Mutations of *Chlamydomonas reinhardtii* affecting the cytochrome *bf* complex

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The properties of some photosynthetic mutants of *Chlamydomonas reinhardtii* which were known to have impaired intersystem electron transport were examined. A new mutant, F18, is described which lacked all the redox centres of the cytochrome *bf* complex. The known mutant *ac*21 was shown to contain a cytochrome *bf* complex that lacked the Rieske iron-sulphur centre. Cytochrome b-559_{LP} was present in all strains examined at about the same concentration as cytochrome *f* in the wild ype and so is unlikely to be an integral component of the cytochrome *bf* complex.

Cytochrome bf *complex* *Cytochrome* b-*559* *Iron-sulfur center* *Photosynthetic mutant* (Chlamydomonas reinhardtii)

1. INTRODUCTION

Chlamydomonas reinhardtii has proved to be a useful organism from which to generate mutants for the study of photosynthetic energy conversion and the biogenesis of the photosynthetic apparatus. Mutant cells can be grown heterotrophically on acetate and their fluorescence properties provide a quick and simple method for screening and characterizing mutant colonies [1,2]. Levine and coworkers isolated a series of mutants with deficiencies in electron carriers; for example, strains *ac*206 and *ac*208 were shown to lack cytochrome *f* and plastocyanin, respectively [3,4]. Garnier and Maroc [5–7] obtained mutants (*Fl*9 and *Fl*15) which lacked cytochrome *f* and were also deficient in cytochrome *b*-563. Levine [8] described another mutant, *ac*21, in which the deficiency could not be identified, although it was clear that it related to an essential component of the intersystem electron-transport chain acting before cytochrome *f* [8,9].

We report here on the cytochrome content and rates of electron transfer of a series of nuclear mutants which were characterized by fluorescence induction curves as having low rates of intersystem electron transfer, and which we show to be due to various effects on the cytochrome *bf* complex.

2. MATERIALS AND METHODS

2.1. *Algal strains and cultures*

Mutant strains were isolated as described by Lemaire et al. [10]. Mutants F18, F37, UV20 and F38 were characterized by the lack of a decay phase in their fluorescence induction curves. Cells were grown in the heterotrophic medium of Gorman and Levine [3] in a Gallenkamp orbital incubator at a shaking speed of 110 rpm and 25°C with continuous illumination at 200 lx.

2.2. *Preparation of chloroplast fragments*

Cells were harvested during late exponential phase by centrifuging at 1500 × g_{av} for 5 min at

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room temperature, washing and resuspending in buffer containing 10 mM NaCl and 20 mM Hepes-NaOH, pH 7.5, and were then broken in a Yeda press (Lamon Instrumentation, Tel Aviv) at 4°C under a pressure of 8.3 MPa of nitrogen. The homogenate was layered onto 30% (w/v) Percoll and centrifuged at 3000 × g_{av} for 10 min at 4°C. The material remaining at the interface after centrifugation, was collected and resuspended in a small volume of either 50 mM Mes, pH 6.0, for cytochrome analyses or 20 mM Hepes, pH 7.5, containing 50 mM NaCl for electron-transport assays.

2.3. *Preparation of detergent extracts of chloroplast membranes*

Cells in late exponential phase were harvested from 1.25 l of culture and then washed and resuspended in a buffer containing 0.15 M NaCl and 10 mM Tricine-NaOH, pH 8.0, at 4°C. All subsequent steps were carried out at this temperature. The cells were resuspended in 10 ml of the same buffer containing in addition 1 mM phenylmethylsulphonyl fluoride and 10 mM EDTA and were broken by passage through the Yeda Press as above. The homogenate was diluted with 300 ml of buffer containing 0.15 M NaCl and 10 mM Tricine, pH 8.0, and centrifuged at 3000 × g for 10 min. The pellet was resuspended in 2 M NaBr, 0.15 M NaCl, 10 mM Tricine, pH 8.0, to give a chlorophyll concentration of 0.5 mg/ml. The suspension was stirred for 45 min, and then diluted with an equal volume of cold water and centrifuged at 32000 × g for 15 min. The pellet was washed by resuspension in 0.15 M NaCl, 10 mM Tricine, pH 8.0, and centrifuging again at 32000 × g for 5 min. The washed membranes were then extracted by modifications of the methods of either Hurt and Hauska (cholate/octyl glucoside) [11] or Nelson and Neumann (digitonin) [12].

For cholate/octyl glucoside extraction the washed membranes were resuspended with a glass homogenizer in a medium containing 20 mM 1-*O*-*n*-octyl-β-D-glucopyranoside, 0.33% sodium cholate, 0.4 M sucrose, 0.4 M $(NH_4)_2SO_4$ and 20 mM Tricine buffer, pH 8.0, and the volume adjusted to give a chlorophyll concentration of 2 mg/ml. The homogenate was stirred for 1 h and then centrifuged at 100000 × g for 1 h. The pellet was discarded and the supernatant concentrated by ultrafiltration with an Amicon filter (PM30 membrane) at a pressure of 240 kPa.

For digitonin extraction washed membranes were resuspended in a buffer containing 0.4 M sucrose, 0.15 M NaCl, 0.01 M Tricine, pH 8.0, 1 mM phenylmethylsulphonyl fluoride and 10 mM EDTA, and the volume was adjusted to give a chlorophyll concentration of 1 mg/ml. Solid digitonin was added to a final concentration of 1.25% and solid NaCl to 0.1 M; the suspension was homogenized with a glass homogenizer and stirred overnight. After addition of protamine sulphate (2 mg/ml; 1 vol./10 vols homogenate) the mixture was stirred for 1 h and centrifuged at 32000 × g for 20 min. The pellet was discarded and the supernatant concentrated by ultrafiltration.

2.4. *Measurement of electron-transport activities*

Photochemical activities were measured as rates of O_2 uptake or evolution with a Hansatech oxygen electrode illuminated with saturating red light (Schott RG 610 filter) from a 150 W tungsten/halogen projector lamp. Photosystem I activity was measured as O_2 uptake with a medium containing 10 mM Hepes, pH 7.5, 5 mM NaCl, 0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 5 mM sodium *iso*-ascorbate, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 30 μg/ml methyl viologen and 1 mM NaN_3. Photosystem II activity was measured as O_2 evolution with a medium containing 10 mM Hepes, pH 7.5, 5 mM NaCl, 1 mM 2,6-dimethyl-*p*-benzoquinone and 1.5 mM $K_3Fe(CN)_6$. Whole chain activity was measured as for photosystem II, but with the omission of dimethylbenzoquinone and the addition of 2.5 mM NH_4Cl as uncoupler. In all cases the chlorophyll concentration was 30 μg/ml.

Plastoquinol-cytochrome *c*-552 oxidoreductase activities were measured by following cytochrome reduction at 552 nm in a Varian Cary 219 spectrophotometer [13]. The reaction mixture contained 10 mM Mes-NaOH buffer, pH 6.2, 90 mM NaCl, 8 μM plastoquinol-1 and 1 μM ferricytochrome *c*-552 purified from *Euglena* [14], and in addition 0.1% digitonin when membrane preparations were being assayed. The uncatalyzed rate was subtracted from all activities.

2.5. *Spectral measurements*

Membrane-bound cytochromes of chloroplast fragments were assayed with a Johnson Foundation split-beam spectrophotometer as described by Bendall et al. [15] using absorption coefficients (reduced minus oxidized) of 19.3 $mM^{-1} \cdot cm^{-1}$ for cytochrome *f* and 20 $mM^{-1} \cdot cm^{-1}$ for *b*-type cytochromes at the α-band maxima.

2.6. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed with a 10–20% gel gradient overlaid by a 5% stacking gel [16]. Haem-containing polypeptides were detected by staining with tetramethylbenzidine [17]. Cytochrome *f* run as a standard was purified from wild-type cells by the method of Wood [18].

3. RESULTS AND DISCUSSION

Measurements of photochemical activities of broken chloroplasts (see table 1) confirmed that the mutant strains F18, *ac*206, F37, UV20, F38 and *ac*21 had impaired intersystem electron transport. As shown in table 2, the wild type fragments contained a complement of cytochromes similar to that of higher-plant chloroplasts.

The mutants could be classified into three groups according to their concentration of cytochrome *f* (table 2): (i) those with none detectable (F18 and *ac*206); (ii) those with about 20% of the normal concentration (F37 and UV20); (iii) those with about 50% of the normal concentration (F38 and *ac*21). Observations on cytochrome *b*-563 were complicated by the discovery that membranes from F18 contained a *b*-type cytochrome that was reducible by dithionite but not by hydroquinone or menadiol, even though cytochrome *b*-563 was undetectable. We have called this new component cytochrome *b*-560, according to its α-band position, and its properties will be reported in more detail elsewhere. The concentrations given in column 5 of table 2 refer to the composite peak of the two components and the peak positions indicate which is likely to be predominant. Thus the peak at 559–560 nm in F18 corresponds to pure cytochrome *b*-560 but an intermediate position, as in UV20 and *ac*21, to mixtures of the two. Spectra of cytochrome *b*-563 relatively free from *b*-560 could be observed in cholate/octyl glucoside extracts of membranes from wild type, UV20 and *ac*21 (not shown). Thus the concentration of cytochrome *b*-563 followed that of cytochrome *f*. The lack of both these components in F18 was confirmed by the failure to detect the corresponding polypeptides in detergent extracts by electrophoresis in SDS-polyacrylamide gels which were stained with H_2O_2 and tetramethylbenzidine (fig.1).

Activities of the cytochrome *bf* complex were measured as rates of reduction of *Euglena* cytochrome *c*-552 by plastoquinol-1, and values obtained are given in table 3. No activity could be detected in chloroplast fragments from F18 and

Table 1

Photochemical activities of chloroplast fragments

Algal strain	Photosystem I	Photosystem II	Whole chain
Wild type	300–408	99–152	62–88
F18	200–300	30– 40	ND
*ac*206	200–210	60– 65	ND
*F*37	300–506	80– 91	ND
UV20	200–250	100–110	<8
F38	200–250	99–152	ND
*ac*21	220–280	90–110	ND

Rates are expressed as μmol $O_2 \cdot$(mg chlorophyll)$^{-1} \cdot h^{-1}$ and represent the range or the average of 3 experiments. ND, not detectable

Table 2

Concentrations of cytochromes

Algal strain	Cytochromes			
	f	*b*-559$_{HP}$	*b*-559$_{LP}$	*b*-560/*b*-563 (λ_{max},nm)
Wild type	0.98	2.43	0.94	2.02(561.5)
F18	ND	2.58	0.92	1.06(559–560)
*ac*206	ND	4.41	1.56	1.04(559–560)
F37	0.25	2.80	1.11	0.84(559–560)
UV20	0.18	2.20	1.07	1.06(561)
F38	0.50	2.00	1.18	1.43(561)
*ac*21	0.50	2.48	1.23	1.90(561)

Concentrations are given as nmol·(mg chlorophyll)$^{-1}$ and are the average of at least 3 experiments. ND, not detectable

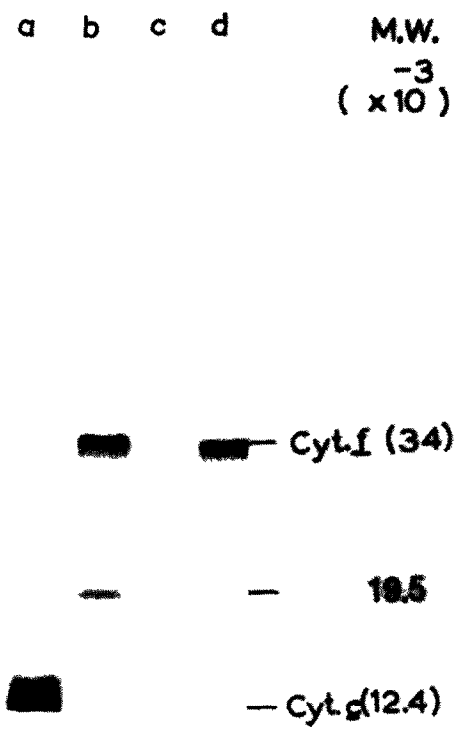

Fig.1. Haem-containing polypeptides of cholate/octyl glucoside extracts. The gel was stained with tetramethylbenzidine and H_2O_2 and labelled as follows: a, mammmalian cytochrome *c*; b, wild-type extract containing 40 μg protein; c, F18 extract containing 40 μg protein; d, purified *Chlamydomonas* cytochrome *f* (80 pmol).

Table 3

Plastoquinol-cytochrome *c*-552 oxidoreductase activities

Algal strain	Chloroplast fragments (μmol cytochrome reduced · (mg chlorophyll)$^{-1}$ · h^{-1})	Detergent extracts (μmol cytochrome reduced · (nmol cytochrome *f*)$^{-1}$ · h^{-1})
Wild type	25.2	21.9[a], 20.5[b]
F18	ND	–
UV20	1.6	19.2[b]
F38	1.4	–
*ac*21	1.3	1.0[b]

[a] Digitonin extract
[b] Cholate/octyl glucoside extract

ND, not detectable

only low rates with UV20, F38 and *ac*21 when they were expressed on a chlorophyll basis. To see whether the low rates might be due to low concentrations of the cytochrome *bf* complex, activities were measured again with preparations of the complex partially purified from detergent extracts of the membranes. In the case of UV20 the activity of the crude complex on a cytochrome *f* basis was similar to that of wild type, so that it seems likely that in this mutant a relatively small amount of a normal complex is produced. On the other hand, the activity of *ac*21 remained very low when expressed in this way.

The possibility that the low activity of *ac*21 might be due to the lack of the Rieske iron-sulphur

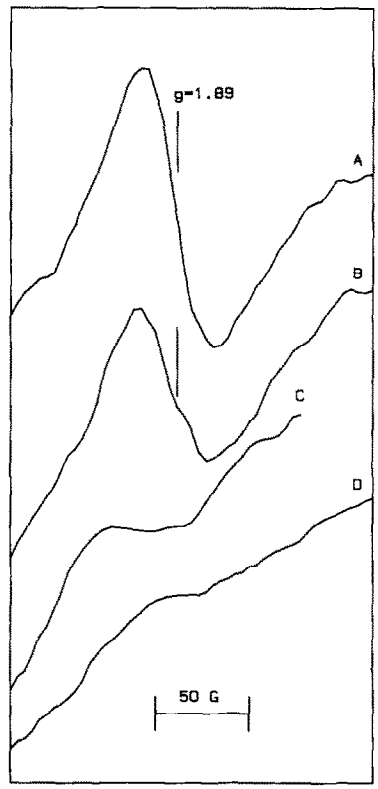

Fig.2. EPR spectra of cholate/octyl glucoside extracts. Each sample contained 0.3 ml of extract to which 1 mM hydroquinone and 10 mM EDTA had been added. A, wild type (8 μM cytochrome *f*); B, UV20 (5 μM cytochrome *f*); C, *ac*21 (8.5 μM cytochrome *f*); D, F18 (containing extract prepared from 1.5 l culture and no detectable cytochrome *f*). EPR conditions: field strength, 350 ± 50 mT; microwave power, 10 mW; modulation amplitude, 1 mT; temperature, 14.5 K.

centre was tested by measurement of EPR spectra of cholate/octyl glucoside extracts in the presence of hydroquinone. The spectra shown in fig.2 demonstrate that in the case of UV20 the intensity of the signal at $g = 1.89$, characteristic of the Rieske centre, was similar to that of wild type when related to the concentration of cytochrome *f*, in agreement with the conclusion that UV20 contains only a small amount of a functionally normal cytochrome *bf* complex. On the other hand little or no signal could be detected in extracts of either F18 or *ac*21.

We conclude that F18 lacks all redox components of the *bf* complex and that assembly of the complex may depend on a nuclear gene product. Mutant *ac*21 contains about 50% of the wild-type number of copies of a complex which lacks the Rieske centre, which is the one component of the complex that has been identified as being encoded in the nucleus [10,19]. A further notable fact was that cytochrome $b\text{-}559_{LP}$ was present in all the mutants (including some not reported here) at about the same concentration as cytochrome *f* in wild type, and is therefore probably not an integral component of the complex.

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