Plant Physiol. (1978) 61, 961-966

# Purification and Characterization of the Photoreducible *b*-type Cytochrome from *Dictyostelium discoideum*[1]

Received for publication November 14, 1977 and in revised form January 13, 1978

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## ABSTRACT

**The photoreducible cytochrome (Cyt) *b* from *Dictyostelium discoideum* was purified by differential precipitation with ammonium sulfate and chromatography over Sephadex G-100, diethylaminoethyl-cellulose, and calcium phosphate. The purified Cyt is composed of a single subunit of 15,000 daltons including a noncovalently bound protohaem, and exhibits in the reduced form alpha absorption bands at 555.5 and 560 nm at room temperature and 551 and 558.5 nm at 77 K. This Cyt is similar in some respects to Cyt $b_{557.5}$ from complex II of beef heart mitochondria, and to Cyt $b_{555}$ from the microsomal fraction of mung bean seedlings. Photoreduction by blue light of the purified Cyt *b* requires the addition of flavin; flavoprotein isolated from *D. discoideum* was the most active of four flavoproteins tested in catalyzing the photoreduction while diaphorase and L-amino-acid oxidase were inactive.**

A large number of diverse physiological processes in plants and animals are influenced by blue light (2). The similarity in the action spectra for these processes has been used as an argument that they are under the control of a common photoreceptor pigment (17-19). The characteristics of the action spectra have led to the suggestion that the photoreceptor pigment is a flavoprotein or a carotenoprotein, but provide inadequate evidence to decide between these alternatives (24).

An *in vivo* blue light-induced absorbance change has recently been described in *Phycomyces blakesleeanus* and *Dictyostelium discoideum* (19), and *Neurospora crassa* (17). The spectral characteristics of the absorbance change indicate that this change results from the photoreduction of a *b*-type Cyt (18, 20). The action spectrum for the light-induced absorbance change is similar to the physiological blue light action spectra and was accepted as evidence that the absorbance change might be used as an assay indicator for the physiological blue light photoreceptor pigment (20).

Although there is no evidence that the blue light photoreceptor pigment controls any physiological responses in *D. discoideum*, the organism is a convenient source of relatively large quantities of the photoreducible Cyt. This work was undertaken to purify and characterize the photoreducible Cyt from *D. discoideum*, and to study the Cyt *b* photoreduction in a known *in vitro* system.

## MATERIALS AND METHODS

**Organism.** *D. discoideum* strain A-3 was grown with rapid swirling in liquid suspension culture in a medium containing 5 g of Difco Proteose Peptone, 5 g of BBL Trypticase Peptone, 5 g of yeast extract, and 10 g of glucose in 1,000 ml of 2 mM K-phosphate buffer (pH 6.5). Following growth to 1.2 to 1.6 $\times 10^7$ cells/ml of medium (maximum stationary phase), the cells were collected by centrifugation, and washed three times with a salt solution containing 0.6 g of NaCl, 0.75 g of KCl, 0.15 g of $CaCl_2$, 0.93 g of $KH_2PO_4$, 0.54 g of $K_2HPO_4$, and glass-distilled $H_2O$ to 1,000 ml of solution (3). Following the final wash, the cell pellet was resuspended in an equal volume of 10 mM K-phosphate buffer containing 1 mM EDTA and 1 mM DTT, and the cells were broken by freezing in liquid $N_2$ and thawing with agitation in a water bath at 30 C followed immediately by cooling to about 0 C. Subcellular particles were eliminated by centrifugation at 16,000*g* for 10 min and centrifugation of the supernatant at 205,000*g* for 1.5 hr. The transparent, reddish yellow supernatant was used in the subsequent purification procedures; these were carried out in a cold room at 3 C.

**Spectroscopy.** Absorption spectra in the visible region were taken with a single beam spectrophotometer similar to that described by Davis *et al.* (8) on line with a Hewlett-Packard 2108 MX minicomputer. A cylindrical cuvette and Dewar with optical windows on the bottom (6) were used for absorbance measurements at the temperature of liquid $N_2$. Absorbance measurements in the UV region were made with a Cary 15 spectrophotometer using a quartz cuvette with a 1-cm light path.

Light-induced absorbance changes were monitored by recording in the memory of the computer a spectrum of a given sample exposed only to the measuring beam, irradiating the sample with 3 mw of actinic light at 470 nm (using a Varian prefocused xenon lamp with 3 cm of 10% $CuSO_4$ and a Baird-Atomic interference filter with a 10 nm bandwidth at half-maximum transmission) and immediately (within 10-30 sec) recording into the memory of the computer a second spectrum of the sample following irradiation. The light-minus-dark difference spectrum calculated by the computer represents the change induced by the actinic irradiation.

Flavins and Cyt *b* were measured using dithionite-reduced-minus-air-oxidized difference spectra and the extinction coefficients reported below (see Table II). Cyt *c* concentrations were measured from the spectrum of the reduced form using an extinction coefficient of 25 $mM^{-1}$ $cm^{-1}$ at 550 nm (21).

**Gel Electrophoresis.** SDS gel electrophoresis was run using the methods of Fairbanks *et al.* (10) with 12.5% acrylamide gels containing 1% SDS. Samples were boiled with 1% SDS and 1% 2-mercaptoethanol (or 5 mM DTT) for 2 min. Coomassie brilliant blue was used as a tracking dye. The gels were stained by acidic Coomassie blue and destained by diffusion in 10% acetic acid.

**Enzymes.** Glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) were obtained from Sigma Chemical Co. Diaphorase (EC 1.6.4.3), D-amino-acid oxidase (EC 1.4.3.3), and L-amino-acid oxidase (EC 1.4.3.2) were obtained from Boehringer Mannheim Corp.

## RESULTS

**Purification of the Photoreducible Cyt *b*.** The crude supernatant fraction from broken *D. discoideum* cells showed the same blue light-induced reduction of a *b*-type Cyt as was reported by Poff

[1] This work was supported by the U.S. Energy Research and Development Administration Contract EY-76-C-02-1338.

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and Butler (19, 20). Fractions from a preliminary separation of the crude supernatant by chromatography on Sephadex G-100 were assayed for Cyt *b*, Cyt *c*, and flavins. Two separate peaks of material containing Cyt *b* were eluted from the Sephadex G-100. The Cyt *b* in the second peak off the column was eluted concomitant with the Cyt *c* and was spectrally the same as the photoreducible Cyt in the crude supernatant. This Cyt could be precipitated from the crude supernatant by ammonium sulfate at 80% of saturation but not at 40% of saturation. In contrast, the *b*-type Cyt in the first peak off the G-100 column was precipitable from the crude supernatant by ammonium sulfate at 40% of saturation. The Cyt *b* precipitated along with flavin from the crude supernatant by an 80% ammonium sulfate precipitation following a 40% ammonium sulfate precipitation (hereafter referred to as a 40–80% ammonium sulfate fraction) was photoreducible when redissolved in phosphate buffer whereas the fractions from the G-100 column containing the same Cyt *b* were not concomitant with flavin and showed no photoreducibility without the readdition of flavin (see below). The elution profile from the column of Sephadex G-100 was unchanged by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF) at the time of cell breakage. These data indicate that flavin is required for the photoreducibility of the Cyt and that the bonding between flavin and Cyt is extremely weak if present at all.

The 40 to 80% ammonium sulfate fraction was redissolved in 10 mM phosphate buffer and chromatographed through Sephadex G-100. Material from the peak of Cyt *b* eluted from the column of Sephadex G-100 was dialyzed against 10 mM phosphate buffer (pH 7) for 2 hr, and chromatographed on DEAE-cellulose which had been preequilibrated with 10 mM phosphate buffer. Cyt *c* was excluded from the DEAE-cellulose while Cyt *b* was absorbed in the upper few cm of the column bed. The column was washed with 10 mM phosphate buffer and the *b*-type Cyt then eluted with 10 mM phosphate buffer with 0 to 300 mm of NaCl in a linear gradient. Two peaks of the Cyt *b* were eluted from the DEAE-cellulose (Fig. 1). The *b*-type Cyt first eluted from the DEAE-cellulose was spectrally similar to the photoreducible *b*-type Cyt reported by Poff and Butler (20) whereas the *b*-type Cyt eluted second from the DEAE-cellulose differed from the photoreducible *b*-type Cyt in the shape of the $\alpha$ peak of the reduced form at 77 K. Fractions of the Cyt first eluted from the DEAE-cellulose and having an $A_{280}/A_{413}$ in the oxidized form less than 3 were collected, concentrated by ultrafiltration using a Diaflo A-10 membrane, and rechromatographed on Sephadex G-100. Fractions with an $A_{280}/A_{415}$ of less than 0.6 were collected and used for the further purification.

The *b*-type Cyt was finally chromatographed through a preequilibrated Ca-phosphate column (28). The Cyt was washed with 10 mM phosphate buffer and eluted with phosphate buffer (pH 7) of 10 to 30 mM in a linear gradient. The purified Cyt, eluted from the column at about the middle of the gradient, had a 280/413 *A* ratio of 0.23 in the oxidized form. The entire purification procedure (Table I) gave a yield of about 15% and a purification of 1,500 compared to the crude high speed supernatant from broken cells. Electrophoresis in SDS gels showed a single band. However, weak contaminating bands were observed with disc gel electrophoresis. More than 80% of the Coomassie staining in the disc gel was within the major band.

**Characterization of the Photoreducible Cyt *b*.** Absorption spectra of both the oxidized and the reduced form of the purified Cyt *b* at room temperature are shown in Figure 2. The oxidized form shows a major peak at 413 nm with smaller bands at 531 and 560 nm in addition to the protein band at 280 nm. The reduced form shows $\alpha$ bands at 555.5 and 560 nm, a $\beta$ band at 527 nm and a Soret peak at 424 nm.

An absorption spectrum of the Cyt at 77 K shows $\alpha$ peaks at 551 and 558.5 nm and $\beta$ peaks at 510, 517, 525, and 532 nm (Fig. 3), with a 555.5/560 nm *A* ratio close to 1. An oxidized-minus-reduced difference spectrum of the alkaline pyridine *ferro* hemochrome of the purified Cyt has an absorption maximum at 558 nm, confirming the presumption (made on the basis of spectral properties) that the Cyt is a *b*-type with protohaem (11, 21). Extinction coefficients of the Cyt are given in Table II.

The mol wt of the Cyt was estimated at 13,000 to 16,000 on the basis of chromatography on Sephadex G-100 and sucrose density ultracentrifugation using ovalbumin, myoglobin, and Cyt-*c* as mol

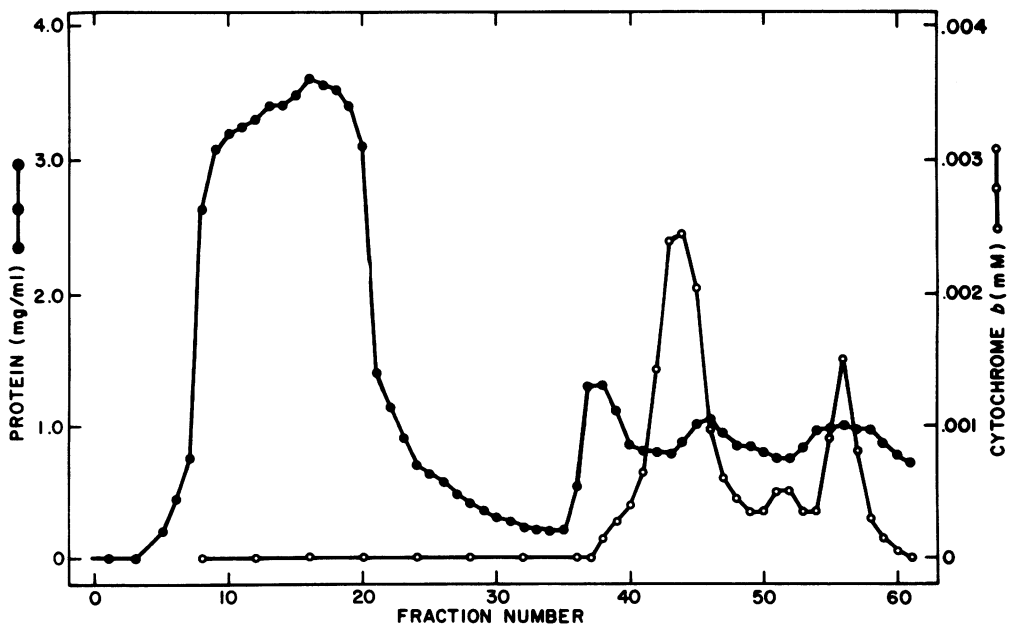


FIG. 1. Elution profile of protein and Cyt *b* following chromatography through DEAE-cellulose. The slowly eluted fraction of Cyt *b* from chromatography over Sephadex G-100 was dialyzed against 10 mM phosphate buffer and applied onto a column (2.5 × 20 cm) of DEAE-cellulose. The column was washed with 50 ml of 10 mM phosphate buffer and then eluted with 10 mM phosphate buffer containing a 0 to 300 mM NaCl in a linear gradient. Fraction volume was 7 ml. Protein (●——●) was measured by the method of Lowry (16) and Cyt *b* (○——○) was measured as $A_{555\,nm} - A_{580\,nm}$ and concentrations calculated using an extinction of 26.6 $mM^{-1}\ cm^{-1}$.

Table I. Purification procedure for the photoreducible b-type cyt from D. discoideum

| Fraction | Total protein | Total b-type cyt[2] | Specific activity[3] | Purification | Yield |
|---|---|---|---|---|---|
| | mg[1] | µmol | cyt/mg protein | fold | % |
| Crude supernatant | 11320 | 0.315 | 2.78 | 1 | 100 |
| 1st Sephadex G-100 | 792 | 0.253 | 31.9 | 11.5 | 80.3 |
| DEAE chromatography | 42.5 | 0.125 | 294.1 | 105.8 | 39.7 |
| 2nd Sephadex G-100 | 5.6 | 0.076 | 1357 | 488.2 | 24.1 |
| Brushite chromatography | 1.0 | 0.041 | 4100 | 1473 | 13.0 |

[1]Protein was measured by the method of Lowry (16).

[2]Measured from a dithionite reduced-minus-oxidized difference spectrum at room temperature as $A_{555}-A_{580}$ and calculated using an extinction of 26.6 $mM^{-1}$ $cm^{-1}$.

[3]µmol b-type cyt/mg protein x $10^5$.

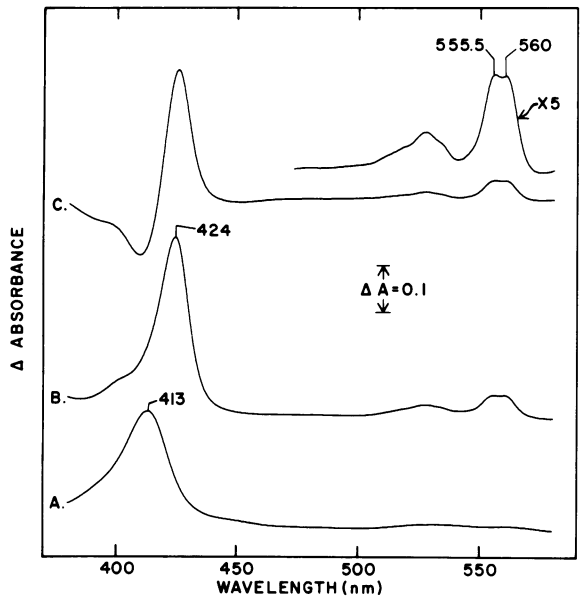


FIG. 2. Absorption spectra of the purified photoreducible Cyt *b*. A: oxidized; B: reduced by sodium dithionite; C: reduced-minus-oxidized difference spectrum.

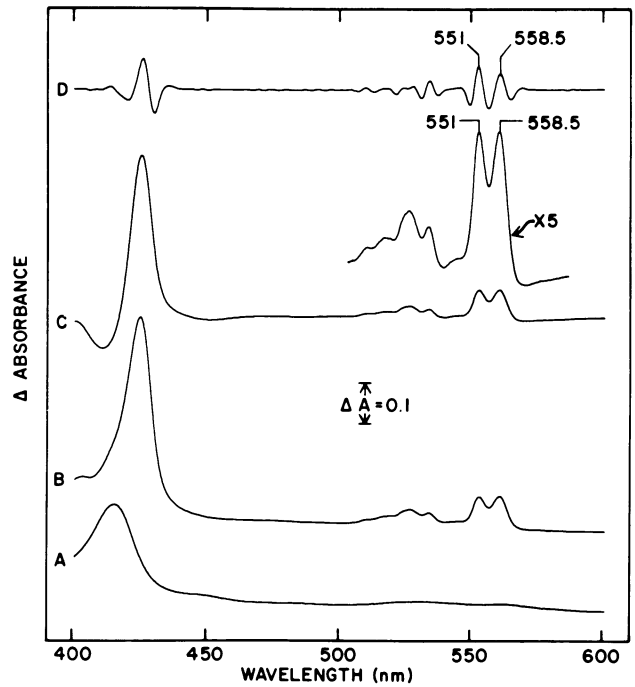


FIG. 3. Absorption spectra of the purified photoreducible Cyt *b* at 77 K. A spectrum was measured of the oxidized form of the Cyt (A), the sample was thawed, reduced with sodium dithionite, and refrozen and a spectrum measured of the reduced form of the Cyt (B). The reduced-minus-oxidized difference spectrum (C) was calculated by the computer. (D): a fourth derivative of the reduced-minus-oxidized difference spectrum.

wt markers. Subunit mol wt was estimated at 15,000 using SDS acrylamide (12.5%) gel electrophoresis. Thus, the Cyt has a single subunit of about 15,000 daltons.

The purified Cyt is reduced by dithionite but not by ascorbate, DTT, NADH, or NADPH. The dithionite reduced form of the purified Cyt can be directly oxidized by air.

**Reconstitution of the Cyt *b* Photoreduction System.** Photoreduction of the *b*-type Cyt by blue light could be accomplished easily in the crude supernatant. However, the photoreducibility was eliminated by any purification (*e.g.* chromatography on Sephadex G-100) which separated the Cyt from the flavins. Chromatography over Sephadex G-25 gave a single peak which contained the *b*-type Cyt and 80% of the flavins, and in which the Cyt was photoreducible. This co-chromatography of the Cyt and of the flavin indicated either a weak flavin-Cyt bonding, or that the photoreceptor for the photoreduction was a relatively high mol wt flavoprotein, and not a free flavin. The possibility that free flavin was released from the flavoprotein during the measurement of the photoreduction was eliminated since no free flavins could be isolated from such a photoreduction mixture by rechromatography over Sephadex G-25.

Initial attempts at reconstituting the photoreduction system using the purified Cyt *b* and partially purified flavoprotein from *D. discoideum* were unsuccessful. However, those fractions in which the Cyt photoreduction had been obtained (crude supernatant) were all observed to have a greatly reduced $O_2$ concentration (measured with a Clarke electrode) while the reconstituted system had a relatively high $O_2$ concentration. When the $O_2$ concentration of the recombined fraction was reduced by glucose and glucose oxidase with catalase, the potential for photoactivity was restored.

Table II. Extinction coefficients of the purified b-type cyt

The molecular extinction coefficients were calculated from the absorption spectra of both reduced and oxidized forms of the b-type cyt and concentration of cyt determined from the reduced minus oxidized difference spectrum of the pyridine haemochrome of the cyt. ± indicates standard error.

| Form | Peak | Extinction coefficient (A $mM^{-1}$ $cm^{-1}$) |
|---|---|---|
| reduced | α | 26.6 ± 0.4 |
| | β | 16.4 ± 0.3 |
| | γ | 196.9 ± 3.2 |
| oxidized | γ | 132.5 ± 2.8 |

The "active" flavoprotein fraction obtained from chromatography over Sephadex G-25 was further chromatographed over Sephadex G-100 (Fig. 4). The various fractions tested for the ability to catalyze the photoreduction of the purified Cyt *b* using the glucose-glucose oxidase-catalase system for reducing the $O_2$ tension in the reaction mixture. Only those fractions containing

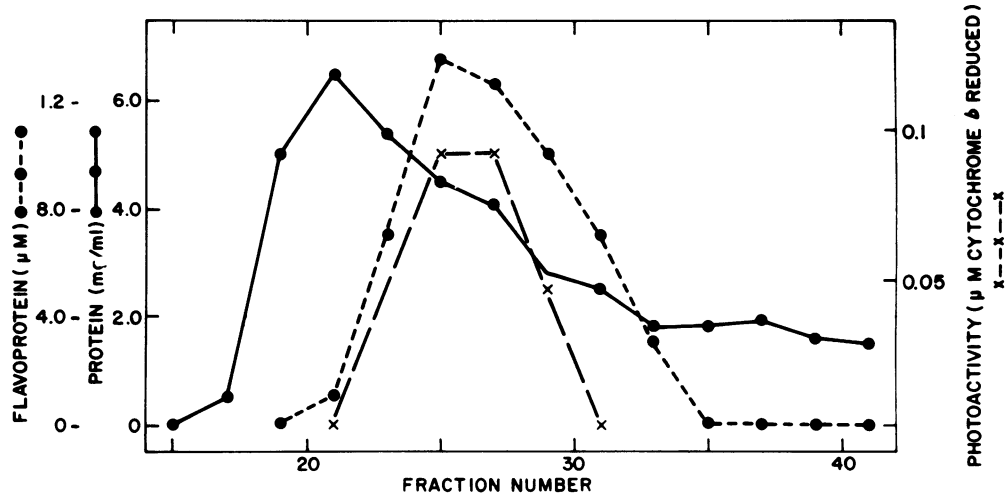


FIG. 4. Elution profile from a Sephadex G-100 filtration. A 5-ml aliquot of the high mol wt fraction following Sephadex G-25 chromatography of the concentrated crude homogenate was chromatographed through Sephadex G-100 (column size, 2.5 × 60 cm) and eluted with 10 mM K-phosphate buffer (pH 7). Fraction size was 4 ml. Protein (●--●) was measured by the method of Lowry (16); flavoprotein (●——●) was measured spectrophotometrically; and photoactivity (x— — —x) was measured as the relative ability of each fraction to catalyze the photoreduction by a 60-sec irradiation with broad band blue light at 20 mw/cm$^2$·sec of purified Cyt *b* in a reaction mixture containing 0.4 μM purified Cyt *b*, 5 mM EDTA, 10 mM K-phosphate (pH 7), 0.1 mg/ml glucose, 1 μM glucose oxidase (0.15 mg/ml), 0.125 mg/ml catalase, and 0.5 ml of the fraction to be assayed for photoactivity. The total volume for each photoactivity assay was 2 ml.

Table III. Components required for the reconstituted photosystem.

The complete reaction mixture contained 1 mM potassium phosphate buffer, pH 7.0; 0.4 μM b-type cyt; 0.6 μM, flavoprotein; 5 mM EDTA; 1 μM, glucose oxidase; 0.25 mg of catalase and 2 mg D-glucose. Total volume was 2 ml.

| Reaction Mixture | Cyt-b photoreduced |
|---|---|
| | % of total |
| Complete | 73 |
| less flavoprotein | 0 |
| less EDTA | 11 |
| less anaerobic system | 0 |

flavin could regenerate the "photoactivity" (Table III). The flavoprotein fraction from the chromatography on Sephadex G-100 was further purified by chromatography over DEAE-cellulose, from which the flavoprotein fraction catalyzed the Cyt *b* photoreduction. The requirements for the photoactive system are summarized in Table III. The flavoprotein is clearly the photoreceptor pigment mediating the photoreduction of the purified Cyt *b* on the basis of the action spectrum for this photoreduction in the reconstituted system (Fig. 5).

Not all flavins are equally efficient in catalyzing the photoreduction of the purified Cyt. The partially purified flavoprotein from *D. discoideum*, D-amino-acid oxidase, L-amino-acid oxidase, and diaphorase were assayed for their activity in this photoreduction system. The flavoprotein from *D. discoideum* was more active than D-amino-acid oxidase, and L-amino-acid oxidase and diaphorase were almost completely inactive (Table IV). It should be noted that the reaction mixture contained glucose oxidase, catalase, and glucose to reduce the $O_2$ concentration. Thus, the control contained a flavoprotein (glucose oxidase) which itself did not catalyze the photoreduction of the Cyt *b*.

## DISCUSSION

The original identification of the photoreducible Cyt from *D. discoideum* as a *b*-type Cyt was based on the spectral characteristics of the Cyt in a crude preparation (20). The same procedure has since been used for a similar identification in preparations from other plants and animals (5, 18). The data presented here (*e.g.* the absorption spectrum of the pyridine hemochrome formed from the purified Cyt) substantiate this preliminary identification.

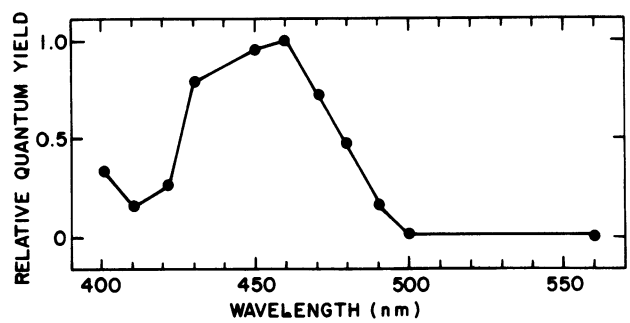


FIG. 5. Action spectrum for the photoreduction of the purified Cyt *b* in a reconstituted system. The reaction mixture contained 10 mM K-phosphate buffer (pH 7); 5 mM EDTA, 0.4 μM purified Cyt *b*, 0.6 μM partially purified flavoprotein from *D. discoideum;* 1 mg/ml glucose; 1 mM glucose oxidase; 0.125 mg/ml catalase. Total volume was 2 ml. Relative quantum yield was calculated as the reciprocal of the incident quantum flux density required for 25% photoreduction of the Cyt relative to that required at 460 nm.

Table IV. Relative ability of various flavoproteins to catalyze the photoreduction of cyt-b purified from D. discoideum.

The complete reaction mixture contained 1 mM potassium phosphate buffer pH 7.0, 0.4 μM cyt-b, 5 mM EDTA, 1 mM glucose oxidase, 0.25 mg catalase, 2 mg D-glucose.

| Flavoprotein | Concentration of test flavoprotein | Cyt-b photoreduced |
|---|---|---|
| | μM | |
| Partially purified flavoprotein from Dictyostelium | 0.6 | 73 |
| | 0.12 | 27 |
| D-Amino-acid oxidase | 4 | 74 |
| | 2.5 | 64 |
| L-Amino-acid oxidase | 2.5 | 23 |
| | 0.5 | 5 |
| Diaphorase | 0.5 | 7 |
| Control | 0 | 0 |

Although most of the characteristics of the photoreducible Cyt *b* are not unusual for *b*-type Cyt, the one characteristic which is surprising is this Cyt's relatively high solubility or low binding constant. An extremely gentle breakage and extraction of cells are required to isolate any significant portion of the Cyt in its membrane-bound form (data not shown). In contrast, the photo-

reducible Cyt *b* from corn and from *Neurospora* is isolated bound to a membrane (4). This apparent difference should be interpreted cautiously, however, since we cannot say that all of the photoreducible Cyt *b* is easily solubilized, and the photoreducible Cyt *b* from corn and from *Neurospora* may not all be membrane-bound. It is possible that we are studying the same Cyt in different environments (soluble *versus* membrane-bound).

The split $\alpha$ bands of the photoreducible Cyt at 77 K are quite similar to those of several described Cyt. Cyt $b_{557.5}$, characterized in complex II from beef heart mitochondria (8), has a split $\alpha$ *A* band with peaks at 77 K at 557.5 and 551 nm for an $\alpha$ peak separation of 6.5 nm. The major difference between the photoreducible Cyt *b* and Cyt $b_{557.5}$ is in the ratio of the heights of the two $\alpha$ bands. The $A_{\alpha 1}$ to $A_{\alpha 2}$ ratio for Cyt $b_{557.5}$ is about 1.6; $A_{\alpha 1}/A_{\alpha 2}$ for the photoreducible Cyt *b* from *D. discoideum* is close to 1. However, attempts to solubilize Cyt $b_{557.5}$ have consistently resulted in a decrease in $A_{\alpha 1}/A_{\alpha 2}$ to about 1 (Davis and Poff, unpublished data). Cyt $b_{555}$ from the microsomal fraction of mung bean seedlings also has a split $\alpha$ band at 77 K with peaks at 559 and 552 nm (26). This Cyt has been isolated in a soluble form for which the $A_{\alpha 1}/A_{\alpha 2}$ is close to 1 (25, 27). Cyt $b_5$ from microsomes of animal cells and Cyt $b_2$ from yeast also show split $\alpha$ bands at 77 K, but the peak separation (4.6 nm) is less than that of the photoreducible Cyt (7). Unfortunately, the relatively high noise level of the spectra of Cyt *b* from plasma membrane preparations (14) will not permit a rigorous comparison of plasma membrane-band Cyt with the photoreducible Cyt *b* from *D. discoideum*. Clearly, the subcellular localization of this photoreducible Cyt must precede its final identification.

Schmidt and Butler (22) have recently described an artificial system which they suggest as a model for the study of blue light-induced absorbance changes. They describe light-induced changes in the oxidation-reduction state of horse heart Cyt *c* mediated by free flavin via the superoxide anion in aerobic conditions and directly by flavin under anaerobic conditions. They have also studied the blue light-induced absorbance changes in cell free preparations from *Neurospora* and conclude that the photooxidation of Cyt is mediated directly by flavin (23). The data presented here support this suggestion, demonstrating that a flavin can indeed mediate the photooxidation or photoreduction not only of Cyt *c* as reported by Schmidt and Butler (22, 23), but also the photoreduction of Cyt *b*. It is not clear, however, why a single Cyt *b* is photoreduced *in vivo* while the other Cyt change their oxidation reduction state little if any.

The artificial system of Schmidt and Butler (22) necessitates a particular note of caution with respect to blue light-induced absorbance changes *in vitro*. As noted by Schmidt and Butler (23) these may consist of nothing more than the fortuitous combination of normally (*in vivo*) separate Cyt and flavin, such that the flavin can mediate the reduction of the Cyt. One must carefully compare the light-induced absorbance changes seen *in vitro* with those obtained in the *in vivo* system. We have carried out such a comparison and the results enable us to say that the photoreducible Cyt *b* isolated from *D. discoideum* is probably the same as the Cyt which is photoreducible *in vivo* (24).

The blue light-induced Cyt *b* reduction would be of most value if it were a specific assay for a single flavin (flavoprotein) which is also the physiological blue light photoreceptor pigment. Such a specificity, as conjectured by Poff and Butler (24), might be provided by a covalent binding between the flavin and Cyt as in Cyt $b_2$ from yeast (1). However, we have not been able to find any evidence to support such an absolute specificity of a particular flavin with the photoreducible Cyt *b*. Thus, one cannot say which flavin or flavoprotein mediates the Cyt *b* photoreduction *in vivo*. Not all flavins are equivalent in the mediation of the photoreduction. The most efficient mediator for the *in vitro* photoreduction of the *D. discoideum* Cyt is the flavoprotein isolated from *D. discoideum* itself.

The action spectrum is virtually the only characteristic of the blue light photoreceptor pigment which the physiologist can use in its identification. Other characteristics such as the dichroic nature of the receptor for phototropism in *Phycomyces* (13) exist but they are characteristics of specific systems and may not be characteristics of the photoreceptor pigment in general. Based on the similarities in action spectra for a wide variety of blue light-dependent physiological responses, the simplest conclusion is that these processes are under the control of a common photoreceptor pigment—the blue light photoreceptor pigment. On this basis—the similarity of action spectra—the blue light-induced reduction of a Cyt *b* was proposed as an assay for the blue light photoreceptor pigment (18, 19).

If one assumes a single blue light photoreceptor pigment, the described Cyt *b* photoreduction may be either the initial biochemical step leading to the blue light-induced physiological response; a convenient indicator for the physiological photoreceptor pigment; or totally separate from the flavin (flavoprotein) which is the physiological photoreceptor pigment. There is, however, the uncomfortable possibility that there are several blue light photoreceptor pigments which are indistinguishable by action spectroscopy because of similar microenvironments. Thus, the Cyt *b* photoreduction may be related to some of the "blue light physiological responses" (4) but not related to others (15).

The suggestion that a flavin is involved in the blue light physiological responses is not novel (12), nor are the recent observations that flavins can catalyze the reduction of Cyt (29). There is now very good evidence to conclude at least for phototropism in *Phycomyces* that the photoreceptor pigment is indeed a flavin (9). The primary question which now must be resolved is: which flavin? Several lines of work such as the relative activity of various flavins in catalyzing the Cyt *b* photoreduction as reported in this paper, or the action of specific inhibitors of various blue light physiological responses (24)—each insufficient for answering this question—may together lead to the identification of a single flavoprotein for a specific physiological response.

## CONCLUSIONS

The photoreducible Cyt purified from *D. discoideum* is characterized as a Cyt *b* (contains protohaem) of 15,000 daltons with maxima of the $\alpha$ absorption bands of the reduced form at 558.5 and 551 nm, and an $A_{\alpha 1}/A_{\alpha 2}$ ratio of close to 1. The easy solubilization of the Cyt inhibits its subcellular localization and identification.

Flavin is the photoreceptor for the photoreduction of the Cyt *b* in the reconstituted system. Flavoprotein partially purified from *D. discoideum* is most active in such a system while diaphorase is inactive. The existing data do not permit an identification of the specific flavoprotein which is the photoreceptor for the *in vivo* Cyt *b* photoreduction.

The reconstituted Cyt *b* photoreduction system contains: Cyt *b*, flavin or flavoprotein, EDTA as an electron donor, and a glucose-glucose oxidase-catalase system for the maintenance of the proper semianaerobic condition in which the photoreduction can be studied.

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