Identification of the Polypeptides in the Cytochrome b_6/f Complex from Spinach Chloroplasts with Redox-Center-Carrying Subunits[1]

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Abstract

An improved procedure for the isolation of the cytochrome b_6/f complex from spinach chloroplasts is reported. With this preparation up to tenfold higher plastoquinol-plastocyanin oxidoreductase activities were observed. Like the complex obtained by our previous procedure, the complex prepared by the modified way consisted of five polypeptides with apparent molecular masses of 34, 33, 23, 20, and 17 kD, which we call Ia, Ib, II, III, and IV, respectively. In addition, one to three small components with molecular masses below 6 kD were now found to be present. These polypeptides can be extracted with acidic acetone. Cytochrome f, cytochrome b_6, and the Rieske Fe-S protein could be purified from the isolated complex and were shown to be represented by subunits Ia + Ib, II, and III, respectively. The heterogeneity of cytochrome f is not understood at present. Estimations of the stoichiometry derived from relative staining intensities with Coomassie blue and amido black gave 1:1:1:1 for the subunits Ia + Ib/II/III/IV, which is interesting in ⁊ of the presence of two cytochromes b_6 per cytochrome f. Cytochrome f titrated as a single-electron acceptor with a pH-independent midpoint potential of $+339$ mV between pH 6.5 and 8.3, while cytochrome b_6 was heterogeneous. With the assumption of two components present in equal amounts, two one-electron transitions with $E_{m(1)} = -40$ mV and $E_{m(2)} = -172$ at pH 6.5 were derived. Both midpoint potentials were pH-dependent.

Key Words: Cytochrome b_6/f complex; heterogeneity of cytochrome f; isolation of cytochrome f; isolation of cytochrome b_6; redox titration; pH-dependent midpoint potential of cytochrome b_6.

Introduction

A cytochrome b_6/f complex of five polypeptides (Ia, Ib, II, III, and IV) has been isolated from spinach chloroplasts (Hurt and Hauska, 1981). Two cytochromes b_6 and somewhat substoichiometric amounts of the Rieske Fe-S center and of plastoquinone were found to be present per cytochrome f. The preparation retained plastoquinol-plastocyanin oxidoreductase activity and also showed the phenomenon of oxidant-induced reduction of cytochrome b (Hurt and Hauska, 1982). These properties are similar to the ones of cytochrome b/c_1 complexes from mitochondria, which, however, have a more complex polypeptide composition (Marres and Slater, 1977; Siedow et al., 1978; Weiss and Kolb, 1979; Engel et al., 1980; Gellerfors and Nelson, 1975). The three larger polypeptides Ia, Ib, and II of the cytochrome b_6/f complex (34, 33, and 23 kD) were shown to carry heme, but it remained unclear which of the subunits represented cytochrome f and which represented cytochrome b_6, although it was speculated that the 34-kD polypeptide might correspond to cytochrome f and the 33- and 23-kD polypeptide might constitute cytochrome b_6, each contributing one heme (Hurt and Hauska, 1981). In a subsequent investigation the 20-kD subunit III was identified as the Rieske Fe-S protein (Hurt et al., 1981). Subunit IV (17 kD) could not be identified with a redox center.

In this paper we demonstrate that, contrary to the previous speculation, cytochrome f is heterogeneous in our cytochrome b_6/f preparation, being represented by the subunits Ia and Ib, and that cytochrome b is identical with subunit II. In addition, we describe an improved procedure for the isolation of the complex. Also estimations of the subunit stoichiometry and the redox behavior of the cytochromes in the isolated complex will be described.

## Materials and Methods

Preparations

The cytochrome b_6/f complex was prepared as described by Hurt and Hauska (1981) with the omission of Triton X-100 from the final sucrose density gradient. The procedure remained the same until the ammonium sulfate fractionation. The pellet precipitating between 45 and 55% ammonium sulfate was resuspended to 50 μM cytochrome f in 0.5% cholate (w/v)/30 mM octylglucoside/30 mM Tris-succinate, pH 6.5, and then dialyzed against 0.5% cholate/30 mM Tris-succinate for 45 min at 4°C, to remove most of the residual ammonium sulfate. After dialysis 30 mM octylglucoside was added again. The dialysate was loaded on sucrose gradients from 7 to 30% (w/v) in cholate/octylglucoside/Tris-succinate buffer which additionally contained

0.1% soybean lecithin (w/v), and then centrifuged in a Beckmann SW-41 rotor at 40,000 rpm for 20 hr at 2°C. The brown zones were collected.

Cytochrome f was isolated from the complex following the method developed for the isolation of cytochrome c_1 from the mitochondrial cytochrome b/c_1 complex (Koenig et al., 1980). Mercaptoethanol was added to 15% (v/v) to 1 ml of the cytochrome b_6/f preparation (35 μM in cytochrome f, in cholate/octylglucoside/Tris-succinate buffer). After 30 min the solution was loaded to a DEAE-cellulose column equilibrated with 1% cholate/0.1% mercaptoethanol/20 mM phosphate, pH 7.4. The column was washed with 10 ml of the same buffer, then a gradient of 0–500 mM NaCl in the same buffer (total volume 50 ml) was applied. First the Rieske Fe-S protein was eluted, followed by cytochrome f. At the end of the gradient some cytochrome b_6 was removed from the column. The remaining yellowish material could be eluted with 1 M NaCl/1% Triton X-100 and contained cytochrome b_6 and the 17-kD polypeptide (subunit IV). The cytochrome f-containing fractions were still contaminated by the Rieske Fe-S protein as revealed by SDS-PAGE (Laemmli, 1970). The combined fractions were dialyzed against water overnight, adjusted to 0.5% Triton X-100/20 mM phosphate, pH 7.4, and applied on hydroxyapatite column equilibrated with the same buffer. The Rieske Fe-S protein was removed by washing with equilibration buffer. Then pure cytochrome f was eluted with 0.05% Triton X-100/200 mM phosphate, pH 7.4. Cytochrome b_6 was isolated from the complex by incubating the complex in urea/Triton X-100 (Stuart and Wasserman, 1975), followed by chromatography on hydroxyapatite. Triton X-100 was added to 0.5% (w/v), urea to 5 M, and phosphate to 20 mM, pH 6.8, to 1 ml of the cytochrome b_6/f complex (25 μM in cytochrome f, in cholate/octylglucoside/Tris-succinate buffer). After 15 min incubation at room temperature the solution was loaded on a hydroxyapatite column equilibrated with 0.5% Triton X-100/20 mM phosphate, pH 6.8. The column was washed with about 30 ml of the same buffer to remove the Rieske Fe-S protein (Hurt et al., 1981) and residual chlorophyll. Cytochrome b_6 was eluted from the column at 60 mM phosphate, pH 6.8, in the presence of 0.1% Triton X-100. Under these conditions cytochrome b_6 is only partially and slowly eluted; however, cytochrome f, subunit IV, the low-molecular-weight subunits, and the residual cytochrome b_6 remain on the column, and these can be eluted with 200 mM phosphate. The yellowish cytochrome b_6 fraction (about 15 ml) was dialyzed overnight against 5 mM phosphate, pH 6.8, and loaded again on a hydroxyapatite column equilibrated with 0.1% Triton X-100/5 mM phosphate, pH 6.8. Cytochrome b_6 was eluted from the column with 200 mM phosphate, pH 6.8/0.05% Triton X-100 with a concentration of 20 μM. The yield of cytochrome b_6 was about 10%.

The small polypeptides of molecular masses below 6 kD (see Fig. 1) could

be removed by treatment with acidic acetone in the cold, a procedure designed for the removal of heme b from mitochondrial preparations (Rieske, 1967). The Rieske Fe-S protein was isolated from the complex obtained by the modified procedure as described before (Hurt *et al.*, 1981).
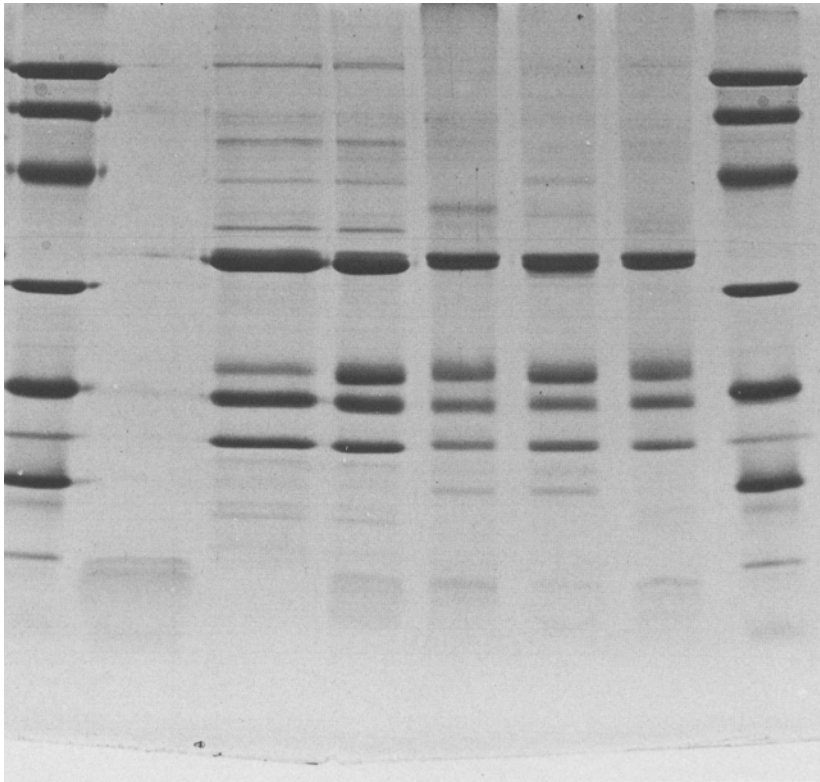
Fig. 1. SDS polyacrylamide gel electrophoresis patterns of different cytochrome b_6/f preparations. Slab electrophoresis was run on 15–18% linear gradient polyacrylamide gels after Laemmli (1970). Lanes 1 and 8 show standard proteins of 94, 68, 45, 30, 21, 17 (degradation product), 14.3, 6.0 (degradation product), and 3.4 kD. Lanes 4–7 show the patterns of different preparations of the cytochrome b_6/f complex (0.25, 0.12, 0.16, and 0.16 nmol cytochrome f were applied on lanes 4, 5, 6, and 7, respectively). Lane 3 shows the residue of the acidic-acetone-extracted cytochrome b_6/f complex, and lane 4 the corresponding nonextracted one. An amount equivalent to 0.5 nmol cytochrome f was extracted, and the residue was dried under a stream of nitrogen and resuspended in the sample buffer containing 12% SDS by boiling for 5 min (after Laemmli, 1970). On lane 2 the organic acetone extract resulting from 1.75 nmol extracted cytochrome f in the form of the cytochrome b_6/f complex was applied. The acetone phase was dried and the residue was resuspended in the sample buffer.

Assays

SDS polyacrylamide gel electrophoresis was performed after Laemmli (1970), or in the presence of urea after Harms *et al.* (1978). Gel staining with Coomassie blue was carried out after Cabral and Schatz (1979), and with amido black after Tombs (1966), with the modification that the staining solution contained 0.1% amido black in 20% methanol/7.5% acetic acid and the destaining solution contained 20% methanol/7.5% acetic acid. The silver stain was performed after Merril *et al.* (1981). Gel concentrations and other details are specified in the legends. Immunodiffusion after Ouchterlony was carried out by the standard procedure (Ouchterlony, 1962). Redox titrations of cytochromes were performed essentially after Dutton (1978). An Aminco DW 2 spectrophotometer was fitted with an anaerobic, stirred cuvette with a redox electrode installed and a reference cuvette. The cuvettes contained 2.5 ml 2.5 μM cytochrome f in the form of the cytochrome b_6/f complex, in 30 mM Tris-succinate, pH 6.5/0.05% cholate/2.5 mM octylglucoside. The following redox mediators were present: 20 μM 2,3,5,6-tetramethyl-p-phenylenediamine (DAD), 40 μM N-methylphenazonium methosulfate (PMS), 40 μM N-ethylphenazonium ethosulfate, 15 μM 2-hydroxy-1,4-naphthoquinone, 15 μM 2,3,5,6-tetramethyl-p-benzoquinone, 15 μM anthraquinone-2-sulfonate, 15 μM anthraquinone-2,6-disulfonate, and 10 μM benzylviologen. Redox titration for cytochrome f and b_6 could be performed consecutively in one assay. For cytochrome f measurements both cuvettes were first oxidized by a slight excess of ferricyanide, and reductive titration was performed in steps of 5 to 10 mV by addition of a concentrated ascorbate solution. After stabilization of the ambient redox potential, difference spectra were recorded between 500 and 600 nm. The height of the absorption at 554 nm was taken for estimation of the cytochrome f redox state. Then an excess of ascorbate was added to both cuvettes, and cytochrome b_6 was titrated by addition of dithionite. Between 0 and -100 mV ambient redox potential, the measurements were complicated by an overshoot of cytochrome b_6 reduction following addition of dithionite, so that longer equilibration times were required.

Plastoquinol-plastocyanin oxidoreductase activity was measured as described by Hurt and Hauska (1981) with the exception that 20 mM MES buffer, pH 6.5, was used and the plastoquinol-1 concentration in the cuvette was 20 μM. Other assays are described by Hurt and Hauska (1981).

Results and Discussion

Omitting Triton X-100 from the preparation of the cytochrome complex allows much better preservation of plastoquinol-plastocyanin oxidoreductase activity. When cytochrome c-552 from *Euglena gracilis* is used as electron

acceptor, rates of 30–40 μmol cytochrome c-552 reduced per nanomole cytochrome f per hour were observed with no significant loss of activity per cytochrome f from solubilized chloroplasts to the purified cytochrome b_6/f complex. Separation of residual chlorophyll from the cytochrome complex during gradient centrifugation was less complete, but with careful ammonium sulfate fractionation, ratios of chlorophyll to cytochrome f of about 1 could be obtained. Other analytical data are the same as reported for the older preparation (Hurt and Hauska, 1981).

On SDS polyacrylamide gel electrophoresis this modified preparation gave the same pattern of five polypeptides. Figure 1 shows this pattern for four different preparations (lanes 4–7) by the new procedure. Some variation is observed with regard to contaminative polypeptides which probably originate from different pooling of the sucrose density fractions. It can be seen that the relative intensity of the bands for subunit Ia (34 kD) and Ib (33 kD) varies. The contaminant of about 14 kD, which is seen on lanes 5 and 6, is probably a degradation product, because it is absent in fresh preparations but is formed on standing at room temperature. Oxidoreductase activity was similar in each case. All preparations shown in Fig. 1, in addition to the five polypeptides described before (Hurt and Hauska, 1981), exhibit one to three bands of very low molecular masses between 3 and 5 kD, the one corresponding to 4.8 kD being the most prominent (see Fig. 2, silver stain). These small polypeptides migrate diffusely and stain weakly with Coomassie blue in a 12–18% gradient gel after Laemmli (1970); they are better seen in a 15 to 18% gradient gel (see Fig. 1). Care must be taken not to destain the gel too long, otherwise the small subunits are partially destained also. The small polypeptides seem to be subunits of the cytochrome b_6/f complex. The polypeptide analysis of the fractions of the final sucrose density gradient clearly show that the small polypeptides co-migrate and culminate with the five polypeptides of the cytochrome b_6/f complex, being absent in fractions that do not contain the complex (data not shown). These low-molecular-mass proteins can be removed from the complex by extraction with acidic acetone (lanes 2 and 3 of Fig. 1). Extraction of lyophilized cytochrome b_6/f complex with acetone alone removes only plastoquinone and chlorophyll, but not the small subunits. The residue of the acidic acetone extraction can be resolubilized only by SDS, so that its oxidoreductase activity cannot be tested. Therefore, we are not able to discern whether the small, hydrophobic components play an obligatory functional role in the cytochrome b_6/f complex or are just degradation products. However, there is the possibility that they correspond to small polypeptides in the mitochondrial cytochrome b/c_1 complexes (Marres and Slater, 1977; Engel et al., 1980; Gellerfors and Nelson, 1975; Siedow et al., 1978), which are somewhat larger and stain better. The residue of the acidic acetone extraction (Fig. 1, lane 3) has lost part of cytochrome b_6, probably by
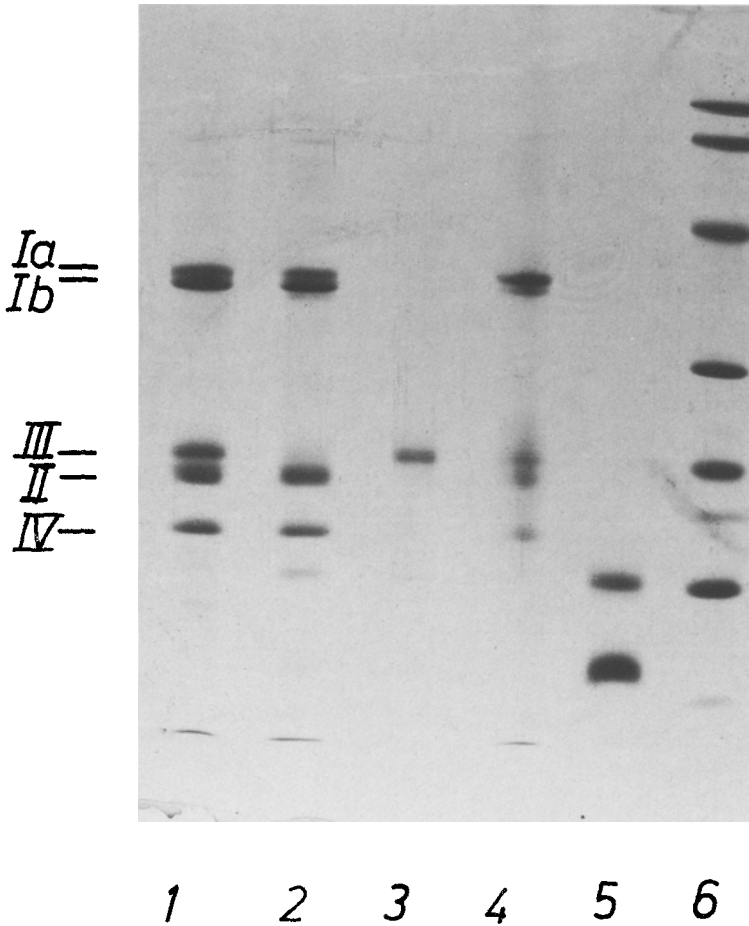
Fig. 2. Urea/SDS polyacrylamide gel electrophoresis patterns. The acrylamide concentration in the gel was 15%. Lanes 1 and 4 show the pattern for two different cytochrome b_6/f preparations (in lane 1, 0.15 nmol cytochrome f was applied, and in lane 4, 0.075 nmol). On lane 3, 2 μg of the Rieske Fe-S protein was applied, and on lane 2, 0.15 nmol of cytochrome f in form of the Rieske Fe-S-depleted cytochrome b_6/f complex was applied. Lane 5 shows protein standards of 12.5 kD (cytochrome c) and 6.5 kD (aprotinin), and lane 6 shows standards run also for Fig. 1 except insulin (3.4 kD).

irreversible aggregation which is indicated by material not migrating into the resolving gel.

On Fig. 2 polypeptide patterns obtained by urea/SDS gel electrophoresis are shown, demonstrating that the relative mobilities of the Rieske Fe-S protein (subunit III) to subunit II (cytochrome b_6 as documented in Fig. 6)
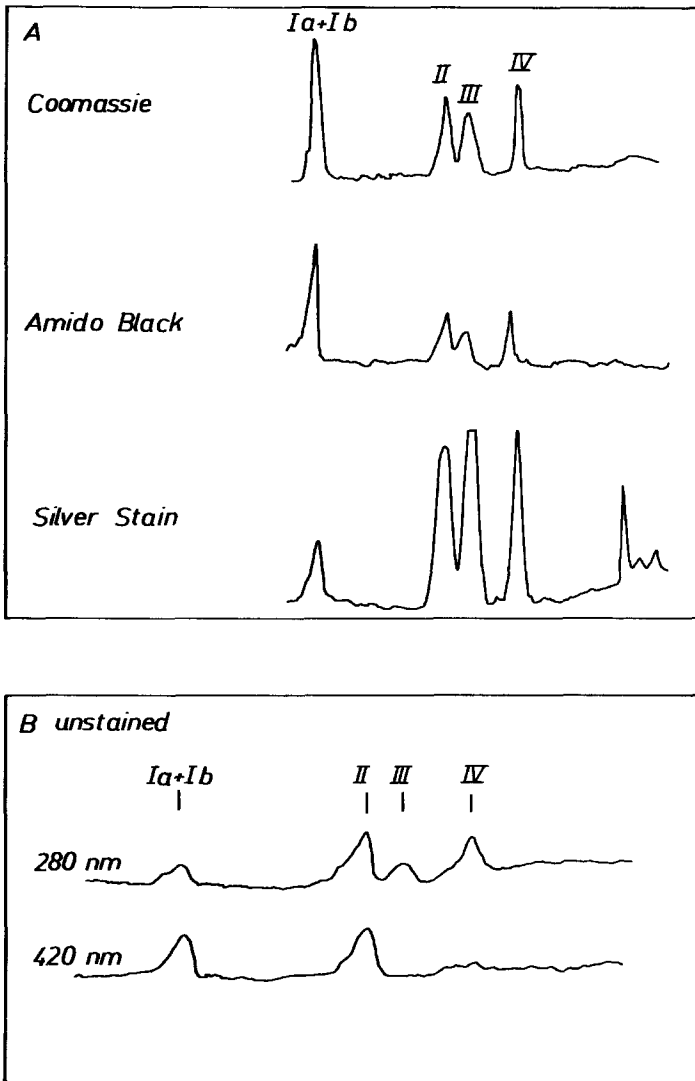
Fig. 3. Scans of unstained and differently stained gels. Sliced gel strips resulting from a 12–18% gradient gel after Laemmli (1970) were mounted into the cuvette of a Gilford gel scanner. Unstained strips (B) were scanned at 280 or 420 nm, and stained ones (A) at 570 nm, at 0.5 cm/min. Staining was carried out as described in Materials and Methods. On the Coomassie-stained gel 0.088 nmol, on the amido black stained gel 0.176 nmol, and on the silver stained gel 0.105 nmol cytochrome f in the form of the cytochrome b_6/f complex were applied. On the unstained gel 0.7 nmol cytochrome f in form of the cytochrome b_6/f complex was applied.

are reversed in this system compared to the one of Laemmli (Hurt *et al.,* 1981). Abnormal mobility is also known for mitochondrial cytochrome *b* (Marres and Slater, 1977). It is also possible that the mobilities of subunits Ia and Ib are reversed, their separation being better in the urea/SDS system. The small components observed in Fig. 1 were migrating in the front on the 15% gel of the urea/SDS system.

Figure 3 depicts scans of the subunit pattern on the gel system after Laemmli (1970), in (B) unstained at 280 nm for protein and at 420 nm for heme, and in (A) stained in three different ways, measured at 570 nm. Only in the case of the silver stain are the small components between 3 and 5 kD seen. Estimations of the stoichiometry of the five larger polypeptides are summarized in Table I. The estimations were obtained by dividing the areas below the protein peaks of the stained gels by the apparent molecular masses of the subunits, normalizing to the value obtained for subunit II (cytochrome b_6). Coomassie blue and amido black stained the polypeptides Ia + Ib/II/III/IV with relative intensities of about 1:1:1:1, while staining with silver gave a different ratio. Silver stain intensities, however, are known not to be proportional to molecular masses (Oakley *et al.,* 1980). Estimations of the stoichiometry from the scan at 280 nm of unstained gels (Fig. 3B) could not be obtained with accuracy due to the small areas below the peaks, but it is obvious that subunits Ia/Ib and III have relatively less absorption than subunits II and IV.

Of course this could mean that subunits II and IV contain a relatively higher amount of aromatic amino acids (and therefore show relatively more UV absorption) than subunits Ia + Ib and subunit III. On the other hand, it could be that subunits II and IV are present in more than one copy in the complex compared to the other subunits, but then one has to assume that Coomassie blue or amido black are poorly bound to subunits II and IV

Table I.   Estimation of Subunit Stoichiometries of the Cytochrome b_6/f Complex with Different Stains of Electrophoresis Slabs[a]

	Coomassie blue	Amido black	Silver
Subunits Ia + Ib			
(cytochrome *f*; 34, 33, kD)	1.01	1.10	0.35
Subunit II			
(cytochrome b_6; 23 kD)	1.00	1.00	1.00
Subunit III			
(Rieske Fe-S; 20 kD)	0.95	1.07	1.02
Subunit IV			
(17 kD)	0.92	0.95	0.47

[a]The peaks of the scans shown in Fig. 3A were cut out from the chart paper and were weighed. The weights were divided by apparent molecular masses (Hurt and Hauska, 1981), and the obtained values were related to the one for cytochrome b_6 which was normalized to 1.00. The values for the Coomassie blue stain represent the average of six estimations, and the ones for amido black, two estimations.
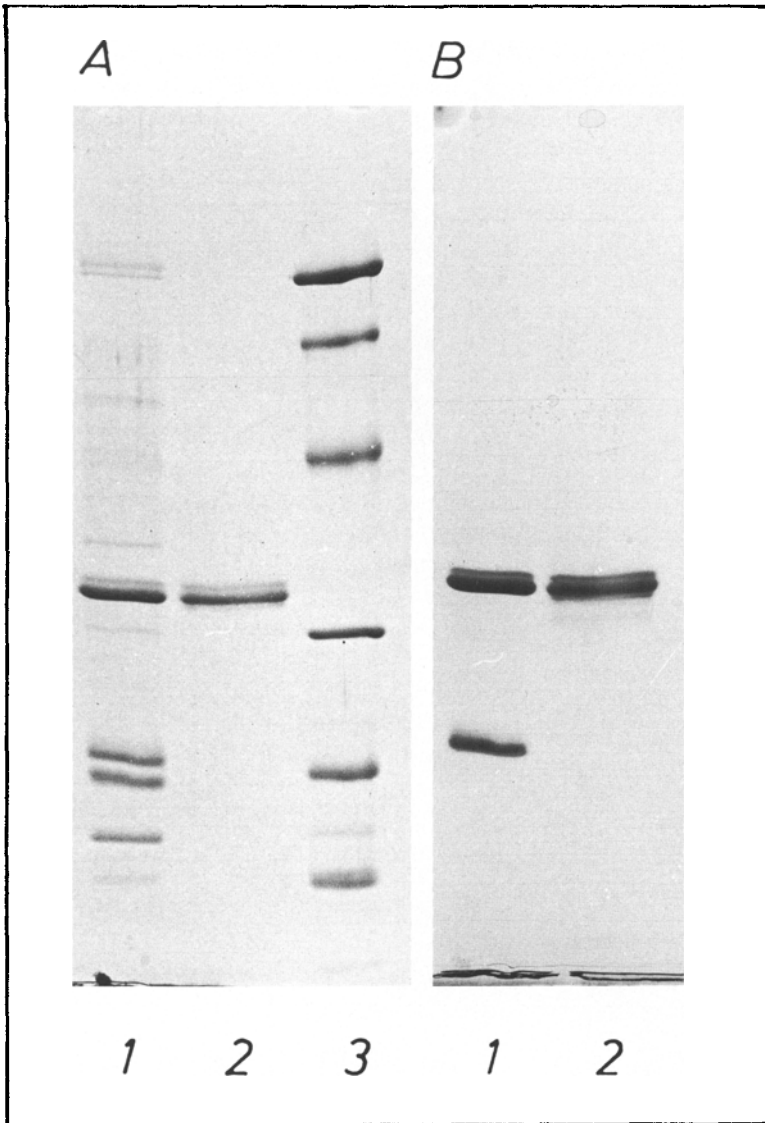
Fig. 4. Electrophoresis and spectrum of isolated cytochrome f. (A) SDS polyacryl-amide gel (12–18%) stained with Coomassie blue. Lanes 1, 2, and 3 show the cytochrome b_6/f complex, isolated cytochrome f, and protein standards, respectively. (B) Lanes 1 and 2 of the same gel stained for heme (Thomas *et al.*, 1976). (C) Absolute spectrum of cytochrome f isolated from the complex, in the presence of 0.1% mercaptoethanol (addition of dithionite did not change the cytochrome spectrum).
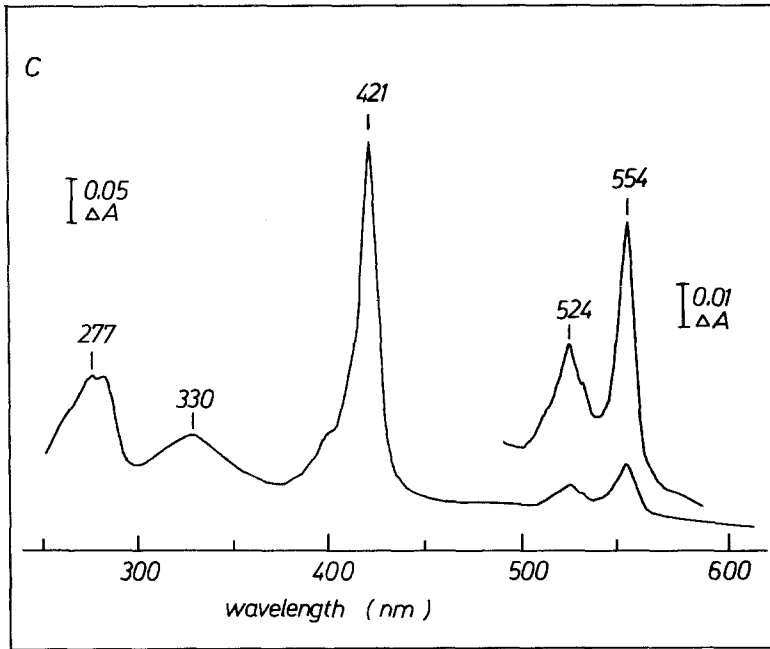
Fig. 4. Continued.

compared to subunits Ia/Ib and III. Similar results were obtained for mitochondrial cytochrome c_1 and cytochrome b (Marres and Slater, 1977). There also the UV absorption of cytochrome b (and a 15-kD subunit always associated with cytochrome b) is relatively higher than the absorption of cytochrome c_1, whereas the scan of the Coomassie stained gel reveals that cytochrome c_1 binds relatively more dye than cytochrome b.

The scan of the unstained gel for heme at 420 nm in Fig. 3B shows about equal peak areas for cytochromes f and b_6. This suggests that a considerable amount of the heme b which is not covalently bound to the apoprotein of cytochrome b_6 stays together with subunit II during gel electrophoresis. The stoichiometry of the polypeptides cannot be defined with certainty on the basis of the results represented here. However, the stoichiometry of cytochrome b_6 to cytochrome f of 1:1 suggested by the relative staining in Fig. 3A and Table I would lead to the conclusion that cytochrome b_6 is a protein carrying two heme groups on one polypeptide. It is interesting that cytochrome b of the cytochrome b/c_1 complex of mitochondria is probably a two-headed heme protein (von Jagow *et al.*, 1981).

The identity of subunit Ia and Ib with cytochrome f is demonstrated in Figs. 4 and 5. Cytochrome f can be purified from the cytochrome b_6/f complex. The purified cytochrome f gave a polypeptide pattern corresponding

$$Ia + Ib$$

$$|$$

$$Ib —$$ $$—Ib$$

$$Ab_{cyt} f ————————$$

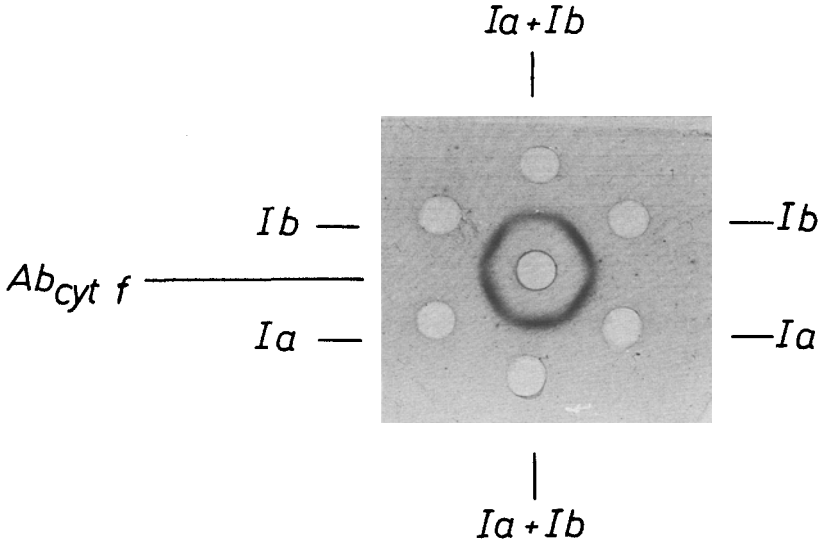$$Ia —$$ $$—Ia$$

$$|$$

$$Ia + Ib$$

Fig. 5. Immunodiffusion of subunits Ia and Ib against cytochrome f antibodies. Subunits Ia and Ib were cut out from urea/SDS polyacrylamide slabs (15% gel) after electrophoresis and staining with Coomassie blue. The gel pieces were filled into the peripheral holes in the agarose gel for immunodiffusion, which was prepared in 0.15 M NaCl/0.1% SDS. Antiserum against pure cytochrome f was filled into the central hole. After 48 hr diffusion the agarose gel was washed and stained with Coomassie blue.

to subunits Ia and Ib of the cytochrome b_6/f complex, plus a faint third component of slightly higher mobility than Ib, probably a degradation product formed during the isolation procedure (see Fig. 4A, lane 2). Figure 4B shows the corresponding heme stain; both subunit Ia as well as subunit Ib (and the third faint component) are heme-carrying polypeptides. From the absolute spectrum of the purified cytochrome f in Fig. 4C it is obvious that there is no cytochrome b_6 present, because addition of dithionite to the cuvette does not alter the spectrum. Immunological identity of subunits Ia and Ib is demonstrated in Fig. 5. Immunodiffusion of subunit Ia and of subunit Ib against an antibody for cytochrome f gave precipitation lines without crossing spurs, so that the "line of identity" indicates that subunits Ia and Ib are both cytochrome f. Finally proteolytic fingerprints of isolated subunits Ia and Ib were identical (N. Nelson, personal communication). Therefore our previous speculation (Hurt and Hauska, 1981) that the 33-kD polypeptide together with the 23-kD subunit represents the cytochrome b_6 does not hold.

The predominance of subunit Ib either in the complex (Fig. 4A, lane 1) or in the purified cytochrome f (Fig. 4A, lane 2) is obvious, but we observe a variation of the relative amounts of subunits Ia and Ib in different preparations (shown in Fig. 1), both polypeptides sometimes showing the same

staining intensity on SDS-PAGE. Boiling the cytochrome b_6/f sample in SDS prior to gel electrophoresis did not change the distribution between subunits Ia and Ib (Krinner *et al.*, 1982), demonstrating that the migration behavior of cytochrome f in the cytochrome b_6/f complex of *Anabaena variabilis* is changed on SDS-PAGE when the sample was boiled prior to electrophoresis. The reason for the microheterogeneity of cytochrome f from spinach is not known. It could be due to a genetic heterogeneity, or a proteolytic cleavage of physiological importance, or it could be caused by isolation. Some observations indicate that proteolytic effects could play a role. So we recognized that a cytochrome b_6/f preparation having the predominant subunit Ib was modified during 3 days incubation at room temperature in 20% ammonium sulfate. The resulting polypeptide pattern showed subunits Ia and Ib in approximately equal amounts. Another degradation product at 14 kD was also formed (Fig. 1, lanes 5 and 6). On the other hand, we observe the presence of subunits Ia and Ib already in early stages of the isolation of the cytochrome b_6/f complex, for example, in washed chloroplasts. During the following isolation procedure the relative distribution of subunits Ia and Ib was not changed. Also isolated cytochrome f from the green algae *Scenedesmus acutus* purified to homogeneity reveals two bands on SDS-PAGE with a molecular mass of 33 and 31 kD, respectively (Böhme *et al.*, 1980).

Cytochrome b_6 could be isolated from the complex in pure form and could be identified with subunit II (Fig. 6A and B). Against a previous speculation (Hurt and Hauska, 1981) we must conclude that cytochrome b_6 is represented solely by subunit II with an apparent molecular mass of 23 kD. The low-molecular-weight subunits of the cytochrome b_6/f complex reported in this paper are missing in the cytochrome b_6 preparation (they stay absorbed on hydroxyapatite column; see Materials and Methods, gel not shown). Therefore, we conclude that isolated cytochrome b_6 is composed of a single polypeptide with an apparent molecular mass of 23 kD, which is in agreement with Lach and Böger (1977) but in disagreement with Stuart and Wasserman (1975), who found also smaller polypeptides in their cytochrome b_6 preparation.

The role of subunit IV (17 kD) is not clear. So far it cannot be identified with a redox center, but it is of course one of the sites where bound plastoquinone might be located (Hurt and Hauska, 1981). In this context it is worth mentioning that this subunit is probably very hydrophobic, because it has high UV absorption (see Figure 3B) and sticks on DEAE-cellulose column during isolation of cytochrome f and can be eluted only with high salt in the presence of Triton X-100. Under these conditions subunit IV co-purified with cytochrome b_6. A similar association of a 15- to 17-kD polypeptide with cytochrome b (34–37 kD) is known for the mitochondrial b/c_1 complex (Gellerfors and Nelson, 1975; Marres and Slater, 1977; Yu *et al.*, 1974). Both
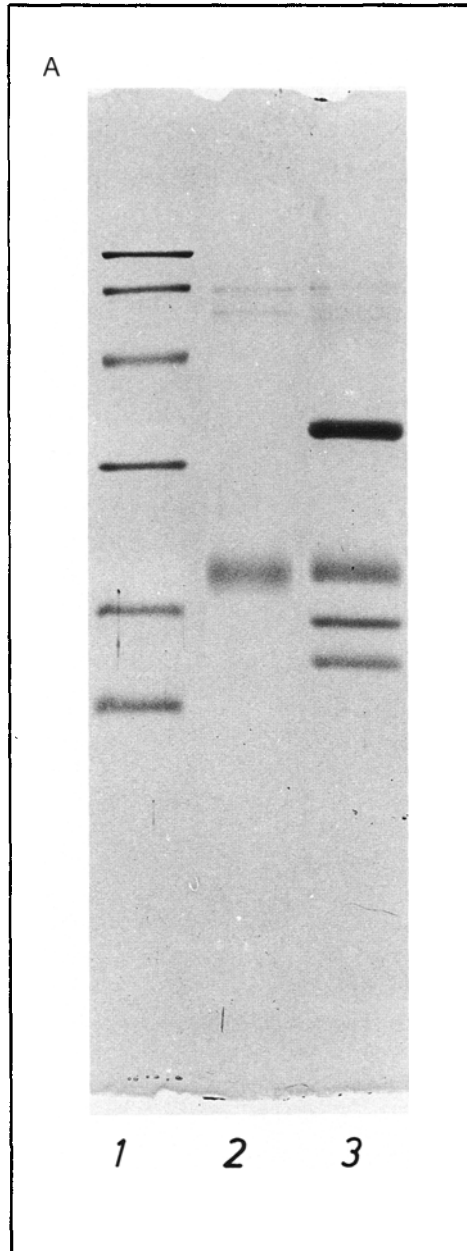
Fig. 6. Electrophoresis and spectrum of isolated cytochrome b_6. (A) Electrophoresis on SDS polyacrylamide gels (15%) stained with Coomassie blue. Lanes 1, 2, and 3 show protein standards, isolated cytochrome b_6, and the cytochrome b_6/f complex, respectively. (B) Spectrum of the isolated cytochrome b_6 in the presence of ascorbate (dashed) and in the presence of dithionite (solid).
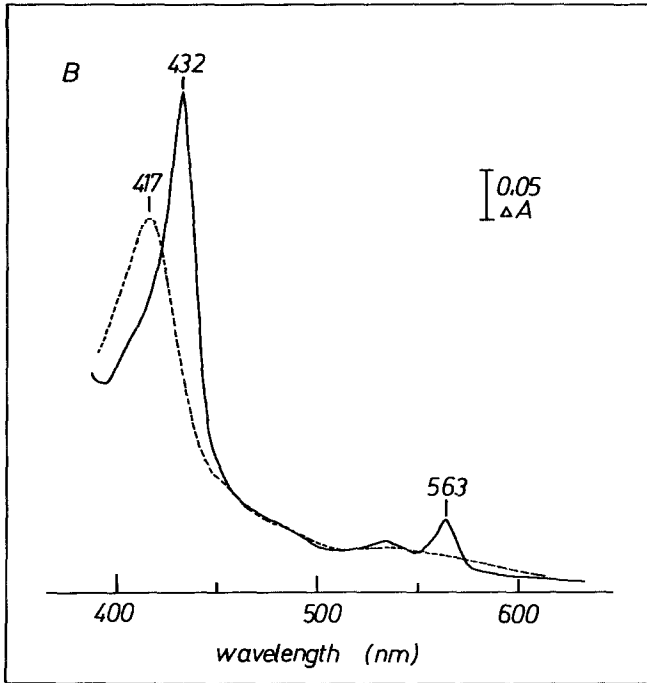
Fig. 6. Continued.

proteins exhibit a high content of hydrophobic amino acids (Yu *et al.,* 1975), and it has been shown that they are ubiquinone-binding proteins (Yu and Yu, 1981). Recently Wang and King (1982) isolated a 15-kD ubiquinone-binding protein from the mitochondrial cytochrome b/c_1 complex which restores ubiquinone cyt *c*-oxidoreductase activity. It is also interesting that cytochrome b_6 and subunit IV of the cytochrome b_6/f complex are coded by adjacent genes in the chloroplast genome, possibly with overlapping nucleotide sequences (Herrmann *et al.,* in preparation).

The redox potential of cytochrome *f* in the complex has been found to be similar to the one for isolated cytochrome *f* (Cramer and Whitmarsh, 1977) and to the one for cytochrome *f* in the cytochrome b_6/f preparation of Nelson and Neumann (1972). A single one-electron transition with an average E_m of $+339$ mV from three independent measurements was obtained (Fig. 7). The redox potential was pH-independent in the range of pH 6.5–8.3. Such a pH-independence of the midpoint potential for isolated cytochrome *f* from *Brassica komatsuna* in the range of pH 5.0–8.9 was shown by Takabe *et al.* (1980). Rich and Bendall (1981) reported an E_m of 370 mV, with a pK of 9.0 for the oxidized form of cytochrome *f*, in a less purified preparation of the cytochrome complex.

The redox titration of cytochrome b_6 in the cytochrome b_6/f complex is
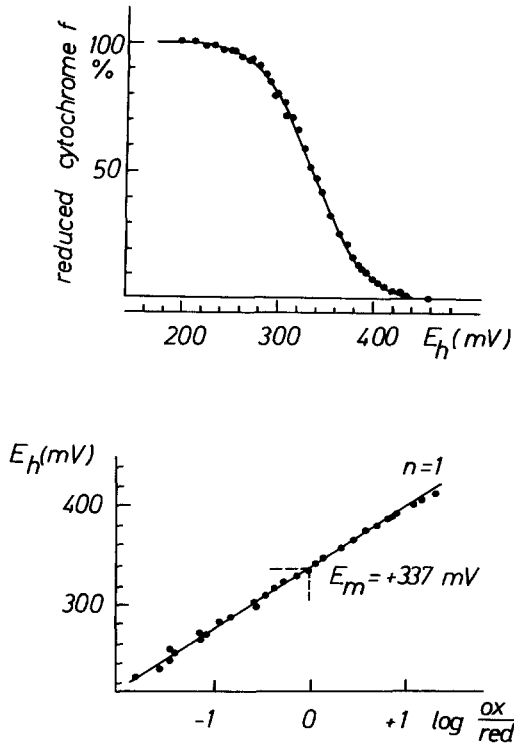
Fig. 7. Redox titration of cytochrome f in the complex. The titration is described under Assays. The upper part of the figure shows the relative absorption at 554 nm plotted versus the redox potential. 100% reduced cytochrome f corresponds to 2.5 μM cytochrome f. In the lower part the logarithmic ratio of oxidized to reduced cytochrome f versus the redox potential is shown. A theoretical line for a one-electron transition is drawn through the experimental points, and the midpoint potential is given.

shown in Fig. 8. Cytochrome b_6 did not titrate as a single one-electron transferring component. The sigmoidity in the lower picture of Fig. 8, where the log of oxidized cytochrome b_6 to reduced b_6 versus the E_h is plotted, suggests that cytochrome b_6 is composed of at least two components. Assuming that two cytochromes b_6 are present in equal amounts, two components can be separated arithmetically with the procedure of Nelson and Gellerfors (1974). The points of the two components fit to two theoretical lines with a slope $n = 1$, which corresponds to two one-electron acceptors with oxidation–reduction midpoint potentials of $E_{m(1)} = -50$ mV and $E_{m(2)} = -170$ mV at pH 6.5, respectively. The average of the two midpoint potentials derived from four independent titrations at pH 6.5 were -40 and -172 mV. So it seems that
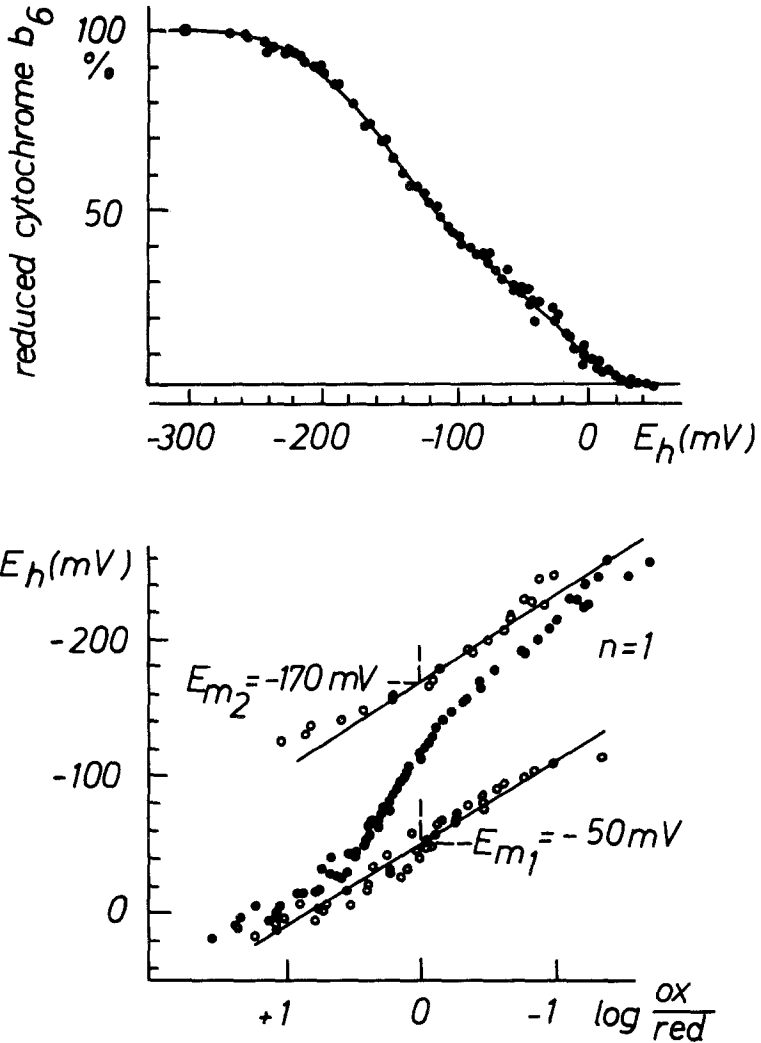
Fig. 8. Redox titration of cytochrome b_6 in the complex. The upper part of the figure shows the relative absorption at 563 nm with changing redox potential. 100% reduced cytochrome b_6 corresponds to 5 μM cytochrome b_6. The lower part represents the equivalent graph as described for cytochrome f in Fig. 7. The sigmoidal pattern of the filled circles is arithmetically resolved into two series of points (open circles) which fit in two theoretical lines with a slope $n = 1$; the midpoint potentials for both cytochrome b_6 components are given.

cytochrome b_6 is potentiometrically heterogeneous, as is known for mitochondrial cytochrome b of complex III (Nelson and Gellerfors, 1974), but spectroscopically cytochrome b_6 is a single species with an absorption maximum of the α-band at 563 nm, which is in contrast to the mitochondrial cytochrome b (Nelson and Gellerfors, 1974).

Both midpoint potentials of cytochrome b_6 were pH-dependent (Table II), as observed for cytochrome b from the mitochondrial b/c_1 complex (von Jagow et al., 1981). This property is the basis for the speculation that cytochrome b, in addition to quinone, might be involved in proton translocation through the membranes of mitochondria (von Jagow et al., 1981) and of chloroplasts in the region of the cytochrome b/c_1 (b_6/f) complex.

Isolated cytochrome b_6 also titrates very similarly to cytochrome b_6 in the intact complex (data not shown), which is in contrast to Stuart and Wasserman (1975) who found for their cytochrome b_6 a single one-electron transient. The redox titration curves we obtained are reminiscent of a titration carried out for cytochrome b_6 in the chloroplast membrane under uncoupled conditions (Böhme and Cramer, 1973); therefore we think that the potentiometric heterogeneity of cytochrome b_6 in our complex is not the result of damage during the isolation but is indicative of two forms of cytochrome b_6 with different midpoint potentials. Rich and Bendall (1981) reported somewhat different results recently. They obtained single-component E_m values around -100 mV for cytochrome b_6 in chloroplasts and in a less purified prepartion of the cytochrome complex. Only in the latter case was the E_m found to be pH-dependent (15 mV per pH unit between pH 5 and 11). Their redox titrations were complicated by the presence of cytochrome b-559, however.

Recently we demonstrated transient reduction of cytochrome b_6 when the complex is oxidized in the presence of plastoquinol, either by ferricyanide or by light in the presence of plastocyanin/photosystem I (Hurt and Hauska, 1982). Prince et al. (1982), in addition, showed that cytochrome b_6 could be photoreduced in a mixture of the complex with bacterial reaction centers. This

Table II. pH-Dependence of Redox Potentials for Cytochrome b_6[a]

pH	$E_{m(1)}$ (mV)	$E_{m(2)}$ (mV)
5.6	-24	-156
6.5	-40	-172
7.4	-60	-188
8.3	-112	-215

[a]Redox titrations shown in Fig. 8 and described under Assays were performed at the indicated pH values. At the two higher pH values Tricine-NaOH replaced Tris-succinate as the buffer.

reduction was dependent on the Rieske Fe-S center being oxidized in the cytochrome complex. These observations are in line with a "Q-cycle" mechanism (Mitchell, 1975) for electron transport through plastoquinol-plastocyanin oxidoreductase of chloroplasts as discussed by Hurt and Hauska (1982) with detailed references. Whether the finding of two different E_m values for cytochrome b_6 reported in this paper is another indication for this mechanism needs further experimentation.

Acknowledgments

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