% (v/v). For comparison the inhibition of the oxidoreductase activity of thylakoid membranes from *A. variabilis* is shown. These assays contained thylakoids with 17 nM cytochrome b_6. The pI_{50} value is defined as the negative logarithm of the molar concentration giving 50% inhibition.

Inhibitor	Cytochrome f-b_6 complex		Membranes	
	pI_{50}	Inhibitor/cytochrome b_6 (mol/mol)	pI_{50}	Inhibitor/cytochrome b_6 (mol/mol)
2,5-Dibromomethylisopropyl-*p*-benzoquinone	6.5	7	6.4	23
2-Iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether	4.9	251	5.4	236
Bathophenanthroline	5.9	25	4.8	943
5-(*n*-Undecyl)-6-hydroxy-4,7-dioxobenzothiazole	5.1	158	4.7	1188
3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea	3.9	2518	3.9	7499

'pull-push' reaction as induced by oxidation with ferricyanide (Fig. 3B) possibly via a semiquinone [34], or, less expected in the absence of ferredoxin and ferredoxin-NADP oxidoreductase [36], by electrons from the reducing side of Photosystem I.

In conclusion, our results demonstrate that *A. variabilis* contains a cytochrome *f*-b_6 complex, with plastoquinol-cytochrome *c* oxidoreductase activity, which is very similar in composition and properties to the complex from spinach chloroplasts [1]. The polypeptide pattern of both preparations is simpler than that for the cytochrome *b*-c_1 complex from mitochondria [1–4]. Especially the 'core proteins' are absent. The complex from *Anabaena* contains four major polypeptides, cytochrome *f*, cytochrome b_6, the Rieske Fe-S protein and a 16.5 kDa protein, plus two small polypeptides which could be subunits of cytochrome b_6 [37], contaminants or degradation products. Like the cytochrome *b*-c_1 complex from Rhodospirillaceae [6], the complex from *Anabaena* could be involved in photosynthetic as well as in respiratory electron transport, which would comprise NADPH-dehydrogenase, plastoquinol, the cytochrome *f*-b_6 complex, plastocyanin or cytochrome *c*-553 and cytochrome oxidase (see Ref. 7). In this context, it is noteworthy that there is no evidence for another membrane-bound cytochrome *b-c* complex in *A. variabilis*, and that the cytochrome *f*-b_6 complex measured as plastoquinol-cytochrome *c* oxidoreductase activity, strictly follows the chlorophyll distribution during sucrose gradient centrifugation of membranes (Lockau, W., unpublished results).

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