Isolation of a Five-polypeptide Cytochrome b-f Complex from Spinach Chloroplasts*

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A simple rapid purification of a cytochrome b-f complex from spinach chloroplasts is described. Novel features of the method include: 1) EDTA treatment of thylakoids prior to detergent extraction; 2) affinity chromatography over equine cytochrome c linked to Sepharose 4B; and 3) inclusion of the protease inhibitor phenylmethylsulfonyl fluoride. Cytochrome b-f complex is obtained in good yield, free of exogenous lipid, and with high plastoquinol-plastocyanin oxidoreductase activity. The complex contains 2 eq of cytochrome b-563 per eq of cytochrome f and a Rieske iron-sulfur center. Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate indicates that the complex is composed of five distinct polypeptides of M, = 37,000, 33,500, 22,000, 19,000, and 16,500. Only the M, = 33,500 and 22,000 polypeptides stain for heme. The M, = 37,000 component in this preparation is absent from cytochrome b-f complex isolated by another procedure (Hurt, E., and Hauska, G. (1981) Eur. J. Biochem. 117, 591-599). The complex described here also differs in its spectrum at 77 K, its stability, and its buoyant density.

The presence of cytochromes in photosynthetic tissues was first demonstrated by Hill (1), who identified the c-type cytochrome f in leaves and autodoxidizable cytochrome b-563 (b6) in chloroplasts. Subsequent research (2) also established the presence of at least two forms of cytochrome b-559 in photosynthetic membranes. High potential (ascorbate-reducible) cytochrome b-559 is associated with photosystem II and may be involved in water-splitting reactions or cyclic electron transport around photosystem II (2); cytochrome f, the low potential (dithionite-reducible) cytochrome b-559 and cytochrome b-563 fractionate with photosystem I (3).

An intimate physical association between cytochrome f and cytochrome b-563 was first indicated by their co-purification from lettuce chloroplasts as a 125-kDa ~ 2:1 b-f complex (4). This same 2:1 stoichiometry was found in intact chloroplasts (3). The 2:1 ratio of b- to c-type cytochromes in the b-f complex, along with its nonheme iron content, suggested a functional analogy of the chloroplast b-f complex to the b-c1 complex (Complex III) of mitochondria (5) and of photosynthetic bacteria (6), a hypothesis which drew strong support from the demonstration by Wood and Bendall that cytochrome f is directly involved in catalyzing the reduction of plastocyanin by plastoquinol (7). This reaction appeared to require cytochrome b as does the ubiquinolcytochrome c oxidoreductase activity of the b-c1 complex. It was subsequently shown that nonheme iron in the b-f complex and in the b-c1 complex is organized in very similar high potential iron-sulfur centers (Reiske Fe-S) (5, 8). The striking similarities in composition of the two complexes led Mitchell to propose that both catalyze electronographic "Q" cycles during quinol oxidation (9). Hurt and Hauska isolated (10) a cytochrome b-f complex from spinach thylakoids that catalyzed electron transport from plastoquinol to plastocyanin or algal cytochrome c-552. After reconstitution into phospholipid vesicles, this complex generated a membrane potential during plastoquinol oxidation (11), thereby extending the analogy with mitochondrial cytochrome b-c complex (5).

Here we report a novel procedure for isolating cytochrome b-f complex from spinach. Higher yields are obtained in comparison to the procedure of Hurt and Hauska (10, 12). The product also contains less chlorophyll and carotenoid and is free of added asolectin. It is equally active in the PQH2:PC oxidoreductase assay, but differs in composition and in some physical characteristics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Spinach (Spinacia oleracea, Hybrid 424, Park Seed Co.) was grown under a combination of fluorescent (5160 watts) and incandescent (3000 watts) lights at an incident intensity of 60 watts/m². The growth chamber was maintained on a 10-h (27°C)/14-h (24°C) light/dark cycle. Spinach was harvested from 6- to 10-week-old plants; leaves were washed and deveined and then stored overnight at 4°C before use.

Equine heart cytochrome c (Sigma) was coupled (13) to cyanogen bromide-activated Sepharose 4B-CL purchased from Sigma or prepared according to Cästelasaas (14). The gel was poured and the column bed washed with 0.4 M NaCl, 0.2 M Tris-chloride, 1% Triton X-100 (pH 8.0) until no more color eluted, then with H2O. The bed was resuspended in water and the gel washed batchwise with 80 mM sodium Tricine containing 2% cholate (pH 7.5), water, and 20 mM sodium Mes containing 0.5% cholate (pH 6.5). The gel was reduced by inclusion of sodium ascorbate in the last batchwise wash, then poured and washed free of ascorbate with Mes/cholate buffer before use. The same washing procedure was used to regenerate the gel after each use. Cytochrome c-Sepharose was stored in buffer containing 0.04% NaN3.

Plastocyanin was purified from spinach by a modification of the method of Borchert and Wessels (15). crude cytochrome c-552 from Euglena was a gift from H. Lyman, State University of New York at Stony Brook, and was further purified according to Mitsui (16).

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1 The abbreviations used are: PQH2, plastoquinol; PC, plastocyanin; DNP-INT, dinitrophenyl ether of iodonitrothymol; Mes, 2-[N-morpholino]ethanesulfonic acid; Mops, 3-[N-morpholino]propane-sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Tricine, N-tris(hydroxymethyl)methylglycine; SDS, sodium dodecyl sulfate.
Asolectin was obtained from Associated Concentrates, Woodside, New York. Plastoquinol-1 was a gift from G. Hauska. DNP-INT was provided by A. Trebst. Other inhibitors and buffers were obtained from Sigma. Octylglucoside was purchased from Calbiochem.

Preparation of Cytochrome Complex—All steps were carried out at 0–5°C unless otherwise indicated. 250-g deined spinach leaves were homogenized in 1 liter of 0.4 M sucrose, 50 mM sodium Tricine, pH 7.8, and 0.1% Triton X-100 (to a slush). The resulting brei was filtered through one, then through eight, layers of wet cheesecloth. The filtered homogenate was centrifuged for 10 min at 3000 × g and the supernatant discarded. The pellet was resuspended and homogenized in 250 ml of 5 mM EDTA, 2 mM Tricine (pH 7.8), and then centrifuged for 20 min at 25,000 × g. The supernatant was centrifuged and washed with the associated chloroplasts resuspended in 250 ml of 0.5 mM EDTA, 2 mM sodium Tricine (pH 7.8). After incubation at 4°C for 15 min the chloroplast suspension was centrifuged for 20 min at 25,000 × g and the supernatant discarded. The pellet was taken up in 10 mM sodium Tricine, 10 mM MgCl2 (pH 8.2) to a chlorophyll concentration of 1.4–1.6 mg/ml.

EDTA-washed chloroplasts were diluted 1:1 with 60 mM octylglucoside, 1% sodium cholate, 100 mM Tris chloride (pH 8.2). The mixture was stirred at 0°C for 1 h and then centrifuged for 90 min at 300,000 × g. The pellet was discarded and enough solid PMSF added to the supernatant to give a terminal concentration of 0.1 mM. The extract was then centrifuged 2- to 3-fold using an Amicon concentrator fitted with an XM-100A membrane. The dialysate was discarded and 0.5 M Mes added to the retentate to a concentration of 20 mM (pH 6.5).

The concentrated extract was passed through an ascorbate-reduced equimolar cytochrome c-Sepharose column (bed volume 8–10 ml) pre-equilibrated with 20 mM sodium Mes, 0.5% sodium cholate (pH 6.5). A flow rate of 1–2 ml/min was maintained; 10- to 20-ml aliquots were run through the column at room temperature, with the eluant collected on ice, so that the extract was warmed for no more than 20 min. The column was equilibrated with 10 ml of 20 mM sodium Mes, 0.5% sodium cholate (pH 6.5) to elute b-f complex retained in the column bed. Eluant from the cytochrome c-Sepharose column was brought to 40% saturation by the addition of solid ammonium sulfate. After stirring for 20 min at 0°C the suspension was centrifuged for 20 min at 11,000 × g and the supernatant brought to 60% saturation by the addition of solid ammonium sulfate. After stirring for 20 min at 0°C, precipitated protein was collected by centrifugation at 12,000 × g and the supernatant discarded. The pellet from the 60% ammonium sulfate cut was resuspended in 30 mM octylglucoside, 0.5% sodium cholate, 30 mM Tris succinate, pH 6.5 (to obtain maximal yield of cytochrome and oxidoreductase activity, the volume at this step should not exceed 4 ml). The suspension was kept at 0°C overnight after addition of one grain of solid PMSF, and then reduced with sodium ascorbate and applied to a Bio-Gel P300 column (1.8 × 17 cm). This column had been pre-equilibrated with 20 mM sodium Tricine, 0.5% sodium cholate, 0.1% Triton X-100 (10 pg of asolectin, and 20 pg of Triton X-100 in 0.5 ml. Asolectin and Triton X-100 were added as a 1:2 aqueous suspension; after mixing by inversion, b-f complex (2–10 pmol of cytochrome c, 10 pg of asolectin, and 20 pg of Triton X-100 in 0.5 ml. Unless otherwise indicated the assay contained 0.3 nmol of PC, 5-10 nmol of equine cytochrome c, 10 Mg of asolectin, and 20 M of Triton X-100 in 0.5 ml. Asolectin and Triton X-100 were added as a 1:2 aqueous suspension; after mixing by inversion, b-f complex (2–10 pmol of cytochrome f) was added and the cuvette again mixed by inversion. The reaction was initiated by the addition of 0.5 Ml of 15–20 mM plastomycin-1 in ethanol. Reduction of cytochrome c was followed at 550 nm in a dual wavelength spectrophotometer at 20°C, taking 540 nm as the reference wavelength and assuming a differential extinction coefficient of 18 mM−1 cm−1. All activities given are corrected for the rate of cytochrome c reduction by plastomycin observed in the absence of b-f complex. Kinetic constants were calculated from double reciprocal (PC) or Edad-Hofstee plots (PQH-1) (17) by least squares regression analysis. Inhibitors were added in ethanolic solution; in no case did the ethanol content of the assay mixture exceed 0.5% (v/v).

Chlorophyll and carotenoids were extracted into acetone and the chlorophyll concentration determined according to Arnon (18). Carotenoids were quantitated after methanolysis (3). Protein was determined according to Lowry et al. (37) after precipitation from deoxycholate with trichloroacetic acid (19), using bovine serum albumin as standard.

Room temperature spectra were obtained on a Cary 14 spectrophotometer. Cytochrome concentrations were determined from difference spectra with samples in 20 mM sodium Tricine (pH 7.5) containing 0.5% sodium cholate or 0.1% Triton X-100; the latter detergent was necessary to effect complete penetration of ferricyanide and ascorbate into chloroplasts. Aliquots of cytochrome fractions were oxidized with 1 mM ferricyanide after dilution and were divided between sample and reference cuvettes. The spectra were taken between 500 and 600 nm after addition of sodium ascorbate to the sample cuvette. The differential extinction coefficient of cytochrome f at 554 nm versus 540 nm was taken as 19,000 M−1 cm−1 (20); where necessary a base-line was interpolated between 540 and 560 nm (20, 21). Cytochrome b-563 was determined from spectra taken after addition of ascorbate to the reference cuvette and dithionite to the sample, assuming a differential extinction coefficient of 21,000 M−1 cm−1 at 563 versus 575 nm (20). The sample was allowed 2–3 min after dithionite addition to reach full cytochrome b reduction. For dilute samples, e.g. gradient fractions, aliquots were diluted into 20 mM sodium Tricine, 0.5% sodium cholate, 1 mM sodium ascorbate (pH 7.5) and divided between sample and reference cuvettes. The sample was then reduced with dithionite and scanned from 380 to 460 nm 2–3 min after dithionite addition. A differential extinction coefficient of 131,000 M−1 cm−1 at 432 nm with respect to 446 nm was assumed, based on the spectrum of the purified complex.

Low temperature spectra were obtained in a vertical optics spectrophotometer of our own design. The cuvettes used were 25 mm in diameter with an optical pathlength of 2–3 mm. Digitized spectra were stored and manipulated by interfacing the spectrophotometer with a PDP 11/34 computer.

Low molecular weight cytochrome c-Sepharose gel electrophoresis was carried out according to Chua and Bennoun (22). Gels were stained for heme and protein as described in Ref. 23.

RESULTS

Purification of Cytochrome b-f Complex from Spinach—The isolation procedure of Hurt and Hauska (10, 12) for cytochrome b-f complex is optimized for application to European market spinach. Applying this protocol to fresh spinach from growth chambers yielded complex containing over 5 mol of chlorophyll per mol of cytochrome f and only 120–160 nmol of cytochrome f per kg of leaves processed. The oxidoreductase activity in these preparations was also low and unstable, hence an alternative procedure was sought for application to fresh spinach (Hybrid 424). The following method gives cytochrome b-f complex with a chlorophyll content of 0.9–1.4 mol of chlorophyll per mol of cytochrome f at a yield of 200–360 nmol of cytochrome f per kg of leaves. Lower yields may result if market spinach is used, though the product is qualitatively the same. It is stable for at least 6 months at 77 K or 4 days at room temperature.

Chloroplasts were isolated from leaves by centrifugation of homogenized tissue and washed twice with dilute EDTA to remove stromal proteins and deplete coupling factor 1 (24). Cytochromes were then extracted into 30 mM octylglucoside, 0.5% cholate at moderate ionic strength. The compositions of this and subsequent fractions are summarized in Table I. The detergent extract was concentrated and then passed through an equine cytochrome c-Sepharose column to remove P700 (25) which otherwise persisted throughout the purification. Subsequent ammonium sulfate fractionation served three purposes: to reduce the free chlorophyll in the complex, to concentrate the cytochrome, and to remove complex deficient in cytochrome f (Table I). Gel chromatography using Bio-Gel P300 removed residual chlorophyll and extraneous protein, notably polyepptides of molecular masses 58 kDa, 54 kDa, 23.5 kDa, and 17 kDa. The polyepptide profile of the purified complex is shown in Fig. 1. The complex is most stable in cholate buffer in concentrated partially reduced form; hence it is important that the ammonium sulfate-precipitated protein be resuspended in a minimal volume of buffer and reduced with ascorbate before application to the Bio-Gel column. The leading and trailing edges of the brown elution peak have less catalytic activity and are discarded.
The cytochrome b-f complex carried to, and PC in the presence of plastoquinol-1 (Fig. 2) but only for complex carried to, or beyond, the ammonium sulfate precipitation stage of purification. This stimulation may reflect restoration of lipid to delipidated enzyme, i.e. a lipid requirement for catalytic activity (10, 26). Asolectin may also prevent aggregation of the complex and adsorption of PQLH2 to the walls of the cuvette. Its inclusion provides a defined lipid content to which concentrations of hydrophobic reagents (e.g. inhibitors and plastocyanins) may be referred.

Under the conditions of pH and ionic strength employed here, the two relevant background reactions interfering with the assay are very slow; neither PC nor b-f complex alone will effectively catalyze the reduction of equine cytochrome c by plastocyanin-1 (Fig. 2). Although the reduction of PC by cytochrome f is much faster than the oxidation of PC by equine cytochrome c (27), the former reaction can be kept rate-limiting by exploiting mass action effects, i.e. by inclusion of cytochrome c in great excess over the cytochrome f assayed. For 10 μM cytochrome c in the presence of 0.7 μM PC, the rate of cytochrome c reduction is directly proportional to the amount of b-f complex added up to a rate of 150 nmol/h. The dynamic range of the assay is doubled by doubling the cytochrome c or PC concentrations, but background rates are also increased. The observed limiting rate of electron transfer from PC to equine cytochrome c is in good agreement with the data of Wood (27).

Because plastocyanin acts catalytically in the oxidoreductase assay, it was possible to extend rate determination to very low PC concentrations and determine the affinity of b-f complex for PC. Michaelis constants for PC and plastocyanin-1 are given in Table II, along with the corresponding second order rate constants calculated from them. An increase in activity with increasing pH has already been reported (10); our data show that the Km for PC increases in parallel from 0.18 at pH 6.0 to 0.48 at pH 7.5. The second order rate at limiting PC concentrations (Vm/Km) is independent of pH (Table II). The rate constant obtained for PC reduction was 1.8 × 108 M-1 s-1, in good agreement with previous determinations (7, 27). Cytochrome c-552 from Euglena was found to substitute satisfactorily for PC in this assay (data not shown). The Km for plastocyanin was 9 μM (Table II). Inhibition by DNP-INT was half-maximal below 0.5 μM. Effects of the redox-active quinoid inhibitors 2-heptyl-4-hydroxyquinoline N-oxide and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole on oxidoreductase activity were biphasic; inhibition observed at low concentrations (10-20 μM) was reversed at higher concentrations.

**Poly peptide Composition.—** The electrophoretic polypeptide profile for the isolated cytochrome b-f complex after reductive electron acceptor. Asolectin markedly stimulates the rate of cytochrome c reduction catalyzed by cytochrome b-f complex and PC in the presence of plastocyanin-1 (Fig. 2) but only for complex carried to, or beyond, the ammonium sulfate precipitation stage of purification. This stimulation may reflect restoration of lipid to delipidated enzyme, i.e. a lipid requirement for catalytic activity (10, 26). Asolectin may also prevent aggregation of the complex and adsorption of PQLH2 to the walls of the cuvette. Its inclusion provides a defined lipid content to which concentrations of hydrophobic reagents (e.g. inhibitors and plastocyanins) may be referred.

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Chloroplast Cytochrome b-f Complex

Dissociation in hot SDS (22) is shown in Fig. 1. The major protein bands stained by Coomassie blue migrate at $M_r = 37,000, 33,500, 22,000, 19,000, \text{ and } 16,500$ (Fig. 3). The staining intensity at these five positions, as quantitated densitometrically, accounts for most of the total staining on gels. Furthermore, these five peptides together account for 90% of the total protein applied to gels if the 33.5-kDa band is ascribed solely to cytochrome f (14, 23). Minor polypeptides of molecular mass 58 kDa, 54 kDa, 23.5 kDa, and 17.3 kDa correspond to contaminants incompletely removed by Bio-Gel P300 filtration. The appearance of polypeptides at 13 and 10.5 kDa varies between preparations; the 10.5-kDa band may be plastocyanin. The PQH$_2$-cytochrome c oxidoreductase activity observed in the absence of added PC (0.34 $\mu$mol h$^{-1}$ nmol$^{-1}$ at pH 6.0) sets an upper limit of 0.4 mol of PC/mol of f. Direct reaction of cytochrome b-f complex with equine cytochrome c (7) would reduce this upper limit further.

**Table II**

Kinetic parameters for PQH$_2$:PC oxidoreductase activity of the isolated cytochrome b-f complex

| Variable substrate | Buffer | $K_m$ | $V_{max}$ | $k_2^b$ |
|--------------------|--------|-------|----------|---------|
| Plastocyanin       | Mes, pH 6.0 | 0.15 | 9.0 | $1.7 \times 10^6$ |
| Plastocyanin       | Mes, pH 6.5 | 0.25 | 14.3 | $1.6 \times 10^6$ |
| Plastocyanin       | Mops, pH 7.0 | 0.28 | 16.7 | $1.7 \times 10^6$ |
| Plastocyanin       | Mops, pH 7.5 | 0.36 | c | c |
| Plastoquinol-1     | Mes, pH 6.25 | 9.0 | 6.0$^d$ | $1.9 \times 10^5$ |

*Assayed as in Fig. 2 except as indicated. All values shown were obtained at each of at least two different concentrations of b-f complex.

*Second order rate constants at limiting substrate concentrations were calculated from the Michaelis-Menten parameters. $K_m$, but not $V_{max},$ was independent of concentration of b-f complex at this pH in the accessible kinetic regime.

*These experiments were carried out with somewhat less active preparations of PC and the b-f complex.
Isosbests were at 540 and 560 nm. Maxima in the redox pairs with a ratio of 0.77 reported protein co-migrated on the gradient with the 34-kDa cytochrome f polypeptide (Fig. 3C) and inactive cytochrome b-563 and the same proportion of applied oxidoreductase activity. Presumably the pellet, which was insoluble, disappeared during sedimentation may account for the appearance of the 37-kDa polypeptide (Fig. 3C) and inactive cytochrome b-563, as indicated by oxidoreductase activity and cytochrome f content (Fig. 3). A low density cytochrome f peak at 420 nm was observed in some preparations and in the 60% ammonium sulfate fraction, corresponds to the final product of the Hurt and Haukka preparation (10, 12). The 37-kDa protein co-migrated on the gradient with the 34-kDa cytochrome f polypeptide (Fig. 3C), peaking in concentration at 420 nm. The specific activity of cytochrome b-f complex was not diminished by fractionation on the density gradient; the high density brown band contained 37% of the applied cytochrome b-563 and the same proportion of applied oxidoreductase activity. Presumably the pellet, which was insoluble, contained the balance of the applied catalytic activity. The loss of 60% of the complex during sedimentation on a sucrose gradient makes this an inappropriate preparative step, having the further disadvantage of requiring addition of exogenous lipid (asolectin) (10). Furthermore, b-f complex is less stable in the gradient fractions than in the applied material. Breakdown during sedimentation may account for the appearance of the 37-kDa polypeptide (Fig. 3C) and inactive cytochrome in the lower density gradient fractions.

Spectral Properties—Cytochrome b-f complex prepared by the method described here does not contain exogenous lipid, so an unambiguous spectrum can be obtained in the ultraviolet (Fig. 4A). Cytochrome f in the isolated complex is reduced. The 420:280 nm absorbance ratio is 1.4, which compares with a ratio of 0.77 reported for the digitomin preparation of Nelson and Neumann (4).

The optical spectrum of the dithionite-reduced complex, shown in Fig. 4B, is qualitatively similar to previously published spectra of the complex (4, 10) except that the y peak for cytochrome b at 432 nm is more pronounced. The preparation also contains less carotenoid (Table I) than that of Nelson and Neumann (4). Absorbance maxima for cytochrome f (ascorbate-reduced versus oxidized) at 20 °C were at 553.5 nm (α), 530 and 524 nm (β), and 422 nm (γ) (Fig. 5A). Isosbests were at 540 and 560 nm. Maxima in the redox difference spectrum for cytochrome b (dithionite versus ascorbate-reduced) were at 563 nm (α), 533 nm (β), and 432 nm (γ). In contrast to another report (20), cytochrome f was only partially oxidized by ammonium persulfate at pH 7.5. Based upon difference extinction coefficients of 19,000 and 21,000 M⁻¹ cm⁻¹ for the α peak (19), the corresponding γ-band extinction coefficients were 124,000 M⁻¹ cm⁻¹ at 422 nm for cytochrome f and 131,000 M⁻¹ cm⁻¹ at 432 nm for cytochrome b-563. The γ bands for this preparation are remarkably sharp, with half-widths of 9 and 10 nm for the 422- and 432-nm bands of cytochrome f and cytochrome b, respectively.

The redox difference spectra of the complex at 77 K are shown in Fig. 6. At liquid nitrogen temperature, heme c cytochromes generally exhibit a split α absorption band, as do many heme cytochromes (30). Ascorbate reduction reveals split cytochrome f peaks at 548.1 and 552.0 nm (α) and peaks at 520.5 and 523.3 nm (β); the maximum observed at 530 nm at room temperature is shifted to 529 nm at 77 K (Fig. 6A). In the γ region, a single peak at 420 nm was observed (data not shown). The 77 K difference spectrum was taken at a 2-fold scale expansion.
from complex in which both equivalents of heme b cytochrome have been reduced by prolonged incubation with dithionite shows peaks at 560.8 nm (a) and 551.0 nm (b) (Fig. 6B). Secondary peaks are at 539 and 549 nm, with a shoulder at 557 nm. EPR spectra of the cytochrome b–f complex will be presented elsewhere.

**DISCUSSION**

The protocol formulated by Nelson and Neumann for the isolation of a b–f complex from lettuce chloroplasts (4) yields material without appreciable PQH$_2$:PC oxidoreductase activity (7). The loss of catalytic activity is evidently due to exposure of the complex to Triton X-100 during isolation; this nonionic detergent is a potent inhibitor of the oxidoreductase (7), possibly because it cleaves the Reiske Fe-S center and plastiquinone from the complex (28). Hurt and Hauska developed a procedure starting with European market spinach which, in its revised form (12), avoids the use of Triton X-100 and retains considerable catalytic activity.

In preliminary studies to develop an isolation protocol appropriate to spinach from controlled environment chambers, we observed proteolytic degradation of the complex during isolation, as indicated by loss of activity, smearing on SDS-polyacrylamide gels, and loss of resolved protein band on gels. Inclusion of the protease inhibitor PMSF (30) overcame this difficulty and greatly improved the stability of the isolated complex; this inhibitor has also been used to block degradation of phytochrome in homogenized plant tissue (31). Proteolysis may also account for the apparent absence of a 37-kDa polypeptide (Fig. 1) and the presence of two cytochrome f polypeptides in the b–f complex of Hurt and Hauska (10, 12, 29). Because the 37-kDa component copurifies with b–f complex and comigrates with the complex on sucrose density gradients (Fig. 3), we believe it is an integral part of the complex. Its presence is necessary to give a molecular mass of over 100 kDa to the complex as a whole, as expected on the basis of gel exclusion chromatography (4, 10) and electrophoresis in nondenaturing polyacrylamide gels. It is noteworthy that inclusion of PMSF during purification of a b–c$_3$ complex from *Rhodopseudomonas sphaeroides* also allowed visualization of an "extra" polypeptide (32) beyond the three components identified in a preparation (33) based on the protocol in Ref. 10.

The physical and spectral characteristics of the 5-polypeptide b–f complex described here are similar to those of a catalytically active digitonin extract (7) and the inactive purified complex of Nelson and Neumann (4). No cytochrome b–f was detectable in our preparation, either by menadion reduction (34) or by potentiometric titration. The absorption spectrum obtained at 20 °C in the visible region is very similar to those previously reported except that cytochrome f is reduced in our isolate (Fig. 4) and the cytochrome b/f ratio is higher.

At 77 K, the splitting of the cytochrome f peak in ascorbate-reduced samples (Fig. 6) is identical with that observed in situ and in crude digitonin extracts (3), with the long wavelength peak at 552 nm and a splitting of 4 nm. In contrast, purified cytochrome f (23) and the spectrum of cytochrome b–f complex reported by Hurt and Hauska (10) have the long wavelength peak at 551.5 nm and a splitting of 3.5 nm. Although these represent small quantitative differences, the resulting difference in spectral resolution is considerable (compare Fig. 6 in this work with Fig. 3 in Ref. 10). The high ratio (1.4) of 420 to 280 nm absorbance (Fig. 4) testifies to high purity; the absorbance ratio for b–f complex purified on a sucrose density gradient free of exogenous lipid was actually somewhat lower. Comparison with the low ratio for the preparation of Nelson and Neumann (4) is perhaps inappropriate, since Triton X-100 bound during purification may contribute to absorbance at 280 nm (20).

The oxidoreductase assay developed by Wood and Bendall (7) utilizes the natural acceptor, plastocyanin. The broadness and weak intensity of absorption by oxidized PC can be circumvented by substituting *Pseudomonas* (7) or algal (10) acidic cytochrome c for PC. These are not the natural substrates, however, and are not readily available in large quantities. At least in the latter case, a high rate of spontaneous reduction by quinol interferes with the assay. We have been able to extend kinetic measurements down to 50 nm PC by substituting equine cytochrome c for PC as terminal electron acceptor and relegating PC to a strictly catalytic role, so aiding the determination of kinetic parameters for the component rates of the overall reaction (Table II). The second order rate constant deduced for rate-limiting PC levels was found to be 1.7 × 10$^7$ M$^{-1}$ s$^{-1}$ independent of pH, in good agreement with the values of 2.6 × 10$^7$ M$^{-1}$ s$^{-1}$ determined in sonicated pea thylakoids (7) and 1.8 × 10$^7$ M$^{-1}$ s$^{-1}$ using ascorbate-reduced purified cytochrome f (27). This close agreement with values determined for the complex described here indicates that the isolated b–f complex is fully competent as a plastocyanin reductase and shows that the equine cytochrome c-linked assay is reliable.

The maximal rate of oxidoreductase activity (i.e. the turnover number) of the complex is, however, much lower than the value reported for digitonin extracts (4) (2.5 s$^{-1}$ versus 70 s$^{-1}$, respectively). Low maximal rates were also reported for the b–f complex isolated by Hurt and Hauska (10, 14) (after correction for the different pH and ionic strength they employed). The low K$_m$ values observed for PQH$_2$:1 and for PC (Table II) indicate that interactions between the complex and these substrates are not impaired. Conceivably, the low overall activity of the isolated complex might result from disrupted electron flow within the complex, though any such damage must occur at an early stage in the purification, since a depressed rate is observed even in the initial extract (Table

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*3 R. D. Clark and G. Hind, manuscript in preparation.*

*3 R. D. Clark and G. Hind, unpublished data.*
It is noteworthy that the $b$-$f$ complex described here migrates much further into a sucrose density gradient than does complex isolated as in Ref. (3). One explanation for this is that the complex may be a dimer in situ and remain associated under our isolation conditions. Such dimerization has been proposed to be important in the functioning of the complex isolated as in Ref. (12). Low turnover may also arise from failure to provide conditions appropriate for "natural" turnover of cytochrome $b$-563 in a full Q-cycle (2, 9).

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Additions and Corrections

Vol. 258 (1983) 10348–10354

Isolation of a five-polypeptide cytochrome b-f complex from spinach chloroplasts.

Robert D. Clark and Geoffrey Hind

Page 10350, right column, line 12 from the bottom:

1.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \text{ should be } 1.7 \times 10^7 \text{ M}^{-1} \text{s}^{-1}.

Page 10351, Table II, last column, the first three entries under M$^{-1}$ s$^{-1}$:

10^8 \text{ should be } 10^7.

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