

Purification and two-dimensional crystallization of highly active cytochrome b_6f complex from spinach

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Abstract The purification and two-dimensional crystallization of highly active cytochrome b_6f complex from spinach is described. The preparation shows all spectroscopic characteristics of the pure complex. The electron transfer activity of 450 ± 60 electrons per s is the highest in vitro activity reported to date. Using dimethyl sulfoxide (DMSO) as a solvent for the electron donor enhanced the performance and reproducibility of the assay. The high yield and the high activity of the protein make it an ideal candidate for biophysical and structural studies. Preliminary two-dimensional crystallization experiments yielded several different forms of two-dimensional and thin three-dimensional crystals, exhibiting varying degrees of order.

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Key words: Cytochrome b_6f ; Purification; Two-dimensional crystal; Spinach

1. Introduction

The cytochrome b_6f complex (plastoquinol:plastocyanin oxidoreductase) mediates the electron transport between photosystem II and photosystem I, via plastoquinol to plastocyanin, or in cyclic electron flow around photosystem I, via ferredoxin to plastocyanin. Electron transport is coupled to a translocation of protons across the thylakoid membrane and contributes to the formation of a proton electrochemical gradient used by the chloroplast ATP synthase to produce ATP. The complex consists of at least seven subunits, four of which have an assigned function. The largest subunit is cytochrome f ($M_w = 32$ kDa) with a covalently bound c -type cytochrome. It contains the docking site of the electron acceptor plastocyanin. The cytochrome b_6 subunit ($M_w = 24$ kDa) binds two b -hemes. The Rieske protein ($M_w = 19$ kDa) contains an iron-sulfur cluster. Subunit IV ($M_w = 17.5$ kDa) does not contain a redox active prosthetic group. The latter three subunits are involved in the binding of the electron donor plastoquinol [1,2]. There are at least two small subunits in spinach cytochrome b_6f with a molecular mass around 4 kDa, each with one transmembrane helix [3,4]. Furthermore, a chlorophyll a molecule is part of the complex. It is probably bound to the cytochrome b_6 polypeptide, but its function is yet unknown

[4–6]. The b_6f complex occupies a central position in the photosynthetic electron transfer chain, equivalent to the related bc_1 complex in the respiratory chain. To understand the function of the complex, a detailed structural model is necessary.

Despite the relatedness of bc_1 and b_6f complexes, there are significant differences with respect to the sequences of homologous subunits, binding of inhibitors, subunit composition and dimer organization, which do not allow for a direct superposition of the crystal structures of the bc_1 complex [7,8] and the b_6f complex. This is confirmed by the 9 Å projection map of the *Chlamydomonas reinhardtii* b_6f complex which looks surprisingly different from a calculated projection map of the bc_1 complex at the same resolution ([9] and C. Breyton, personal communication).

So far, information about the structure of the b_6f complex has come from different sources. Thin three-dimensional crystals and tubular crystals were obtained from spinach b_6f complex [10], but the structural information derived from these crystals was limited. Two projection maps of the protein from the unicellular green alga *C. reinhardtii* provide a view of the two-dimensional structure of the complex [9,11]. High-resolution structures of water-soluble fragments of the cytochrome f and the Rieske subunit have been determined by X-ray crystallography [12,13], but a detailed structure of the whole complex is still missing.

This paper describes the purification of highly active cytochrome b_6f complex from spinach chloroplasts which is well-suited for structural studies. Preliminary two-dimensional crystallization trials yielded a variety of crystal morphologies and a preliminary projection map, showing that the spinach complex is structurally similar but not identical to the *C. reinhardtii* complex.

2. Materials and methods

2.1. Purification

Spinach plants were grown in a growth chamber in hydroculture on Hoagland medium [14]. One batch of cytochrome b_6f complex was isolated from ~1 kg of leaves without stalks. The spinach chloroplasts were prepared as in Black et al. [15] with an additional wash of the homogenized leaves for 30 min at 4°C in a buffer containing 10 mM Tris (pH 8.0) and 10 mM EDTA (pH 8.0). The thylakoid membranes were washed twice with 2 M NaBr and the membranes were resuspended in a buffer containing 40 mM Tricine (pH 8.0), 10 mM $MgCl_2$ and 10 mM KCl at a chlorophyll concentration of 3 mg/ml. The subsequent procedure was adapted from a protocol for purification of cytochrome b_6f complex from *C. reinhardtii* [16]. This procedure makes use of the selective solubilization of the complex by the non-ionic detergent, 6-*O*-(*N*-heptylcarbamoyl)-methyl- α -D-glycopyranoside (Hecameg), which preferentially removes the cytochrome b_6f complex from the thylakoid membranes. The supernatant of the solubilization step was loaded onto a 10–30% sucrose gradient containing 40 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 10 mM KCl,

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Abbreviations: Hecameg, 6-*O*-(*N*-heptylcarbamoyl)-methyl- α -D-glycopyranoside; DDM, dodecyl- β -D-maltoside; PC, 1- α -phosphatidylcholine; DMSO, dimethyl sulfoxide; DOPG, 1- α -dioleoylphosphatidylglycerol

20 mM Hecameg and 0.1 mg/ml egg phosphatidylcholine (egg PC) and centrifuged at $180\,000\times g$ for 16 h. The brownish band containing the b_6f complex was then loaded on a hydroxylapatite column (Bio-Rad), washed with 2.5 column volumes of 125 mM ammoniumphosphate, 20 mM Hecameg. The purified protein fraction was eluted with a buffer including 400 mM ammoniumphosphate, 20 mM Hecameg and the following protease inhibitors: 0.15 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 5 mM amino-caproic acid. All purification steps were performed at 4°C and in the presence of 0.1 mg/ml egg PC.

2.2. Spectroscopy

Absorbance spectra were recorded at 23°C on a Perkin-Elmer Lambda Bio 40 spectrophotometer, using an extinction coefficient for the cytochrome b_6f complex of $\epsilon = 20\text{ mM}^{-1}\text{ cm}^{-1}$ [17]. The buffer contained 0.3 mM dodecyl- β -D-maltoside (DDM) and 20 mM Tricine, pH 7.5. For the redox difference spectra, the cytochromes were reduced with Na-ascorbate (cytochrome f) or dithionite (cytochrome f and b_6) and oxidized with ferricyanide (cytochrome f and cytochrome b_6). After recording the baseline, a grain of the reducing or oxidizing reagent was added to the sample cuvette. The reduction of cytochrome b_6 takes a few minutes, after which a difference spectrum can be recorded.

2.3. In vitro activity

Activity measurements were performed using the spectrophotometer and the buffer described above. It was favorable for spectroscopy and especially for the activity assay to use a buffer containing DDM as detergent, because additional lipid was not required to prevent monomerization of the complex [18]. In the assay, 0.5 nM purified b_6f complex was used, together with 15 μM decylplastoquinol (Sigma) as electron donor and 5 μM spinach plastocyanin as electron acceptor [16]. The reaction was started by addition of the electron donor and monitored for 2 min as decrease of absorbance of plastocyanin at 600 nm, using an extinction coefficient of $\epsilon = 4500\text{ mM}^{-1}\text{ cm}^{-1}$ [19]. The data were plotted as $\ln(\Delta\text{OD})$ against time t (with $\Delta\text{OD} = A_t$ (absorbance at time t) $- A_f$ (final absorbance)). The slope of the corresponding line gives a constant factor k in s^{-1} .

The electron transfer activity is given by:

$$V\left(\frac{\text{mol PC}_{\text{red}}}{\text{s}}\right) = \frac{k \times \Delta\text{OD}}{\epsilon(\text{PC})}$$

The specific activity is:

$$\text{SA} = \frac{V(\text{sample}) - V(\text{without } b_6f)}{\text{mol } b_6f}$$

2.4. Purification of spinach plastocyanin

Plastocyanin was purified by the method described in [20] from spinach bought at a local market.

2.5. Reduction of decylplastoquinone

Decylplastoquinone (Sigma) was reduced as described as in [16].

2.6. Crystallization

The crystallization solution contained 400 mM ammonium phosphate, 2 mM CaCl_2 , 20 mM Hecameg, 1% glycerol, egg PC and dioleoylphosphatidylglycerol (DOPG) in the ratio 1:1 (w/w) and 1 mg/ml purified cytochrome b_6f complex as described for the crystallization of cytochrome b_6f complex from *C. reinhardtii* [11]. The lipid to protein ratio was adjusted for each purification and was in a range of 1:2 to 1:4 (w/w). After setting up crystallization trials, the sample with a final volume of 80 μl was stirred overnight at 4°C. The detergent was then removed by the addition of 10 mg biobeads (SM2 Bio-Beads, Bio-Rad) and stirring for a period of 6 h at 4°C. The turbid supernatant was then transferred to another test tube and left overnight at 4°C. Three freeze-thaw cycles followed: the sample was quickly frozen in liquid nitrogen and then slowly thawed at 4°C. It was then left at 4°C. By this procedure, crystals could be observed after 3 days (Fig. 4a,d and e).

Other crystallization trials were performed in 20 mM Tris pH 8.0, 20 mM Hecameg, 1% glycerol, egg PC and 0.75 mg/ml purified cytochrome b_6f complex at a lipid to protein ratio of 1:2 (w/w). Detergent was removed by 5 mg biobeads at 4°C and crystals were obtained after 6 days (Fig. 4b and c). Prior to use, biobeads were washed in

methanol and stored in water. Egg PC and DOPG were obtained from Avanti Polar-Lipids.

2.7. Electron microscopy and image processing

Samples were negatively stained with 2% uranyl acetate. Images were taken on a Philips CM 12 transmission electron microscope operating at 120 kV in the low dose mode at a magnification of $45\,000\times$. Images were processed using the MRC program suite [21,22].

3. Results and discussion

3.1. Purification

The procedure for purifying cytochrome b_6f complex from plants was adapted from a protocol developed for the isolation of the same complex from the single cell alga *C. reinhardtii* [16]. Growing the spinach in an in-house growth chamber greatly enhanced the reproducibility of the preparation procedure, compared to market spinach. The enzyme was purified from spinach chloroplast membranes in three steps. In the first step, thylakoid membranes were selectively solubilized with the non-ionic detergent Hecameg. For each batch of membranes, it was important to determine the optimal concentration of Hecameg experimentally to ensure a selective solubilization of the b_6f complex. The detergent concentration was generally in the range of 32 mM. In Fig. 1, a comparison of the pellet (lane 1) and the supernatant (lane 2) from the solubilization step shows that the solubilization is indeed selective. The cytochrome f band is the strongest band in the supernatant. In the second step, the supernatant is separated on a 10–30% sucrose gradient and the resulting brownish band contains mainly cytochrome b_6f complex (lane 3). In the final step, this band is loaded onto a hydroxylapatite column which removes contaminants and the protein is eluted as purified b_6f complex (lane 4). In addition to the four major

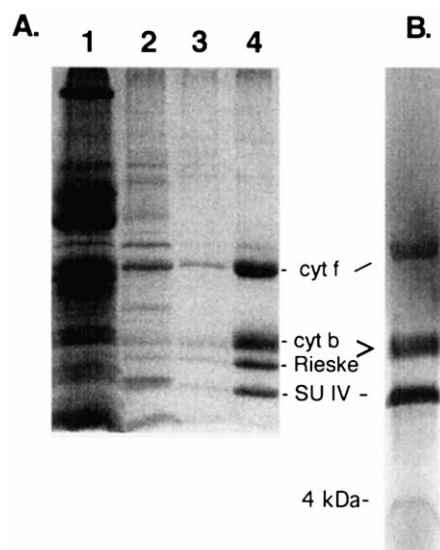


Fig. 1. A: Purification of spinach cytochrome b_6f . Pellet (lane 1) and supernatant (lane 2) after the solubilization of thylakoid membranes with 32.5 mM Hecameg. b_6f -enriched sucrose gradient fraction (lane 3) and hydroxylapatite column eluate (lane 4) on a 12.5% SDS-PAGE. The gel was stained with Coomassie blue. B: Purified b_6f complex with small ~ 4 kDa subunits on a 10–20% gradient SDS-PAGE gel. Under these conditions, the small subunits are resolved, whereas cytochrome b_6 and the Rieske protein appear as one band. The gel was silver-stained.

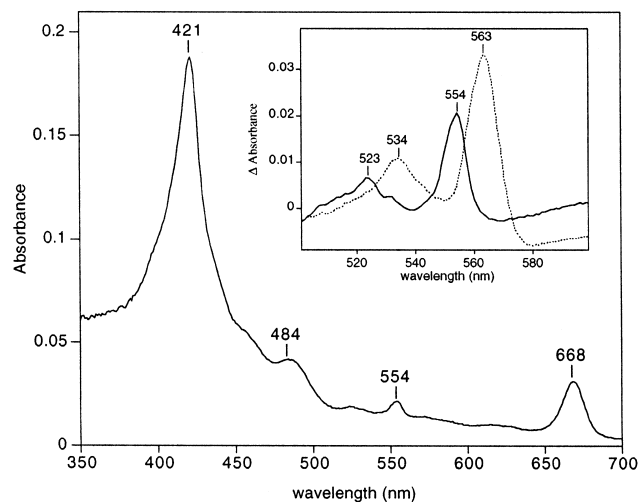


Fig. 2. Absorbance spectra of purified b_6f complex. Ascorbate-reduced UV-visible spectrum of purified b_6f complex. Inset: difference spectra of ascorbate-reduced minus ferricyanide-oxidized (solid line) and dithionite-reduced minus ascorbate-reduced enzyme (dotted line).

polypeptide subunits, the purified complex also contains the 4 kDa subunits which are visible in a 10–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (lane 5). The preparation is largely free of other polypeptides and elutes at a concentration of 5–10 mg/ml. The protein is purified as a dimer. At a higher detergent concentration (> 50 mM), a band of monomeric complex appears during the sucrose gradient centrifugation (data not shown). As the monomeric enzyme tends to lose the Rieske protein and becomes inactive, care was taken to keep the detergent concentration close to the critical micellar concentration in order to obtain fully active, dimeric complex. The overall yield of this preparation is about 20 mg cytochrome b_6f complex per kg of fresh spinach leaves, whereas with *C. reinhardtii*, the yield was ~ 0.3 mg pure complex per l of culture.

3.2. Spectroscopic properties

The absorption spectrum of the purified cytochrome b_6f complex is shown in Fig. 2. At the lower end of the spectrum, the Soret band of the bound pigments (chlorophyll and hemes) can be found at 421 nm. The presence of chlorophyll *a* is indicated by a band at 668 nm. From the spectrum, it can be deduced by comparison of the absorption peaks that there was 1.0 ± 0.14 chlorophyll *a* per cytochrome *f* as shown previously [23]. Redox difference spectra show the presence of cytochrome *f* absorption peaks at 523 and 554 nm and absorption peaks for cytochrome b_6 at 534 and 563 nm (Fig. 2, inset). The calculated ratio of cytochrome b_6 to cytochrome *f* was 1.87 ± 0.2 , in agreement with published data [23]. Altogether, the preparation exhibits all spectroscopic characteristics of the intact cytochrome b_6f complex.

3.3. Activity

To determine the activity of the purified complex, the rate of electron transfer from the synthetic substrate decylplastoquinol (C_{10} -PQH₂) to the natural electron acceptor spinach plastocyanin was measured in vitro. The reaction can be monitored spectroscopically, as oxidized plastocyanin has a maximal absorption at 600 nm, which decreases upon reduc-

tion of the protein [19]. Ethanol was originally used as a solvent for plastoquinol [24], but it was found to interfere with this activity assay and made it less reproducible by inducing an undesirable background reaction. This resulted in an initial increase of absorption followed by a rapid decay, even in the absence of the electron donor, and thus made it difficult to obtain reliable readings for absorption changes. The use of dimethyl sulfoxide (DMSO) as a solvent for the electron donor greatly improved the reproducibility of the assay and no similar background reaction occurred. In six experiments with cytochrome b_6f complex from two different purifications (three experiments each), an electron transport of 450 ± 60 electrons per s was measured using 15 μ M decylplastoquinol and 5 μ M plastocyanin (Fig. 3). This was considerably higher than reported for other preparations. The highest electron transfer rate for a b_6f complex was 270 ± 60 electrons/s for the enzyme from *C. reinhardtii* [16] and 20–35 electrons/s for a spinach preparation [15]. The in vitro assay for our isolated complex shows an even higher turnover number than measured for b_6f in situ in intact chloroplast thylakoids (300 s^{-1}) [25]. This might be due to a better accessibility of the substrates to their protein binding sites in detergent solution. The preparation can be stored at -80°C for several months without loss of activity.

3.4. Crystallization

The aim for optimizing the purification and the activity assay was to have a reproducible high quality and abundant supply of enzyme for crystallization trials. The concentration of the eluted protein is sufficient for two and three-dimensional crystallization, so that a further concentration step is not necessary. As a result, the amount of detergent in the

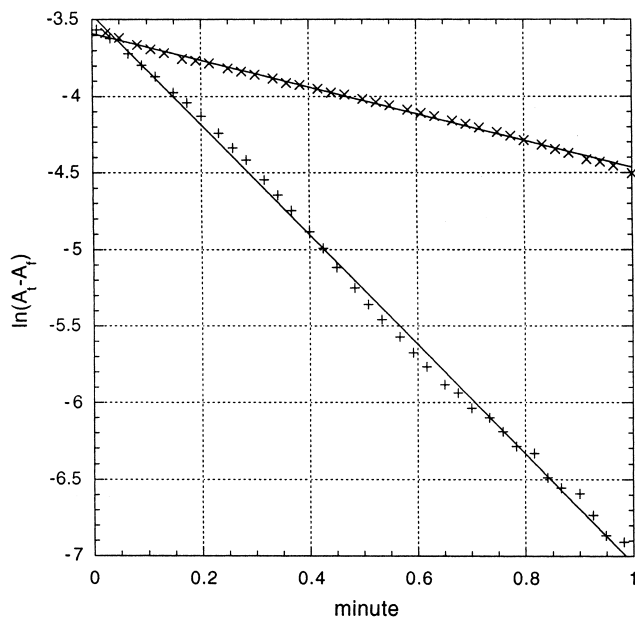


Fig. 3. Electron transfer activity of purified cytochrome b_6f complex. The assay medium contained 0.3 mM DDM, 20 mM Tricine (pH 7.5), 5 μ M plastocyanin and 0.5 nM purified b_6f complex. The reaction was started by the addition of decylplastoquinol (15 μ M) dissolved in DMSO and was detected as decrease of absorbance of plastocyanin at 600 nm (+). The other curve (x) shows the uncatalyzed background reaction without the enzyme.

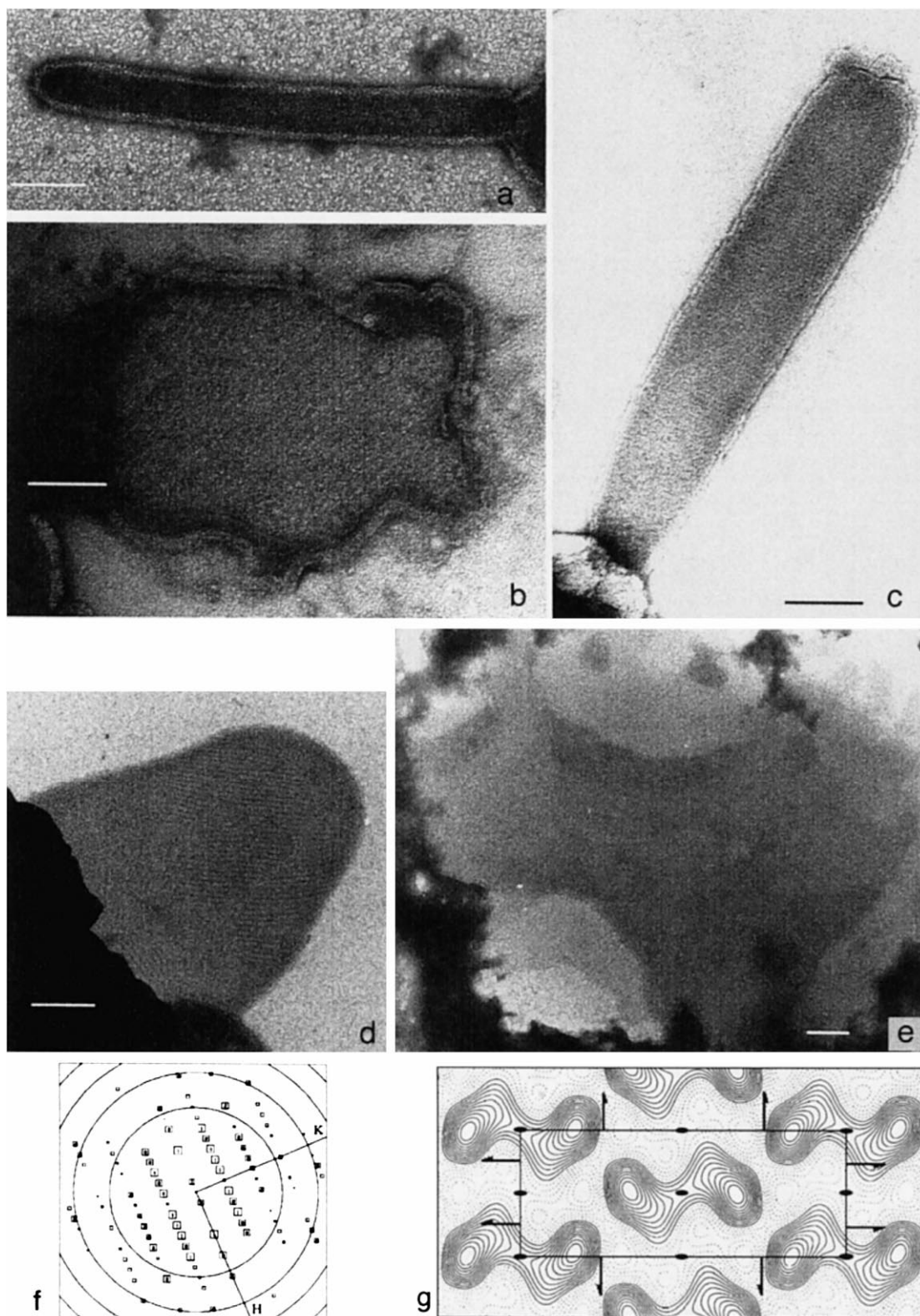


Fig. 4. a–e: Electron micrographs of negatively stained samples of different crystal forms of the spinach cytochrome b_6f complex. Scale bars represents 0.1 μm . f: Computed diffraction pattern of the crystal shown in (e) after unbending of the crystal lattice. The squares indicate the quality of the reflections. Large squares and low numbers stand for a high signal to noise ratio [21]. The circles show the zero position of the contrast transfer function. The resolution at the edge of the plot is 15 $\text{\AA}$. g: Projection map of the cytochrome b_6f complex in negative stain with imposed $p22_12_1$ symmetry at a resolution of 20 $\text{\AA}$. The unit cell is $a=176$ $\text{\AA}$, $b=66$ $\text{\AA}$ and $\gamma=90^\circ$. Solid lines indicate the stain-excluding density of the protein.

sample is well-defined, in contrast to many other preparations of membrane proteins, where a final concentration step often leads to a detergent concentration that is not precisely known. A well-defined detergent concentration is an advantage for crystallization trials, as control over the starting parameters is crucial for reproducibility. The protocol is very efficient and with spinach as an abundant source, the protein can be purified in large amounts. This makes it an ideal starting point for crystallization experiments.

Crystallization trials were conducted and yielded a variety of crystalline structures. Purified protein was reconstituted with egg PC and DOPG solubilized in Hecameg using biobeads for detergent removal [26]. The vesicles obtained were subjected to three cycles of freeze-thaw whereby the sample was quickly frozen in liquid nitrogen and then slowly thawed at 4°C. The procedure follows that for crystallization of the *b₆f* complex for *C. reinhardtii* [11]. Crystals could be observed within 3 days. In general, the crystals formed at a pH of 8.0 and a temperature of 4°C. Two-dimensional crystals had various morphologies: these included tubular crystals which seemed to be growing from non-crystalline vesicles (Fig. 4a). The tubes had dimensions of 50–70 nm in width and up to 1.5 µm in length. They were collapsed and were ordered only to about 35 Å as judged by optical diffraction of samples in negative stain. In trials without the freeze-thaw cycle, tubular vesicles with crystalline areas could be observed after 6 days (Fig. 4b). Images of those vesicles diffract weakly. In the same experiment, sheets and vesicles with ordered arrays and a diameter of up to 0.5 µm were found (Fig. 4c). Large vesicles of a size up to 2 µm in diameter were obtained in freeze-thaw experiments and by the addition of subsolubilizing amounts of detergents after the initial detergent removal with biobeads, which probably facilitated the fusion of smaller vesicles. These vesicles exhibited mosaics of crystalline patches, which appeared ordered in only one direction (Fig. 4d), as indicated by the optical diffraction pattern (not shown). Both the tubular and the sheet-like crystal morphology have not been seen with the *b₆f* complex from *C. reinhardtii*. Multilayered crystals have so far proven to be the best ordered, although these crystals were rare and difficult to reproduce. Fig. 4e is an example of such a crystal in negative stain, which shows its multilayered nature, similar to those obtained for the cytochrome *b₆f* complex from *C. reinhardtii*. It showed optical diffraction to 20 Å.

One electron micrograph of a negatively stained specimen was processed using the MRC program suite. The crystal suggests a $p22_12_1$ symmetry with unit cell dimensions of $a = 176$ Å, $b = 66$ Å and $\gamma = 90^\circ$. Fig. 4f shows a computed diffraction pattern of that crystal. The symmetry was clearly identified as $p22_12_1$ by the MRC program ALLSPACE, even though the expected systematic absences for this symmetry cannot be seen in the diffraction pattern. The reason for this is most probably different staining of the two sides of the crystal during the negative staining. A projection map of the molecule to a resolution of 20 Å reveals a dimeric organization of the complex (Fig. 4g). Comparison with the projection map of cytochrome *b₆f* from *C. reinhardtii* in negative stain shows the same symmetry ($p22_12_1$) and similar unit cell dimensions (*C. reinhardtii*: $a = 175$ Å, $b = 68$ Å, $\gamma = 90^\circ$) [11]. However, the dimers themselves are notably different in the two projections. The spacing between the centers of density of the monomers is smaller in crystals from the spinach complex

and the angle between monomers in the dimer appears to be different.

The multilayered crystals are thin three-dimensional crystals, which are not amenable to three-dimensional structure determination by electron crystallography. However, vesicles with ordered arrays (Fig. 4b,d) can be used for electron crystallography, because they represent true sheet-like two-dimensional crystals. To obtain a three-dimensional map, it will be necessary to decrease the mosaicity and to increase the size of the crystals further.

Our protocol for preparing cytochrome *b₆f* complex from spinach leaves provides a method for a reliable purification of highly active cytochrome *b₆f* complex from spinach by adapting a protocol developed for *C. reinhardtii* [16]. The yield and the concentration of the eluted protein is however significantly higher with spinach. The ratios of the spectroscopically identifiable subunits are in agreement with published data [23]. SDS-PAGE shows the presence of all four major subunits and a band for the 4 kDa subunits (Fig. 1, lanes 4 and 5). The preparation is 10–15 times more active than previously reported preparations from plant leaves and nearly twice as active as the best preparations from *C. reinhardtii*. The change of the solvent for the electron donor in the activity assay from ethanol to DMSO caused a significant improvement in the reliability of the assay. The results presented here indicate the potential for two-dimensional crystallization of this enzyme and for producing sheet-like crystals that will be suitable for electron crystallography. A structural model, even at a medium resolution of about 8 Å, would be most interesting: the related cytochrome *bc₁* complex is functionally similar and has sequence homologies [4,27], but the structures may be quite different.

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