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PURIFICATION OF CYTOCHROME b_6 A TIGHTLY BOUND PROTEIN IN CHLOROPLAST MEMBRANES

ALLAN L. STUART* and AARON R. WASSERMAN

Department of Biochemistry, McGill University, Montreal, P.Q. (Canada)

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SUMMARY

Purification of cytochrome b_6 was pursued to further develop rational technology for purification, proof of purity, and study of properties of membrane proteins. Cytochrome b_6 was purified —the first time from any source— from spinach chloroplast membranes; yield of pure cytochrome b_6 was 30% of that found in ethanol-extracted particles. The three-step procedure (pH 8) employed: (I) extraction in Triton X-100–4 M (optionally 2 M) urea, (II) chromatography in a Bio-Gel A-1.5m Column (Triton X-100–4 M urea). Without this step, subsequent electrophoresis failed. (III) Preparative disc gel electrophoresis.

Properties of cytochrome b_6: Cytochrome b_6 migrated in undenatured form as a single band in disc electrophoresis (pH 8, 7 or 8.9). None of the limited, accepted properties of the cytochrome in particles was altered by the purification procedure: Reduced b_6 has absorption maxima (22 °C) at 434, 536, and 563 nm; at −199 °C the α absorption region shows two peaks of equal intensity at 561 and 557 nm. Cytochrome b_6 is reduced by dithionite (not by ascorbate) and is autoxidizable. The prosthetic group of b_6 is protohaemin and is fully extractable by acid–acetone. No non-haem iron is present. The millimolar extinction coefficient of reduced b_6 (563 − 600nm) per mole of haem is 21. The protein equivalent weight is 40000 g per mole of haem. Cytochrome b_4 is an intrinsically aggregatable molecule. The reduced cytochrome does not react with CO except when Triton X-100 is present.

INTRODUCTION

The purification of tightly bound membrane proteins with retention of their known properties has long hindered and challenged many areas of Biology. The chloroplast grana of higher plants contain three cytochromes —f (a peak at 554 nm), b_{559} (α peak at 559 nm) and b_6 (α peak at 562) — all tightly bound membrane proteins which are not released by extraction with salt solution or by prior delipidation of the membrane. Consequently, purification of the chloroplast cytochromes was pursued with the hope that technology might gradually developed which was applicable generally to the purification, proof of purity, and study of membranes proteins. Purification of cytochrome f (ref. 1) and cytochrome b_{559} (ref. 2) with evidence for

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purity was recently reported from this laboratory though the development of new methods, some of which may be generally applicable to the purification of membrane proteins. However, both procedures relied on an empirical intermediate purification step of binding most of the protein impurities to DEAE-cellulose. Purification of cytochrome b_6 was therefore undertaken with the hope of devising a different, more generally useful intermediate purification step.

The 563-nm peak of cytochrome b_6 was observed in chloroplast by Davenport[3] in 1952; it was designated as b_6 and further examined by Hill[4] in 1954. No success was subsequently reported for nearly 20 years in either the purification of cytochrome b_6 or even its extraction in stable from form particles.

This paper reports the complete purification of cytochrome b_6 and some of its properties. The initial step of the procedure (extraction using aqueous Triton X-100–4 M (or 2 M) urea, pH 8) and the final step (disc electrophoresis in aqueous Triton X-100, pH 8) were derived with some modification from the cytochrome b_{559} and *f* procedures from this laboratory[2,1]. An innovation and critical step in the b_6 procedure is the successful use of a Bio-Gel Z-1.5m column containing Triton–4 M urea as an effective preliminary chromatographic step which enables the following electrophoretic step to succeed.

METHODS

"Total chloroplast particle fraction" was prepared from spinach leaves and stored frozen (−15 °C) in physiologically active form as previously described[1]. Washed "ethanol-extracted particles" were then prepared from thawed suspensions of "total chloroplast particle fraction"[2].

Disc gel electrophoresis with non-ionic detergent was performed as described earlier[1] except that the resolving gel buffer was 0.38 M Tris–HCl, pH 8, rather than 8.9 to increase cytochrome stability; other changes are noted, where appropriate, under Results. Location of protein bands in gels by staining and spectrophotometric location of cytochrome in gel slices were also described previously[1]. Content of cytochrome b_6 (using its α maximum at 563 nm) was assayed in ethanol-extracted particles and during purification by difference spectrophotometry (reduced with dithionite *vs.* ascorbate). (Ascorbate reduces cytochromes *f* and b_{559} but not cytochrome b_4; dithionite reduces all three cytochromes.) Absorption spectra were recorded during purification using a Phoenix–Chance scanning spectrophotometer. The response of the instrument was linear (calibrated with solutions of horse-heart cytochrome *c*) down to its detection limit of about 0.0002 absorbance unit (1-cm cell). Accuracy of the Phoenix–Chance absorbance values was checked by cross-calibration *via* cytochrome *c* with a Cary Model 15 recording spectrophotometer and Beckman DU spectrophotometer. Absorbance spectra at 22 °C of pure cytochrome b_6 were recorded on the Cary model 15 spectrophotometer; spectra at the temperature of liquid nitrogen (−199 °C) were recorded with the Phoenix–Chance instrument equipped with low-temperature accessory (optical Dewar flask, 2-mm cells). One (absorbance) unit of cytochrome b_6 is defined as that amount of reduced protein which when dissolved in 1 ml gives an absorbance at 22 °C of 1 (1-cm light path) at its α maximum at 563 nm relative to the absorbance at 600 nm.

Haem content was assayed as the reduced pyridine haemochromogen by the

method of Appleby[5]. Acid–acetone extraction of the haem of cytochrome b_6 was performed (and assayed as the reduced pyridine haemochromogen[5]) by the method of Basford *et al.*[6]. Biuret protein determinations were performed by the method of Gornall *et al.*[7] with crystalline bovine serum albumin as standard; the biuret color of pure cytochrome b_6 samples was corrected for haem contribution by an alkaline control containing cytochrome b_6. Total iron was analyzed by the method of Doeg and Ziegler[8] with pure iron wire as standard. Chlorophyll content of particles was determined by the method of Arnon[9].

Bio-Gel A-1.5m, a non-ionic Agarose Gel, was purchased from Bio-Rad Laboratories, Rockville Centre, N.Y. Sephadex G-25 and G-200 were purchased from Pharmacia Canada Ltd (Montreal). Diaflo membranes (used in a Diaflo Ultrafiltration Pressure-concentrating Cell, under N_2) were from Amicon Corp., Lexington, Mass. Millipore filters were products of Millipore Corporation, Bedford, Mass.

Sonic disturption: The instrument, manufactured by Blackstone Ultrasonics, Inc., Sheffield, Pa. consisted of probe model BP2 with 5/8-inch probe tip and generator model SS-2 capable of maximum power output of 200 W. During sonic disruption of suspensions, it was set to position 40, out of a possible range of 100, and tuned to maximum pitch. Further details are given in Step 1 of the purification procedure in Results.

RESULTS

Experiments to develop the purification procedure for cytochrome b_6

Quantitative (90–100%) extraction of the cytochrome b_6 content of ethanol-extracted chloroplast particles into an optically clear aqueous dispersion was accomplished by sonication at 0 °C in a medium containing 2% Triton–4 M urea–0.05 M Tris–HCl buffer, pH 8. If the medium was not supplemented with sulfhydryl reagents such as 2 mM dithiothreitol or 10 mM cysteine, b_6 was stable at 0 °C for at least 2–3 days. Using the approach developed in this laboratory[1], the optically clear dispersion of cytochrome b_6 in Triton–urea was applied to an analytical disc-electrophoretic 5 or 7% resolving gel (with or without a 4% stacking gel) with Triton X-100 as supplement in all media. A series of experiments was performed in which the Triton content in disc electrophoresis was varied from a low of 0.1% up to a high concentration of 2%. In all cases, disc electrophoresis failed: the extract did not stack properly and cytochrome b_6 did not penetrate the resolving gel, nor was b_6 adequately separated from other proteins present. It was, thus, uncertain whether the original extract contained cytochrome b_6 as separate molecules or as polydispersed aggregates, perhaps bound to other proteins. It was consistently observed that a brown-protein material formed a layer at the top of the first gel in its path. Operationally, a "trouble fraction" was hypothesized, *i.e.* some protein fraction other than the chloroplast cytochromes which prevented proper electrophoretic resolution in disc electrophoresis containing Triton X-100. In work in our laboratory on cytochrome b_{559} the same phenomenon had previously been observed during the purification of b_{559}: an intermediate complete binding in Triton–urea medium (pH 8) of the brown-protein fraction to DEAE-cellulose was required to allow subsequent proper electrophoresis of b_{559}. It was thus probable that more than a reduction of the total protein content of the original extract had to be accomplished; rather,

complete removal of the brown-protein fraction ("trouble fraction") was required. A variety of attempts were made to purify cytochrome b_6 (in either Triton–urea medium or in 2% Triton alone). Two criteria were applied: (1) at least 2 fold purification and (2) subsequent proper resolution of cytochrome b_6 in analytical disc gel electrophoresis containing 1% Triton X-100. $(NH_4)_2SO_4$ fractionation failed completely; both b_6 and the most of protein in the extract precipitated together between 15 and 20% saturation of $(NH_4)_2SO_4$ at pH 8. Purification *via* fractionation with sequential of calcium phosphate gel also failed. Unlike cytochrome *f* or cytochrome b_{559}[2] which pass through a DEAE-cellulose column in Triton-urea-Tris medium (pH 8), cytochrome b_6 was bound to DEAE-cellulose together with most of the contaminating protein, including all of the brown-protein fraction. Recovery of b_6 from the column by supplementing the eluting medium with NaCl at a fixed concentration (ranging from 0 to 2 M in separate experiments) produced poor recovery and negligible purification. Attempts were then made to bind "trouble fraction" but not b_6 on DEAE-cellulose by increasing the ionic strengh of the applied sample before binding from a low of 0.025 M to a high of 0.125 M in separate column experiments. No purification was achieved, although recovery of b_6 was quantitative with the extracts applied in 0.125 M ionic strength.

On the further assumption that cytochrome b_6 migth exist as separate molecules in the original extraction medium (2% Triton–4 M urea–0.05 M Tris–HCl buffer, pH 8), *i.e.* not bound to other proteins, and that the brown "trouble fraction" might merely be clogging the top surface of polyacrylamide gels, purification experiments were pursued using molecular-sieving materials. Both b_6 and the total protein content (including "trouble fraction") were quantitatively retained by the Diaflo membranes XM50 (50000-dalton exclusion limit), XM100 (100000 daltons), XM300 (300000 daltons), and Millipore filters of 0.05-μm and 0.10-μm porosity. Both cytochrome b_6 and "trouble fraction" quantitatively passed through the more porous 0.22- and 0.45-μm Millipore filters. In a column of Sephadex G-200, containing Triton–urea–Tris medium, b_6 was slightly retarded but the procedure achieved no effective separation of b_6 from the excluded brown-protein fraction. (The exclusion limit for proteins on Sephadex G-200 in aqueous medium is 800000 daltons; the exclusion limit of Sephadex G-200 for proteins in aqueous Triton–urea medium is not known.) All of the above sieving materials failed, but the slight retardation of b_6 on Sephadex G-200 suggested that a molecular-sieving material of higher exclusion limit might suffice. Accordingly, purification of the extract was attempted on a column of Bio-Gel A-1.5m in the same medium. (Bio-Gel A-1.5, has an exclusive limit of $1.5 \cdot 10^6$ daltons; the exclusion limit for proteins in Triton–urea is not known.) The brown-protein fraction was completely excluded and completely separated from the retarded single cytochrome b_6 and single cytochrome *f* fractions. The brown-protein fraction represented about 30–50% of the applied protein but contained no cytochromes. 70–80% of the cytochrome b_6 applied to the column was routinely recovered with purification, on a Biuret-protein basis, of 18-fold. The cytochrome b_4 fraction when applied in Triton–urea medium was then found to stack and separate satisfactorily in analytical disc gel electrophoresis containing Triton X-100; no urea supplements were required for the gel media and electrode buffer. Optimum conditions for efficient electrophoretic purification of cytochrome b_6 were then obtained by controlled experiments in which individual parameters of disc-gel electrophoresis were varied, followed

TABLE I

PURIFICATION OF CYTOCHROME b_6

Fraction	*Color*	*Total volume (ml)*	*Total units* of cytochrome b_6*	*Total protein (mg)*	*Specific content (units/mg protein)*	*Recovery of cytochrome b_6 (%)*	*Purification (-fold)*
1. Unextracted green particles**	Dark green	375	15.3***	6285	0.0024	100	–
2. Ethanol-extracted particles in Triton X-100–4 M urea	Brown	110	15.3	4230	0.0036	100	1.5
3. Concentrated 100000 × *g* supernatant	Dark brownish-green	25	14.0	1555	0.0090	93	3.8
4. Eluent from Bio-Gel A-1.5 m column	Light green	88	9.8	61.3	0.16	65*	67
5. Polyacrylamide gel electrophoresis eluent after concentration	Orange	10	4.6 (4.9)*	9.4	0.49 (0.52)*	30**	200

* Determined by difference spectra (see Methods).

** These particles contained 750 mg of chlorophyll.

*** Determined from ethanol-extracted particles assuming no loss during extraction. Cytochrome content in green particles could not be reliably measured because of optical limitations.

§ 70–80% of the units applied to the column were routinely recovered.

§§ Recovery after electrophoresis was routinely at least 80% of the applied units. Concentrating the eluents after steps 4 and 5 by dry Sephadex G-25 contributed the bulk of the loss in this case, although each concentration can incur, with greater care, less than 10% loss.

§§§ Numbers in parentheses employ units of cytochrome b_6 from absolute spectra (563–600 nm).

by spectrophotometric assay of the resolved b_6 band and by inspection of the stain pattern of resolved protein bands. These optimum conditions are presented in Step 3 of the purification procedure for cytochrome b_6 in the following section.

Purification of cytochrome b_6

All steps were performed at 0–4 °C. The procedure, as described, is for a preparation using chloroplast particles originally containing about 750 mg of chlorophyll and about 6300 mg of protein. A typical preparation is summarized in Table I.

Step 1— extraction of cytochromes. A pellet of washed "ethanol-extracted particles" containing 0.05 M Tris–HCl buffer, pH 8, was resuspended by hand homogenization in about 10 pellet volumes (100 ml) of 2% Triton X-100, 4 M urea, 0.05 M Tris–HCl buffer, pH 8. The Triton to protein ratio (mg/mg) was at least 0.4. Final volume was usually 110–115 ml. The suspension was sonicated for 2 min total time (four 30-s sonication periods in ice interspersed with 1-min cooling intervals) and then centrifuged twice, each time at $27000 \times g$ for 15 min. The supernatant was further centrifuged, this time at $100000 \times g$ for 60 min, to remove non-cytochrome components. After spectrophotometric assay for content of cytochrome b_6 and cytochromes b_{559} and f, the extract was concentrated twice with dry Sephadex G-25 to reduce the volume to about 25 ml. Recovery of b_6 after extraction and concentration with Sephadex was essentially complete.

Step 2— partial purification by chromatography on a Bio-Gel A-1.5m column containing Triton–4 M urea. A 5.0 cm × 84 cm column of Bio-Gel A-1.5m (200–400 mesh) was prepared and equilibrated with the extraction medium (2% Triton X-100, 4M urea, 0.05 M Tris–HCl, pH 8). The 25-ml extract from Step 1 was applied and chromatographed using the same extraction medium in a descending direction at a flow rate of 60 ml/h. Large size, non-cytochrome components —which otherwise severely interfered with purification in Step 3 (disc electrophoresis)— were effectively removed as an excluded front fraction. Cytochrome b_6 moved as a single retarded fraction (peak at 924 ml *vs* V_0 for the column of 528 ml $-K_{AV} = 0.36$) which was virtually completely separated from cytochrome f ($K_{AV} = 0.59$). (Due to the lack of thiols, which stabilize b_{559}[2] but inactivate b_6, no b_{559} is observed in the eluted fractions.) The tubes containing cytochrome b_6 were then pooled, supplemented to 10% glycerol and then concentrated once or twice with dry Sephadex G-25 to achieve a volume of about 15 ml.

Step 3— Final purification via preparative disc-gel electrophoresis (0.5% Triton X-100). A Kontes Glass Co. Preparative Electrophoresis Apparatus (250-ml chamber) was used. Electrode buffer was Tris–glycine, pH 8.3 (0.005 M Tris base, 0.039 M glycine) *plus* 0.5% Triton X-100. The running gel (9%, 100 ml volume) contained 10% glycerol, 0.5% Triton X-100 and 0.38 M Tris–HCl buffer, pH 8.0. Prewashing of the gel with electrode buffer was performed for 2 h at 20 mA, 300 V prior to applying the sample. (Note 1: without prewashing, the b_6 recovery was reduced and significant of a second (artifactual) faster-moving b_6 band were produced. Note 2: because of the prewashing, the usual pH 6.7 stacking gel was necessarily omitted.) The 15-ml sample, greenish brown in color, was then applied (without supplementation with sucrose) and electrophoresis at 20 mA, 300 V was performed for a total time of

about 40 h. After the cytochrome had penetrated the gel (about 12–16 h) the electrophoresis was briefly stopped and a chlorophyll layer (light green) remaining above the gel was carefully pipetted off; electrophoresis was then resumed until the sharp, orange b_6 band had penetrated 1 cm into the gel. After cutting out the orange b_6 band, the gel slice was fragmented in a hand homogenizer together with about 40 ml of 0.05 M Tris–HCl, pH 8, 10% glycerol (no Triton X-100). The suspension was diluted with additional amounts of this medium so that the total amount of fluid added was about 10 times (about 60 ml) that of the original volume of the gel slice. The gel suspension was stirred about 3 h, then centrifuged ($27000 \times g$, 15 min), and the supernatant was concentrated with dry Sephadex G-25. The concentrated b_6 solution contained about 0.3% Triton X-100, estimated from the volume of the gel slice and its Triton concentration of 0.5%, from the known dilution factor during extraction from the slice, and from the known -fold concentration after treatment with dry Sephadex G-25. (Triton X-100 micelles are quantitatively concentrated together wit protein by dry Sephadex G-25; unpublished studies in this laboratory.) Overall yield of pure b_6 was 30% of that present in ethanol-extracted particles.

Stability

Cytochrome b_6 was stable for at least 3 months when stored frozen (−15 °C) in 0.05 M Tris-HCl buffer, pH 8, containing 10% glycerol or stable for at least 7 days in liquid solution at 0 °C. In contrast with cytochrome b_{559}, whose stability is best preserved by thiols such a 1–5 mM dithiothreitol, and with cytochrome *f*, whose stability is enhanced by thiols, cytochrome b_6 (otherwise a relatively stable molecule) is slowly inactivated by 1–5 mM dithiothreitol even at 0 °C. (By inactivation we mean the abolition of the b_6 spectrum; no new absorption peaks are observed.)

Proof of purity

Analytical disc gel electrophoresis (pH 8) of b_6 in undenatured, non-aggregated form in medium containing Triton X-100 showed no contamination by either cytochromes *f* or b_{559} or any other protein (*cf.* Fig. 1 and its legend). Additional

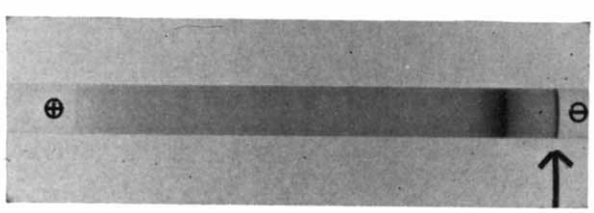

Fig. 1. Analytical disc gel electrophoresis (18 h) of pure cytochrome b_6. No stacking gel; 9% running (pH 8) prerun for 1 h with electrode buffer to wash gel. Electrode buffer was 5 mM Tris base–0.039 M glycine (pH 8.3) *plus* 1% Triton X-100; 9% gel medium was 0.38 M Tris–HCl (pH 8) *plus* 2% Triton X-100, 10% glycerol. (When the applied sample was directly after the purification procedure, an estimated concentration of Triton X-100 of 0.3% was already present. When the applied sample had previously been dialyzed seven days against Tris buffer, it was supplemented to about 0.5% Triton X-100.) Prior to staining the band was visibly orange colored before reduction and gave a typical b_6 spectrum. No protein or cytochrome was seen in the front fraction or anywhere else in the gel. The arrow indicates the top of the gel, where no protein stain was visible.

disc gel electrophoretic experiments performed either at pH 7.0 or 8.9 also showed no impurities. Instability of b_6 in acetate buffer (pH 5.5) precluded a purity test at pH 5.5. The absorption spectrum of reduced cytochrome b_6 (22 °C) also shows

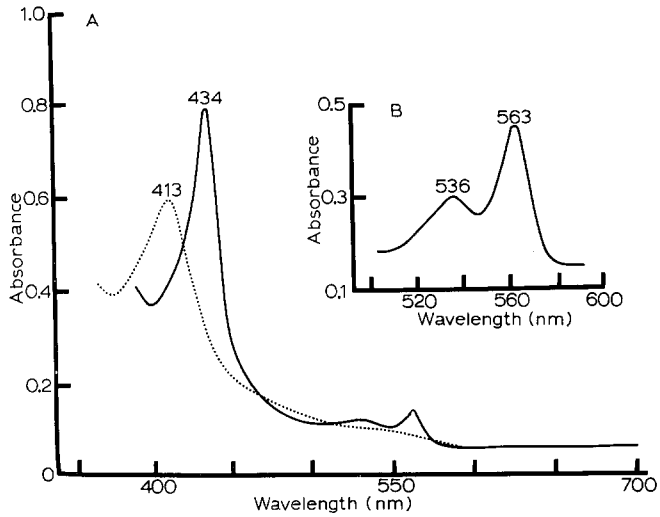

Fig. 2. Absolute reduced (dithionite) (solid line) and absolute oxidized (untreated) absorbance spectra of pure cytochrome b_6 (22 °C). For insert (B), the sample aliquot was 4 times the concentration of that in A, so as to reveal more detail. Solution medium was 0.05 M Tris–HCl, pH 8.

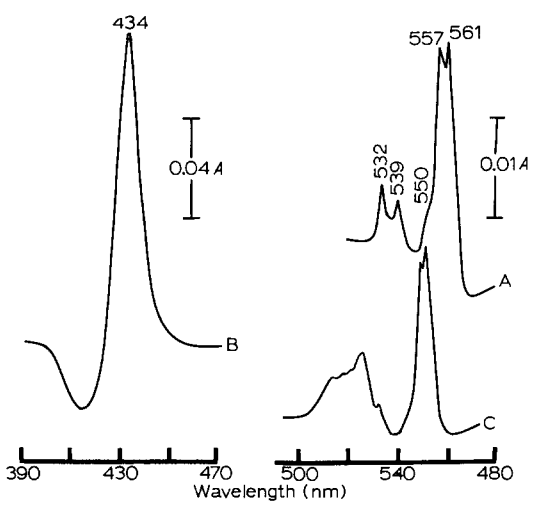

Fig. 3. Absorbance difference spectrum (reduced (dithionite) *vs.* oxidized (untreated)] of pure cytochrome b_6 at the temperature of liquid nitrogen (−199 °C). A and B. Cytochrome b_6. Magnification in A is 4 times that in B. The concentration of the sample was such that it gave an absorbance of 0.040 (563–600 nm) in a 1-cm cell at 22 °C. Dialysis for 5 days removed sufficient Triton X-100 to allow optical measurements at −199 °C; b_6 was stable during this dialysis. C. Horse heart ferrocytochrome *c* spectrum (−199 °C) as reference to demonstrate validity of the technique as performed. Medium in A, B and C was 0.05 M Tris–HCl, pH 8, 50% glycerol. The slit width must be as narrow as possible for good optical resolution.

no contamination by cytochrome *f* and b_{559} (Fig. 2). Furthermore, a difference spectrum (not shown) of ascorbate *vs.* ferricyanide (oxidized) at −199 °C, which would normally reveal any native b_{559} or *f* present (but not b_6), in fact showed no absorption peaks. The absorption difference spectrum of b_6) [reduced by dithionite *vs* oxidized (untreated)] recorded at −199 °C is presented in Fig. 3. Two peaks (561 nm, 557 nm) are seen in the α absorption region. If the peak at 557 nm were contributed by a small contaminating of cytochrome b_{559}, additional peaks of $b_{559}{}^2$ would be expected at 513, 529, and 536 nm (β region) and especially the intense

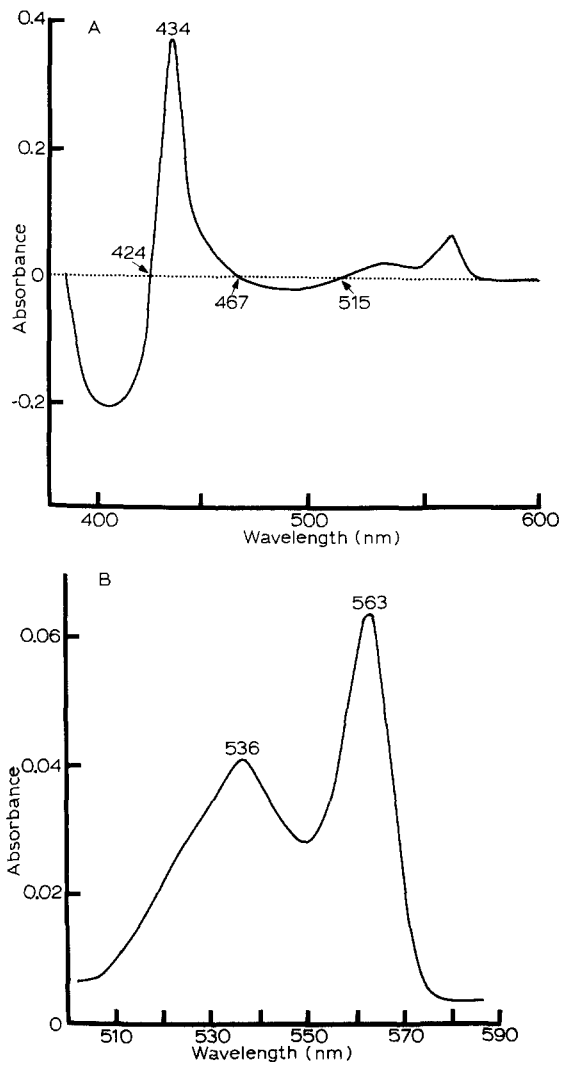

Fig. 4. Difference spectrum at 22 °C of pure cytochrome b_6 (reduced (with dithionite) *minus* oxidized (untreated)). The spectrum in B was recorded at 10 times the magnification of that in A to show the α and β peaks in greater detail. Solution medium was 0.05 M Tris–HCl buffer, pH 8.

Soret peak of b_{559} at 429 nm. The absence of the latter b_{559} peaks would suggest that, at −199 °C, both the peak at 561 nm and that at 557 nm are contributed by b_6 itself.

Absorption spectra

Absolute reduced and absolute oxidized spectra (22 °C) of b_6 are presented in Fig. 2. (The absorption spectra were unaffected by Triton X-100.) The low-temperature difference spectrum (Fig. 3) indicates that b_6 exhibits two peaks at −199 °C in the α region, 561 and 557 nm. This supports the tentative suggestion made by Boardman and Anderson[10] on b_6 in chloroplast particles. The absorption difference spectrum of b_6 (22 °C) is presented in Fig. 4.

Like b_6 in chloroplast particles, purified b_6 is autoxidizable and is reduced by dithionite, but not by ascorbate. (Either ascorbate or dithionite can reduce cytochrome f and cytochrome b_{559}.) No time lag was observed during reduction by dithionite. Reduction was complete within the time required to record the spectra at 22 °C (*i.e.* about 30 s to record the α and β peaks).

Haem prosthetic group, millimolar extinction coefficient, protein equivalent weight, iron content

The absorption spectrum of the reduced pyridine haemochromogen of pure b_6 was identical with that of protohaemin, showing maxima at 556, 526 and 423.5 nm. The haem of b_6 was quantitatively extracted by acid–acetone with recovery of 95% of the haem.

Using the coefficient of the reduced pyridine haemochromogen of $\Delta\varepsilon_{mM}=23.4$ (556 − 539 nm)[5] the millimolar extinction coefficient of cytochrome b_6 was determined as $\Delta\varepsilon_{mM}=21$ (563 − 600nm) per mole of haem. Cytochrome b_6 contained 47.(5) nmoles of haem per A unit and 40000 g of Biuret protein per mole of haem. $\Delta A_{563-600\,nm}$ per mg Biuret protein was 0.52. Total iron content of cytochrome b_6 was 47 (±4%) nmoles of iron per A unit. The equivalence of total iron and haem iron indicates that b_6 contains no significant amount of non-haem iron.

Quantitative (reversible) reaggregation upon depletion of Triton X-100

The intrinsic reaggregatability of cytochrome b_6 was examined by the disc-electrophoretic approach used in this laboratory[1,11]. Pure b_6 containing initially less than 0.3% Triton X-100 was dialyzed against 0.05 M Tris–HCl buffer, pH 8, for an aribitrary dialysis time of seven days with daily changes of the Tris buffer. The cytochrome was quantitatively stable and soluble during this dialysis period. When the dialyzed sample was examined by analytical disc gel electrophoresis (pH 8) using a washed 9% gel (*cf.* legend of Fig. 1 for details) but without Triton X-100 either in the sample or in the electrophoretic system, the cytochrome was quantitatively reaggregated in that none of it was able to penetrate the 9% running gel. When the dialyzed sample was supplemented with 0.5% Triton X-100 and examined by disc gel electrophoresis (pH 8) with 0.5% Triton X-100 in the system, the entire cytochrome b_6 sample dissociated and migrated in the 9% gel as a single electrophoretic band which exhibited the usual b_6 absorption spectrum. Cytochrome b_6 was also non-destructively dissociated by Triton X-100 at pH 7 and pH 8.9.

Non-reaction of cytochrome b_6 with CO after depletion of Triton X-100

Samples of pure cytochrome b_6 — sufficiently dialyzed (as in the above section) so that quantitative reaggregation was observed — showed no reaction with CO. As shown by Hill[4], native cytochrome b_6 in chloroplast particles also showed no CO reaction. However, when the pure sample was dissociated by the addition of 0.5% Triton X-100 (final concentration) complete reaction with CO occurred as shown spectrophotometrically in Fig. 5. The minimum concentration of Triton X-100 needed

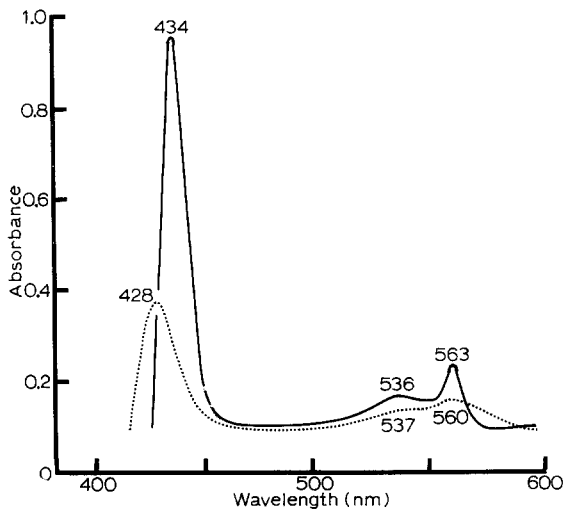

Fig. 5. The effect of CO on the difference spectrum (reduced (with dithionite) *versus* oxidized (untreated)) of pure cytochrome b_6 (22 °C) in the absence and presence of 0.5% Triton X-100. The sample had been dialyzed against 0.05 M Tris–HCl buffer, pH 8, for 7 days to deplete its concentration of Triton X-100. The sample was stable and soluble during this dialysis. After reduction with dithionite, CO was bubbled through the reduced sample for 3 min and the spectrum then recorded. ——, no Triton X-100 added; spectrum is unaltered by CO. · · · ·, 0.5% Triton X-100 (final concentration) added, after bubbling with CO, and the spectrum was recorded 1 min later. Calculated aborbance ratios are $A_{563\text{ nm}}/A_{560\text{ nm}} = 1.9$; $A_{434\text{ nm}}/A_{428\text{ nm}} = 3.2$, thus indicating a drop in absorbance upon reaction with CO. (Addition of Triton X-100 alone (no CO), not shown, had no effect on the normal b_6 spectrum.)

to induce CO reactivity is not known but is probably considerably less than 0.5%. (The results of this section do not establish or imply that monomeric, *i.e.* dissociated cytochrome b_6 is intrinsically CO reactive, except of course in the presence of Triton; nor, conversely, can one infer that the cytochrome in the chloroplast is necessarily in aggregated form.)

DISCUSSION

The purification procedure suffers none of the problems usually encountered in the extraction and purification of most "hydrophobic", tightly bound membrane proteins: (1) Extraction of b_6 was easily accomplished and was complete, and no alteration of the cytochrome's accepted properties was observed. Further, if Bio-Gel A-1.5m is indeed an inert molecular-sieving material, then b_6 was in molecular

form, not bound to protein contaminants, in the original extraction medium of Triton–4 M urea, pH 8. (2) The cytochrome moved as a single fraction either in the Bio-Gel chromatography or in the more-discriminating method of disc electrophoresis. Multiple fractions, multiple forms or streaking of b_6 was not encountered. A second substantial band of b_6 (exhibiting the same absorption spectrum as the normal band) was an artifact not an "isozyme" whose formation was avoided by "pre-washing" the disc-electrophoretic resolving gel. (3) The purification steps were few and essentially quantitative (70–80% recovery, routinely, in the Bio-Gel step and at least 80% recovery, routinely, from preparative disc electrophoresis). (4) As in the work in our laboratory on cytochrome *f*, we have had no success in effectively chromatographing cytochrome b_6 on the ion exchanger DEAE-cellulose (or on any other charged support medium). This difficulty may possibly be attributable to aggregation of b_6 as well as of other proteins present induced by charges in the ion exchanger and/or in the eluting salt solution. To our knowledge, the Bio-Gel step is the first demonstration of chromatography of a desired membrane protein in undenatured form in a crude extract. (5) Purification of cytochrome f[1] in this laboratory demonstrated that disc electrophoresis in non-ionic detergent could serve in effect as a much-needed chromatographic method for a membrane protein both as a final preparative step and as a subtle method for "proof of purity". The procedures for all three chloroplast cytochromes have now successfully employed these methods, thus validating their general promise for other membrane proteins.

The experiments in the first section of Results indicate that removal of a "trouble fraction" from the initial Triton–4 M urea dispersion was required to allow cytochrome b_6 to penetrate disc electrophoretic gels. Only chromatography by molecular sieving in Bio-Gel A-1.5m (Triton–urea) succeeded in removing this apparently large molecular weight fraction despite a variety of attempts by other methods. Since cytochrome b_6 is small enough to penetrate a 9% electrophoretic gel, the requirement for sieving out the "trouble fraction" *via* a large-pore Bio-Gel A-1.5m sieving material (rather than *via* Sephadex G-200) remains at present an enigma. The Bio-Gel procedure and the concept of a "trouble fraction" (or disc-gel plugging fraction) may prove useful for purification of other membrane proteins.

Bendall[12] in a brief report proposed that chloroplasts contained a second b_6 component with an absorption peak at 559 nm (presumably at room temperature) together with the usual 563-nm (b_6) component. (Both were reduced by dithionite Although no definitive comment can yet be made by us, the following observations may be of interest: (1) We have never observed any b_6 component either in particles or soluble extracts other than the conventional b_6 component at 563 nm. (2) Assay of conventional, ascorbate-reducible b_{559} in solvent-extracted chloroplasts has not been reliable (in our laboratory) except in Triton–urea medium, pH 8. Solvent-extracted particles in suspension with or without Triton X-100 (but lacking 4 M or 2 M urea) are not always fully permeable to ascorbate. (Dithionite appears to encounter no permeability barrier.) The same particles in Triton–2 M or 4 M urea — either by suspension in Triton–urea, pH 8, or by adding 2 or 4 M urea during assay to particle suspensions in Triton alone — show their expected full amount of ascorbate-reducible b_{559}. The latter observation also poses a reservation to the proposal of a second, low-potential form of b_{559} in particles (*i.e.* a form not-reducible by ascorbate, *e.g.* see Cox and Bendall[13].)

We have observed two alpha absorption peaks (561, 557 nm) at −199 °C in our cytochrome b_6 preparation. Based on all available evidence, does the 557-nm peak represent an unresolved bound impurity? (1) It is universally accepted that two α peaks at −199 °C can be produced by two different cytochrome molecules whose absorption spectra are otherwise poorly resolved and show considerable asymmetry at 22 °C. However, a single pure cytochrome molecule also can exhibit two major α peaks at −199 °C even though only one α peak is found at 22 °C. One such example is cytochrome *f* which at −199 °C shows both a 551.5 nm peak and a very considerable peak at 548 nm. No one has yet suggested that the latter peak represents a major or minor impurity. Thus, the presence of two α absorption peaks (−199 °C) for a preparation is, by itself, an equivocal criterion for impurity and additional evidence must be examined. (2) No ascorbate-reducible b_{559} was present in the cytochrome b_6 preparation (−199 °C). (3) Pure cytochrome b_{559} has absorbance maxima (22 °C) at 559 nm (α), 530 nm (β) and 429 nm (Soret). At −199 °C it shows absorbance peaks in a difference spectrum at 556 nm (α), 529 (principal β) (as well as minor β peaks at 513 and 536 nm) and an intense Soret Peak at 429 nm. The above preparation of cytochrome b_{559} was completely ascorbate reducible. From additional, more extensive studies on cytochrome b_{559} in this laboratory (Garewal, H. S. and Wasserman, A.R., unpublished) we have no reason to question either the reliability of the above absorption data or whether it is representative (within experimental error) of the cytochrome b_{559} content of chloroplast grana. If the ascorbate-reducible form, unprotected by thiols, is converted artifactually or otherwise to one or more forms of lower potential (*i.e.* forms reducible by dithionite but not by ascorbate), we would expect any such "dithionite-reducible form "either to retain all of the characteristic absorption peaks of cytochrome b_{559} or to have shifts in maxima for all three principal maxima. There is no precedent for any cytochrome retaining the position and sharpness of its α peak while at the same time suffering alteration or elimination of its β and Soret peaks. If all, or even 15% of the height of the 557 nm peak (−199 °C) in Fig. 3 is presumed to be contributed by a "dithionite-reducible form" of b_{559}, we would expect to see the principal beta of b_{559} at 529 nm or at least some shoulder at 529 nm and to see either a peak or shoulder near 429 nm. This, however, is not seen. Further, such contamination by b_{559} would, at 22 °C, either produce a considerable shoulder on the left side of the alpha peak or, for larger contamination, shift the 563 nm peak to a shorter wavelength (perhaps to 560 or 561 nm). Inspection of Figs 2B and 4B shows no such signs of contamination. From currently available data on pure b_{559} as well as the present data on cytochrome b_6, we must therefore conclude that the peaks at 557 and 561 nm (−199 °C) are intrinsic properties of pure cytochrome b_6. A corollary of this conclusion would be that any particulate preparation containing b_6 and presumably free of b_{559} would show both peaks (−199 °C) with almost equal intensity and that particulate preparations containing both b_6 and b_{559} would show greater absorbance at 557 nm than at 561 nm. (4) Using another approach, experiments in progress on the polypeptide subunits of our cytochrome b_6 preparation (Stuart, A. and Wasserman, A. R., unpublished) and of pure cytochrome b_{559} (Garewal, H.S. and Wasserman, A.R., unpublished) show that about 75% of the mass of cytochrome b_6 consists of chains unequivocally absent in cytochrome b_{559}; the remaining 25% of the protein mass of b_6 also appears to consist of a chain not found in b_{559} but this requires further verification.

We have not yet examined the redox potential of pure cytochrome b_6. The redox potential of cytochrome b_6 in particles is in dispute, having been reported variably as -60 mV[4], recalculated later as 0.0 mV[14], -180 (± 20) mV[15] and -100 mV[16].

No acceptable enzyme assay exists for cytochrome b_6; at this time the immediate physiological reductant or oxidant for the cytochrome in photosynthesis is unknown.

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