ELECTRON TRANSPORT BETWEEN PLASTOQUINONE AND CYTOCHROME *c*-552 IN *EUGLENA* CHLOROPLASTS

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Received 3 March 1976

1. Introduction

There is now good evidence that in higher plants a membrane-bound *c*-type cytochrome, cytochrome *f*, conveys electrons between plastoquinone and plastocyanin (see e.g. [1–4]). Plastoquinone is thoroughly lipophilic, whereas plastocyanin is either loosely adsorbed to the inner surface of the thylakoid, or free in solution in the intrathylakoid space [1]. Thus one function of cytochrome *f* is to link carriers which would not normally come into contact [4]. One might expect that other photosynthetic eukaryotes would have a similar entity, and indeed membrane-bound *c*-type cytochromes have been reported in chloroplasts from the green algae *Chlamydomonas* [5], *Scenedesmus* [6], *Chlorella* [7] and the blue-green alga *Phormidium* [8]. The factor which complicates any search for bound *c*-type cytochromes in algae is that all algal chloroplasts contain a soluble *c*-type cytochrome [9] (cytochrome *c*-552 or *c*-553) which is not found in higher plants. Plastocyanin has only been isolated from vascular plants and green algae (in which it is universal), and two species of blue-green algae [10]. In all other algae studied its role is taken by the soluble cytochrome [11–14], which has a similar redox potential and similar kinetic properties [3]. (The role of the soluble cytochrome in algae that also contain plastocyanin is not clear, but an absolute requirement for plastocyanin has been demonstrated in *Chlamydomonas* [15].)

The present study is concerned with electron transport between plastoquinone and the soluble cytochrome in *Euglena*, and the choice of *Euglena* was prompted by two considerations: (i) the existence of a membrane-bound *c*-type cytochrome seemed uncertain [11,16], and (ii) a strikingly different scheme has been proposed for this part of the chain in *Euglena* by Ikegami et al. [16], and adopted by Wildner and Hauska [11]: plastoquinone – cytochrome *b*-563 – cytochrome *b*-558 – cytochrome *c*-552, with 5 μM Antimycin A inhibiting electron transfer to cytochrome *b*-558 [11].

Abbreviations: DBMIB, 2,5-dibromomethylisopropyl-*p*-benzoquinone; MES, 2-(*N*-morpholino)-ethanesulphonate.

2. Materials and methods

Cells of *Euglena gracilis*, strain Z, were grown photo-organotrophically on Hutner's acidic medium [17] at 25°C, with continuous illumination and stirring. The cells were harvested in the log phase of growth. They were washed with 40 mM Tricine-NaOH, pH 7.8, containing 0.3 M sucrose and 5 mM $MgCl_2$, and resuspended in this medium. The cells were broken by French press treatment at 6000 psi, and chloroplasts were separated from larger debris and then sedimented as described by Wildner and Hauska [11]. Chlorophyll was assayed by the method of Arnon [18]. Cytochrome *c*-552 was purified as described by Perini et al. [19], and estimated by extinction coefficients given by Ben-Hayyim and Schejter [20]. Oxidised cytochrome was prepared by adding an excess of ferricyanide, and passing the solution through a Sephadex G25 column.

Cytochrome difference spectra were recorded on the split-beam spectrophotometer described by Jones and Saunders [21]. The same instrument was used for spectra at 77 K [22], with 1 M sucrose added to

the medium to given maximum enhancement [23].

For disruption by sonication the chloroplasts were washed and resuspended at about 130 μg chlorophyll/ml in a medium containing 10 mM NaCl, 1 mM $MgCl_2$ and 10 mM phosphate buffer, pH 7.0. A Dawe 'Soniprobe' was used, and 30 s sonication was given in 10 s bursts. The temperature of the sample was kept close to 0°C. Membraneous material was then sedimented by centrifugation for 15 min at 60 000 g_{av}, with resuspension in the same medium by thorough homogenisation followed by brief sonication.

For acetone extraction the chloroplasts were washed and resuspended at about 1 mg chlorophyll/ml in the medium used for sonication. Four volumes of acetone at −20°C were then added. After homogenisation the particulate material was pelleted by a brief centrifugation and washed with medium. After resuspension in the same medium it was left for 1 h, pelleted by centrifugation, and resuspended. Sonication was found to aid resuspension.

The plastoquinol–cytochrome *c*-552 assay system [4] was monitored with a dual wavelength recording spectrophotometer, the cuvette (1 cm^2) being fitted with an efficient stirrer. Suitable wavelengths were 537 and 552 nm, and measurements were made at pH 6.2. Plastoquinol-1 was synthesised and estimated as described by Wood and Bendall [4].

3. Results

Euglena chloroplasts are known to contain three cytochromes. Cytochrome *b*-563 is not reduced by ascorbate [11,24], and hence has $E_0' < 0.0$ V as in higher plants. For cytochrome *b*-558 a redox potential $E_0' = +0.32$ V has been measured [24]. Cytochrome *c*-552, unlike the other two, is not membrane-bound but is located inside the thylakoids, loosely adsorbed or in solution [25,26]; $E_0' = +0.38$ V has been measured for the purified cytochrome [24]. A hydroquinone–ferricyanide difference spectrum for untreated chloroplasts gave a mixed peak which included cytochrome *b*-558 as well as cytochrome *c*-552 (fig.1A). For the present study it was useful to eliminate *b*-558 from such spectra; it could be converted to a low potential form, no longer reducible by hydroquinone, by treatments that have a similar effect on cytochrome $b\text{-}559_{HP}$ of higher plants [27]. This

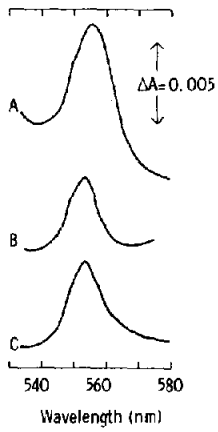

Fig.1. Difference spectra (hydroquinone reduced minus ferricyanide oxidised) for *Euglena* chloroplasts, with 145 μg chlorophyll/ml. The buffer was pH 7.0 phosphate. (A) No further additions. (B) In the presence of 2% Triton. (C) In the presence of 0.25 M sodium perchlorate.

can be seen in Figs. 1B and 1C, which show hydroquinone–ferricyanide difference spectra in the presence of 2% Triton X-100 or 0.25 M perchlorate [27], and in both cases the peak is shifted to about 553 nm.

The French pressure treatment used to break the cells resulted in liberation of much of the soluble cytochrome *c*-552, which remained in the supernatant after the chloroplasts had been pelleted [11]. More cytochrome *c*-552 could be liberated by sonication [11,16], and separated from the membranes by centrifugation. However, after repeated sonication and centrifugation, the resuspended membranes still showed a peak in a hydroquinone–ferricyanide difference spectrum recorded in the presence of Triton or perchlorate. This peak was not at 552 nm, but at about 554.5 nm (fig.2A). The cytochrome: chlorophyll ratio for this cytochrome was determined as 1:1480, by the procedure recommended by Bendall et al. [22] for estimation of higher plant cytochrome *f*.

Membrane-bound *c*-type cytochromes have been detected in chloroplasts from other algae [6,8,28] by extraction with 80% acetone, followed by resuspension in buffer and separation of membrane-bound from soluble components by centrifugation. Treatment of *Euglena* chloroplasts in this way (see Materials and methods) led to a hydroquinone–ferricyanide

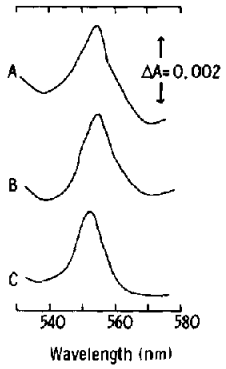

Fig.2. Difference spectra (hydroquinone reduced minus ferricyanide oxidised) for membrane-bound cytochrome *c* in *Euglena* chloroplasts. The buffer was pH 7.0 phosphate, and the temperature, 20°C. (A) Membranes sonicated and washed three times (see Materials and methods), at 121 μg chlorophyll/ml, plus 2% Triton. (B) Acetone extracted and washed membranes. (C) Purified *Euglena* cytochrome *c*-552, for comparison.

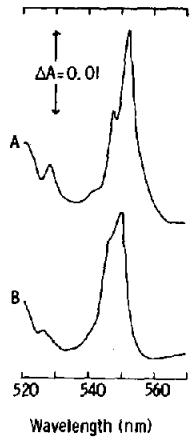

Fig.3. Difference spectra at 77 K (hydroquinone reduced minus ferricyanide oxidised) for membrane-bound and soluble *Euglena* cytochrome *c*. The buffer was pH 7.0 phosphate plus 1 M sucrose. (A) Acetone extracted and washed membranes. (B) Purified *Euglena* cytochrome *c*-552.

difference spectrum similar to that described above for sonicated material. This is shown in fig.2B; there was no need to add Triton since cytochrome *b*-558 is converted to a low-potential form by the extraction. For comparison, fig.2C shows a similar spectrum for purified cytochrome *c*-552.

Fig.3 shows 77 K spectra for the membrane-bound cytochrome and for purified cytochrome *c*-552. At 77 K the peaks were shifted about 2 nm to the violet from their room temperature positions. The bound cytochrome showed a subsidiary peak at 548 nm (c.f. higher plant cytochrome *f* [29]), while the soluble cytochrome, with a more symmetrical α-band at 20°C, showed merely a shoulder on this side of the main band.

Wood and Bendall [4] have shown that the cytochrome *f* region of higher plant electron transport can be studied in a dark reaction in which purified plastocyanin is reduced by plastoquinol-1 in the presence of disrupted chloroplasts: a plastoquinol-plastocyanin reductase system. The reaction took place via cytochrome *f*, and fractionation experiments showed that the cytochrome *f* was only active when bound to a membrane fraction containing other proteins and lipids. The reaction was inhibited by DBMIB, and other 1-electron acceptors could be used in place of plastocyanin, for instance cytochrome *c*-551 from *Pseudomonas fluorescens*.

This reductase system provides a novel way of studying this region of electron transport in other photosynthetic organisms. For *Euglena* the obvious choice of electron acceptor was purified, oxidised, *Euglena* cytochrome *c*-552, and table 1 shows the

Table 1
Catalysed reduction of *Euglena* cytochrome *c*-552 by plastoquinol-1

Addition	nM s^{-1}
No chloroplasts	0.6
No detergent	1.9
0.1% digitonin	17.5
0.1% Brij 35	14.9
0.1% Triton X-100	0.7
0.1% Brij 35 and 0.25 M NaCl	10.9
0.1% Brij 35 and 0.25 M $NaClO_4$	9.9
0.1% Brij 35 and 6 μM Antimycin A	13.3

In each case the initial rate of reduction is given in nM s^{-1}. The buffer was pH 6.2 MES, with NaCl added to give an ionic strength of 0.1. Temperature, 21°C. The reaction mixture included chloroplasts containing 3.6 μg chlorophyll/ml (except in the first run), 3.7 μM plastoquinol-1 and 0.75 μM oxidised *Euglena* cytochrome *c*-552.

results obtained. The first entry indicates the basal, uncatalysed rate. A stimulation was observed when undisrupted chloroplasts were added, and a much greater increase with digitonin as well. This is parallel to results obtained in the higher plant system [4]; given that the natural site at which *Euglena* cytochrome *c*-552 accepts electrons is on the inner face of the thylakoids [26], it is not surprising that the thylakoids must be disrupted before exogenous cytochrome can be reduced. On the other hand, the low but measurable rate in the absence of detergent correlates with the 'leakiness' of *Euglena* thylakoids prepared by the French press; much of the endogenous cytochrome *c*-552 escapes, and rates of photophosphorylation are low [11]. As with the higher plant system (P. M. Wood, unpublished results), Brij 35 [30] (polyoxyethylene-23-lauryl ether) was an acceptable alternative to digitonin, and more convenient in view of its greater stability in solution. Also as with higher plants, Triton X-100 was inhibitory except at very low concentrations. In the presence of 0.25 M perchlorate, which lowers the potential of cytochrome *b*-558 as described above, the rate was about the same as with an equal ionic strength of sodium chloride. 6 μM Antimycin A had little effect (c.f. ref. [11]).

Table 2 shows that the catalysed reaction was inhibited by DBMIB, which is known to inhibit light-driven electron transport in *Euglena* [31]. As was found in the demonstration of analogous inhibition in higher plants [4], the concentrations had to be carefully adjusted for this experiment in order to minimise the tendency for DBMIB (which can act as an electron carrier) to increase the rate of the uncatalysed reaction.

4. Discussion

It has been shown that *Euglena* chloroplasts contain a membrane-bound cytochrome (it seems reasonable to call it '*c*-type' in view of the position of its α-band and the presence of analogous bound *c*-type cytochromes in other algae), and that in a plastoquinol-1 reductase assay they behave in an identical manner to higher plants with respect to: inhibition by DBMIB, insensitivity to Antimycin A or lowering of the potential of cytochrome *b*-558/*b*-559, and effects of the detergents digitonin, Triton X-100 and Brij 35. These properties are hard, if not impossible, to explain by the scheme [16] plastoquinone–cytochrome *b*-563–cytochrome *b*-558–cytochrome *c*-552, with Antimycin A inhibiting between the two *b*-type cytochromes [11]. On the contrary, they strongly suggest that the membrane-bound cytochromes in *Euglena* have properties and roles similar to those in higher plants, the one conspicuous differences between the two electron transport chains being replacement of higher plant plastocyanin by *Euglena* cytochrome *c*-552. The question then arises: can the proposition that cytochromes *b*-558 and *b*-563 have essentially the same properties as cytochromes *b*-559 and *b*-563 in higher plants be reconciled with the experimental observations of Ikegami et al. [16] and Wildner and Hauska [11]? The experiments of Ikegami et al. which led to their formulated pathway were largely concerned with cytochrome *b*-558 photo-oxidation, and the effects on this of added cytochrome *c*-552, DCMU, and prior treatment at pH 9.5 or 55°C. One can find paralleles for their results in studies with higher plants; see [32] and [33] for the effects of high pH and heat treatment on higher plant *b*-559

Table 2
DBMIB Inhibition of reduction of *Euglena* cytochrome *c*-552 by plastoquinol-1

	No chloroplasts	With chloroplasts	Difference
No DBMIB	0.6	15.9	15.3
1.0 μM DBMIB	2.2	3.5	1.3

In each case the initial rate of reduction is given in nM s^{-1}. The medium was as in table 1. Temperature, 22°C. The reaction mixture included 0.02% digitonin, chloroplasts containing 3.7 μg chlorophyll/ml, 3.7 μM plastoquinol-1 and 0.7 μM plastoquinol-1 and 0.7 μM cytochrome *c*-552.

photo-oxidation. Moreover, since the *Euglena* thylakoids were mainly broken, exogenous cytochrome *c*-552 was necessary to couple electron transport to photosystem I, and this added cytochrome would have a general redox poising effect, displaced by illumination especially when DCMU was added to prevent it being reduced via photosystem II. Wildner and Hauska [11] found that Antimycin A increased the extent and rate of photo-oxidation of cytochrome *b*-558. But once again, similar results for higher plant cytochrome *b*-559 can be found in the literature [34,35], and are attributed to a light-stimulated lowering of the redox potential of the cytochrome in the presence of Antimycin A. A further similarity between *Euglena* cytochrome *b*-558 and higher plant *b*-559 is that in both cases oxidation results from illumination with red light after addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [35,36,37].

The differences in the spectra for bound and soluble *c*-type cytochrome can be compared with results for other algae: with *Chlamydomonas* Gorman and Levine [15] found that the α-band was further to the red in the bound cytochrome (553.5 nm instead of 552.5 nm), while in *Scenedesmus* [6] and *Phormidium* [8] there was little difference in the spectra at room temperature, although with *Phormidium* the shoulder on the 77 K spectrum was more pronounced for the bound cytochrome.

These earlier reports of membrane-bound cytochrome *c* were all for algae containing plastocyanin, and detection of the bound cytochrome was made easier by the fact that such algae tend to have low levels of soluble cytochrome, at least during log phase of growth [8], with less than 1 molecule per 10^5 chlorophyll in activity growing *Chlamydomonas* [5]. By contrast, for cytochrome *c*-552 in *Euglena* Wildner and Hauska [11] estimated 1 molecule per 40–50 chlorophylls (a ratio which Böger and San Pietro [38] found varied markedly according to the age of the culture), and the low ratio of bound:soluble cytochrome probably explains why a cytochrome with α-band at 554 nm has not been detected before. In higher plants cytochrome *f*:chlorophyll ratios of less than 1:1000 have been reported [39], and if, as seems likely, electrons can be equilibrated between different electron transport chains both at plastoquinone [40] and at the donor to P700 [41] (plastocyanin or cytochrome *c*-552), there is no reason for any strict stoichiometry between a bound cytochrome and photosystem I.

Acknowledgements

I am grateful to Mrs J. Fielding for assistance in the culture of *Euglena*, and should like to thank Dr A. R. Crofts and Dr O. T. G. Jones for use of their apparatus.

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