

Cytochrome *b₆/f* complex from the cyanobacterium *Synechocystis* 6803: evidence of dimeric organization and identification of chlorophyll-binding subunit

Chiara Poggese^a, Patrizia Polverino de Laureto^b, Giorgio M. Giacometti^a, Fernanda Rigoni^a, Roberto Barbato^{a,*}

^aDipartimento di Biologia, Università di Padova, Via Trieste 75, 35121 Padua, Italy

^bCRIBI, Università di Padova, Via Trieste 75, 35121 Padua, Italy

Received 16 July 1997; revised version received 6 August 1997

Abstract Fractionation of photosynthetic membranes from the cyanobacterium *Synechocystis* 6803 by polyacrylamide gel electrophoresis in the presence of Deriphat-160 allowed the isolation of a number of pigmented bands. Two of them, with molecular masses of 240 ± 20 and 110 ± 15 kDa respectively, showed peroxidase activity and, by means of polypeptide composition, immunoblotting and N-terminal sequencing, were identified as dimeric and monomeric cytochrome *b₆/f* complexes, containing 1.3 ± 0.35 chlorophyll molecules per cytochrome *f*. Further fractionation of monomeric complexes by mild gel electrophoresis in the presence of sodium dodecyl sulfate indicated that it is the cytochrome *b₆* polypeptide which provides the actual binding site for the chlorophyll molecule observed in the complex.

© 1997 Federation of European Biochemical Societies.

Key words: Cytochrome *b₆/f*; Pigment-binding protein; Dimeric/monomeric organization; *Synechocystis* 6803

1. Introduction

The cytochrome *b₆/f* complex is an intrinsic membrane protein complex involved in photosynthetic electron transfer. It functions as an enzyme with plastoquinone-plastocyanine oxidoreductase activity. Electron transfer through this protein complex is coupled with proton transfer across the thylakoid membrane, operating according to a modified Mitchell Q-cycle [1,2]. A homologous complex, cytochrome *b/c₁*, plays a similar role in mitochondria and some prokaryotes [3].

The cytochrome *b₆/f* complex has been isolated from a number of different organisms, such as higher plants [4,5], green algae [6] and cyanobacteria [7,8]. It has been found to contain four main subunits, cytochrome *f* (31 kDa, encoded by the *petA* gene), cytochrome *b₆* (25 kDa, encoded by the *petB* gene), FeS protein (19–22 kDa, encoded by the *petC* gene) and subunit IV (encoded by the *petD* gene). In higher plants and green algae, the complex also contains some lower molecular weight polypeptides such as the products of genes *petG* [9], *petM* [10,11], and *petL* [6], the functions of which are unknown. Some of these genes are also present in cyanobacteria.

In higher plants and green algae, the cytochrome *b₆/f* complex has been isolated both as a dimer [6,12,13] and as a monomer [12]. In cyanobacteria, only the monomeric form has been isolated so far [14]. Whether these different oligomeric forms and/or their interconversion play any physiological role, as is thought to be the case of photosystem (PS) I and PSII [15] and of the homologous mitochondrial *b/c₁* complex [16,17], is not clear.

Quite surprisingly, most of the cytochrome *b₆/f* preparations so far described contain chlorophyll, irrespective of whether they are isolated from higher plants, green algae or cyanobacteria [4,6,7,18]. Although these studies indicate the presence of a single chlorophyll-*a* molecule per cytochrome *b₆/f* complex, this pigment has been considered a contaminant, and only recently has the possibility that it could be a true component of this membrane protein complex been taken into consideration [19,20]. Very recently, biophysical data have been reported suggesting the presence of a chlorophyll molecule near the quinol oxidation site on the *b₆/f* complex [21], thus reinforcing the idea that this pigment is an intrinsic component of the complex.

In a previous paper [22] we reported the adaptation of the Deriphat-PAGE method originally described by Peter and Thornber [23] for separation of higher plant pigment-protein complexes to fractionate the photosynthetic membrane of the cyanobacterium *Synechocystis* 6803. This method results in the resolution of different oligomeric forms of PSI and PSII. In addition, at least two additional pigmented bands have been detected, besides free pigments. In this paper, we show that these two bands contain a cytochrome *b₆/f* complex, and that this complex can be detected in a dimeric form, as previously shown in higher plants and green algae. By using a combination of Deriphat-PAGE and mild SDS-PAGE, we also provide evidence that the cytochrome *b₆* polypeptide is the actual binding site of the chlorophyll molecule associated with this complex.

2. Materials and methods

2.1. Strains, growth conditions and thylakoid isolation

A glucose-tolerant strain of *Synechocystis* 6803 [24] was used in this study. Cells were grown in BG11 as described previously [22] and thylakoids isolated according to [25].

2.2. Electrophoresis, staining and immunoblotting

Deriphat-PAGE was carried out as originally described by Peter and Thornber [23] with modifications reported previously [22]. SDS-PAGE in mild denaturing conditions was performed according to [26], using a 15% acrylamide gel. TMBZ/H₂O₂ staining of heme-associated peroxidase was carried out as described in [27]. After marking relevant

*Corresponding author. Fax: (39) (49) 827 6344.
E-mail: rbarbato@civ.Bio.unipd.it

Abbreviations: Deriphat, *N*-lauryl iminodipropionate, disodium salt; FeS protein, iron-sulfur Rieske protein; TMBZ, *N,N,N',N'*-tetramethylbenzidine

polypeptides, gels were destained by the addition of solid sodium bisulfite and restained with Coomassie blue as usual.

2.3. Molecular weight determination

The molecular weight of pigmented complexes resolved by Deriphat-PAGE was calculated using the Ferguson plot [28]. In this procedure, standard proteins are electrophoresed together with thylakoid membranes in a number of gels with different acrylamide concentrations (4, 4.5, 5.5, 6, 7 and 8%). Relative mobilities are plotted vs. acrylamide concentration yielding a straight line for each standard protein and pigmented complex. The negative slopes of these lines are then plotted against the respective molecular weight on a logarithmic graph, yielding a second straight line, from which the molecular weight of pigment-protein complexes can be calculated. In our study, standard proteins were: hexameric and trimeric urease (545 and 272 kDa respectively), dimeric and monomeric bovine serum albumin (132 and 66 kDa), chicken serum albumin (45 kDa) and carbonic anhydrase (29 kDa). Before electrophoresis, all proteins were treated with 2% dodecyl maltoside for 15 min on ice, as for thylakoid membranes.

2.4. Limited proteolysis, blotting and N-terminal sequencing

Limited proteolysis was carried out according to [29]. In the case of cytochromes *f* and *b₆*, due to their own pigmentation, bands were located either by visual inspection of the gel, or by staining with acid-free Coomassie blue. Bands were loaded on a second gel, and overlaid with desired amounts of protease. When the tracking dye reached the bottom of the stacker, digestion was performed by turning off the current for 2 h. For N-terminal sequencing, proteins or protein fragments resolved by SDS-PAGE were blotted to PVDF membranes [30]. Filters were stained with Coomassie blue and N-terminal sequencing carried out using an Applied BioSystem protein/peptide sequencer (model 477A). Immunodetection of proteins on nitrocellulose filters was carried out as described previously [22].

2.5. Other methods

Chlorophyll concentration was measured in methanol as described in [31]. Reduced-minus-oxidized spectra of cytochromes *b₆* and *f* were obtained as described in [32] using an ϵ of $20 \text{ mM}^{-1} \text{ cm}^{-1}$. Absorption spectra were recorded after elution in 12.4 mM glycine, 48 mM

Tris and 0.03% dodecyl maltoside. Simulation of the absorption spectrum of cytochrome *b₆* was carried out using turnip cytochrome *f* (Sigma) and CP43 from spinach isolated as described in [33].

3. Results

When thylakoids from the cyanobacterium *Synechocystis* 6803 are solubilized with dodecyl maltoside and subjected to Deriphat-PAGE, the typical electrophoretic pattern reported in lane 1 of Fig. 1A is observed. Four green bands are resolved (bands I–IV) which were previously identified as different oligomeric forms of PSI (bands I and III) and PSII (bands II and IV). In addition, three more pigmented bands are resolved (D, M and FP) which have only partially been characterized [22].

Factors affecting the mobility of proteins or protein complexes in Deriphat-PAGE have not been thoroughly investigated. We found that estimation of molecular masses in Deriphat-PAGE requires the use of a Ferguson plot rather than a simple semilog plot, as direct comparison of mobilities of pigmented bands with those of standard proteins gave inconsistent results. Fig. 1B shows a typical Ferguson plot obtained after Deriphat-PAGE of standard proteins. Molecular masses of about 1000 and 310 kDa were calculated for bands I and III, and 270 and 540 kDa for bands II and IV respectively. These data are consistent with the assignment of bands I and III to trimeric and monomeric PSI and of bands II and IV to dimeric and monomeric PSII [22]. Molecular masses of 240 ± 20 and 110 ± 15 kDa were obtained for bands D and M. Band FP had a molecular mass of 30–35 kDa, probably due to free pigments in detergent micelles.

Heme-binding proteins with peroxidase activity can be detected on SDS polyacrylamide gel by TMBZ/H₂O₂ staining

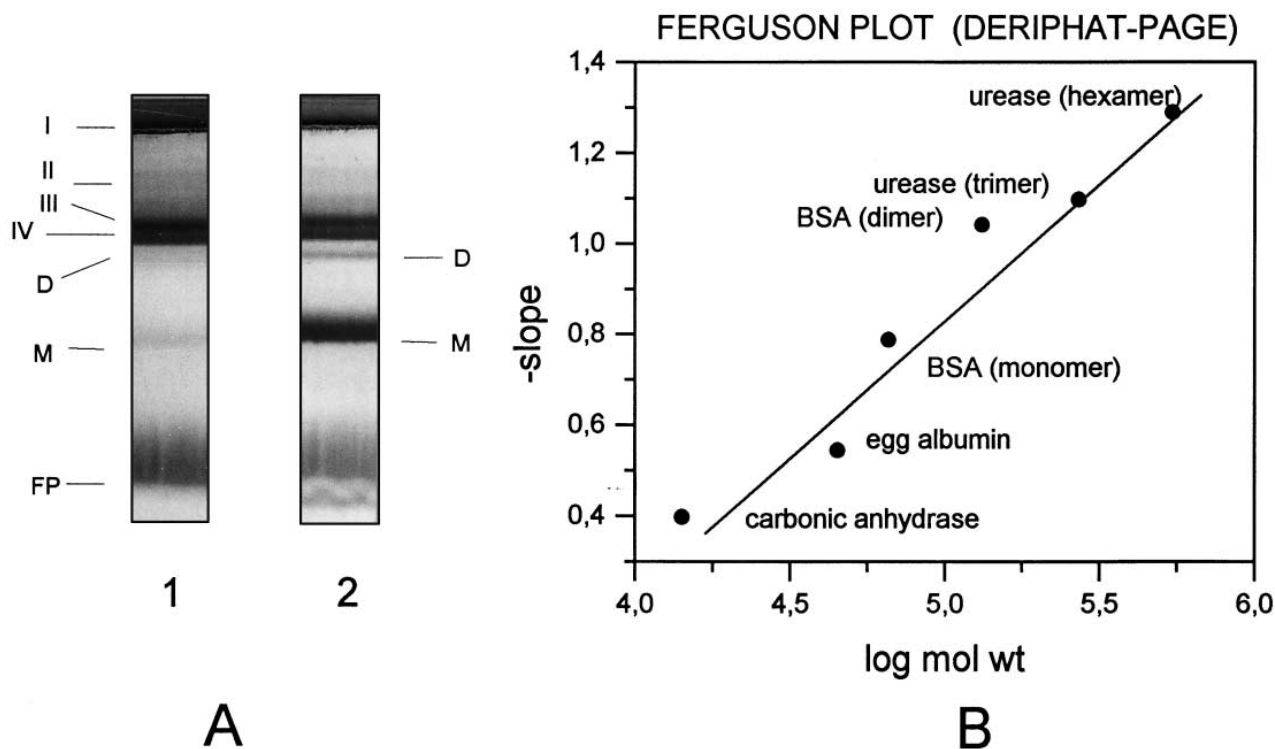


Fig. 1. A: Deriphat-PAGE of *Synechocystis* 6803 thylakoids solubilized with 2% dodecyl maltoside. Lane 1, unstained gel; lane 2, same gel after staining with TMBZ/H₂O₂. B: Calibration of Deriphat gel by means of a Ferguson plot.

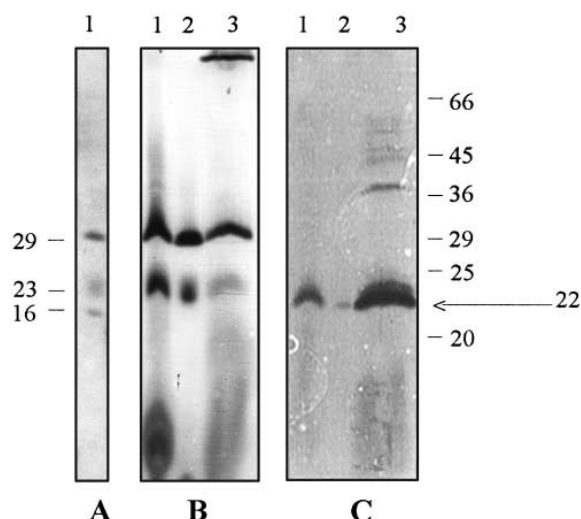


Fig. 2. A: SDS-PAGE of band M from Deriphat gel (Coomassie blue stained gel). B: TMBZ/H₂O₂ staining of SDS gel. C: Immunoblotting with anti-FeS antibody of: band D (lane 1), band M (lane 2) and thylakoids (lane 3).

[27]. The same technique is also applicable with Deriphat gels: as shown in lane 2 of Fig. 1A, bands D and M turned blue, indicating that both of them possess peroxidase activity.

The polypeptide composition of band M is shown in Fig. 2A, lane 1. Three polypeptides were detected, with relative molecular masses of 29, 23 and 16 kDa. They were also detected from re-electrophoresis of band D (data not shown); however, in this case, a number of additional proteins were often observed, indicating that this band may be contaminated by other protein components. Re-electrophoresis in SDS gel of bands D and M, followed by TMBZ/H₂O₂ staining, showed that the 29- and 23-kDa polypeptides possess peroxidase activity (Fig. 2B, lanes 1 and 2). These polypeptides were purified to homogeneity by some rounds of preparative electrophoresis (see for example Fig. 4) and further characterized by N-terminal sequencing. As both of them were blocked at their N-termini, limited proteolysis was used to generate digests suitable for internal sequencing. The 29-kDa band was digested with Glu-C protease; two fragments of about 10 kDa could be sequenced (Table 1), which identified the protein as the *petA* gene product, i.e. cytochrome *f*. Digestion of the 23-kDa band with elastase gave rise to a 16-kDa fragment, the sequence of which identified the protein as the product of the *petB* gene, i.e. cytochrome *b₆*. Immunoblotting and N-terminal sequencing were carried out to test for the presence of other components of the cytochrome *b₆/f* complex such as FeS protein and subunit IV.

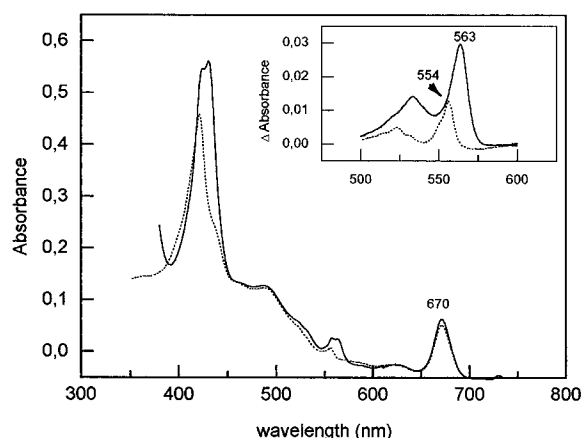


Fig. 3. Absorption spectrum of band M as eluted from gel (dotted line) and after addition of sodium dithionite (solid line). Insert: ferricyanide-minus-ascorbate difference spectrum (dotted line); ascorbate-minus-dithionite spectrum (solid line).

For immunological detection of FeS protein, bands D and M were loaded with such amounts that, after TMBZ/H₂O₂ staining, a comparable intensity was observed, as shown in Fig. 2B, lanes 1 and 2. In Fig. 2C, the FeS protein appears as a 22-kDa band, particularly evident in band D (lane 1) but almost absent in band M (lane 2). Lastly, N-terminal sequencing of the 16-kDa band shown in lane 1 of Fig. 2A identified this protein as the *petD* gene product, i.e. subunit IV; the protein starts with a serine, indicating that, after removal of the initial methionine, no additional post-translational modification occurs (Table 1).

The absorption spectrum of band M, as eluted from the gel (Fig. 3, dotted line) shows a peak at 670 nm, indicative of the presence of chlorophyll, and distinct peaks around 560 and 410 nm, indicative of hemes. Indeed, the spectrum is very similar to that of a highly purified cytochrome *b₆/f* complex purified from *Chlamydomonas reinhardtii* [6] and, after reduction with sodium dithionite (solid line), shows essentially the same spectral changes. Further characterization of our preparation was carried out by difference spectrophotometry (Fig. 3, insert). For this purpose, the sample was oxidized with 0.5 mM potassium ferricyanide and the spectrum recorded after reduction with ascorbate (dotted line) and sodium dithionite (solid line). Assuming a millimolar extinction coefficient of 20 cm⁻¹ [32], a ratio of 2.05 ± 0.14 was found between the two kinds of hemes. When the level of chlorophyll was expressed on a cytochrome *f* basis, a ratio of 1.3 ± 0.35 was found.

While the presence of chlorophyll in the cytochrome *b₆/f* complex is well documented [6,7,13], it is not known to which subunit this pigment is bound. Re-electrophoresis in mild dis-

Table 1
Identification of cytochrome *b₆/f* subunits by N-terminal sequencing

Fragment and/or polypeptide	Source sequence		Assignment
10 kDa, 29-kDa band ^a	band M	NVIVGPLPG	cytochrome <i>f</i>
10 kDa, 29-kDa band ^a	band M	AGEFLTNNPN	cytochrome <i>f</i>
18 kDa, 23-kDa band ^b	band M	ISDDIAS	cytochrome <i>b₆</i>
16-kDa band	band M ^c	SIKKPDLSD	subunit IV
16-kDa band	band D ^c	SIKKP	subunit IV

^aFragments generated by limited proteolysis with 0.5 µg of Glu-C protease.

^bFragments generated by limited proteolysis with 2 µg of elastase.

^cUnblocked polypeptides.

sociating conditions of eluted band M shows the presence of two pigmented bands besides free pigments (not shown). Each band contains a single polypeptide (Fig. 4, CBB), with apparent molecular masses of 29 and 23 kDa respectively, and both stain with TMBZ/H₂O₂ (Fig. 4, TMBZ): they were therefore identified as cytochromes *f* and *b₆* respectively. The absorption spectra of the two pigmented bands indicate that chlorophyll is associated with cytochrome *b₆* (Fig. 5, spectrum A) but not with cytochrome *f* (Fig. 5, spectrum B). In an attempt to measure the ratio between heme and chlorophyll, the cytochrome *b₆* band was eluted and analyzed by difference spectrophotometry. Unfortunately, heme could not be detected by this method, indicating that the chromophore had been damaged by prolonged exposure to SDS during purification of the protein. As an alternative approach, the recorded absorption spectrum was simulated using pure turnip cytochrome *f* and CP43 from spinach (which has its absorption maximum in the red very near that observed for the cytochrome *b₆/f* complex). The result of this experiment indicated that 8–12 hemes should be added to each chlorophyll molecule in order to approach the spectrum of the cytochrome *b₆* (not shown).

4. Discussion

After Deriphat-PAGE of *Synechocystis* thylakoids solubilized with dodecyl maltoside, two pigmented bands with peroxidase activity were detected, D and M, related to the cytochrome *b₆/f* complex. These bands are characterized by molecular masses of 240 and 110 kDa respectively. The only apparent difference between them is the absence, in the latter, of FeS protein. However this protein (19–22 kDa) is too small to account for the large difference observed in the molecular masses of bands D and M. We interpret this finding as evidence that bands D and M, besides FeS protein, also differ in oligomeric organization, band D being a dimer and band M a monomer. Evidence for the presence of monomeric and dimeric cytochrome *b₆/f* complexes in the thylakoid membranes

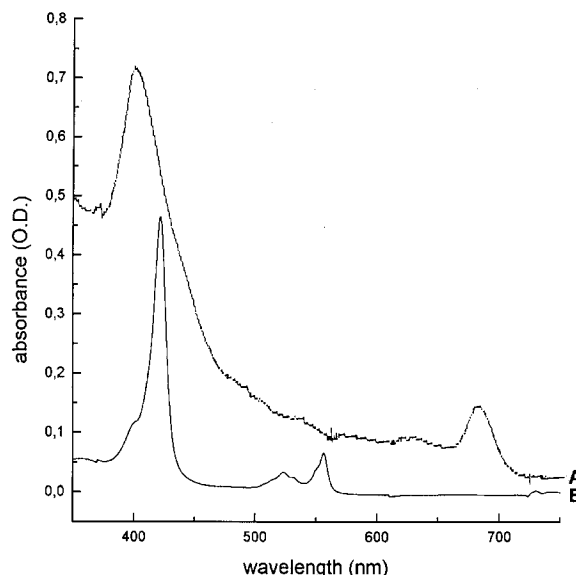


Fig. 5. Absorption spectrum of cytochrome *b₆* (A) and cytochrome *f* (B).

of higher plants and green algae has been obtained using different approaches in other laboratories [12,13] and the dimeric structure was suggested to be the functional form of this complex. In band M, the low level of FeS protein suggests that most of the monomeric complexes have lost this protein. As pointed out by other authors [34,35], there seems to be a relationship between monomerization and loss of the FeS protein.

The combination of Deriphat-PAGE and mild SDS-PAGE allowed the isolation of cytochrome *b₆* in a form in which both heme and chlorophyll are bound to the same polypeptide. The presence of chlorophyll in cytochrome *b₆/f* preparations from spinach has been known for some time [4] and has been confirmed in similar preparations from green algae [6] and cyanobacteria [7]. However, the presence of chlorophyll was usually interpreted as contamination, and only recently has the possibility that this pigment may be a true component of the cytochrome *b₆/f* complex been taken into consideration [14]. Our findings provide evidence that chlorophyll is not only an intrinsic component of the cytochrome *b₆/f* complex but also that the actual binding site for this pigment resides in the cytochrome *b₆* subunit. Based on the fact that one single chlorophyll molecule is usually observed per cytochrome complex ([4,7,18], this work), the cytochrome *b₆* subunit is expected to carry one chlorophyll and two hemes per polypeptide. Although we could not directly measure the exact chlorophyll/heme ratio, simulation of the recorded spectrum by addition of pure chlorophyll and cytochrome indicated a ratio between 8 and 12. This is 4–6 times higher than expected, due to the loss of pigments during purification by SDS-PAGE. Indeed, the fact that some chlorophyll molecules are still associated with the polypeptide reinforces the idea that chlorophyll is specifically bound to cytochrome *b₆*.

Although it might be argued that the chlorophyll molecule observed in the cytochrome *b₆/f* complex and therefore in cytochrome *b₆* represents an unspecifically bound impurity, there are several points against this possibility. Strong evidence supporting the specific binding of chlorophyll to the *b₆/f* complex was given by Popot et al. [36], who showed

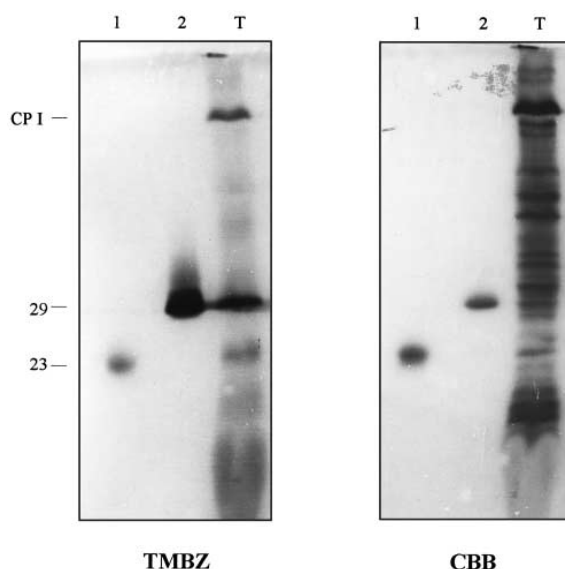


Fig. 4. Re-electrophoresis of bands 29 and 23 of Fig. 2. TMBZ, TMBZ/H₂O₂ staining; CBB, Coomassie staining. Lane 1, 23-kDa band; lane 2, 31-kDa band; lane T, thylakoids (10 µg chlorophyll).

that when radioactive chlorophyll in detergent micelles is mixed with cytochrome *b₆/f* complex, the chlorophyll in the complex is not easily exchanged. Moreover, biophysical evidence has recently been obtained [21] indicating the presence in vivo of a chlorophyll molecule near the quinol oxidation site, inferred from an electrochromic shift called 'red signal', with a peak at 669 nm. This shift is associated with the cytochrome *b₆/f* complex, as it is absent from mutants lacking the complex and is abolished by inhibitors blocking plastoquinol re-oxidation. Thus, it is possible that the chlorophyll molecule detected in the present study is the same pigment giving rise to the 'red signal' of Joliot and Joliot [21].

References

- [1] Crofts, A.R., Meinhardt, S.W., Jones, K.R. and Snozzi, M. (1983) *Biochim. Biophys. Acta* 723, 202–218.
- [2] Rich, P.R. (1986) *J. Bioenerg. Biomemb.* 18, 145–156.
- [3] Trumpower, B.L. and Gennis, R.B. (1994) *Annu. Rev. Biochem.* 63, 675–716.
- [4] Hurt, E. and Hauska, G. (1981) *Eur. J. Biochem.* 117, 591–599.
- [5] Phillips, A.L. and Gray, J. (1983) *Eur. J. Biochem.* 137, 553–560.
- [6] Pierre, Y., Breyton, C., Kramer, D. and Popot, J.-L. (1995) *J. Biol. Chem.* 270, 29342–29349.
- [7] Koppenal, F. and Krab, K. (1991) in: *Light in Biology and Medicine* (Douglas, R.H., Moan, J. and Ronto, G., Eds.) Vol. 2, pp. 111–121, Plenum, London.
- [8] Rögner, M., Nixon, P.J. and Diner, B.A. (1990) *J. Biol. Chem.* 265, 6189–6196.
- [9] Haley, J. and Bogorad, L. (1989) *Proc. Natl. Acad. Sci. USA* 86, 1534–1538.
- [10] Pierre, Y. and Popot, J.-L. (1993) *C.R. Acad. Sci. Paris* 316, 1404–1409.
- [11] Ketchner, S.L. and Malkin, R. (1996) *Biochim. Biophys. Acta* 1273, 195–197.
- [12] Chain, R.K. and Malkin, R. (1991) *Photosynth. Res.* 28, 59–68.
- [13] Huang, D., Everly, R.M., Cheng, R.H., Heymann, J.B., Schägger, H., Sled, V., Ohnishi, T., Baker, T.S. and Cramer, W.A. (1994) *Biochemistry* 33, 4401–4409.
- [14] Boekema, E.J., Boonstra, A.F., Dekker, J.P. and Rögner, M. (1994) *J. Bioenerg. Biomemb.* 26, 17–29.
- [15] Rögner, M., Boekema, E.J. and Barber, J. (1996) *Trends Biochem. Sci.* 21, 44–49.
- [16] Leonard, K., Wingfield, P., Arad, T. and Weiss, H. (1981) *J. Mol. Biol.* 149, 259–274.
- [17] Nalecz, M.J. and Azzi, A. (1985) *Arch. Biochem. Biophys.* 240, 921–931.
- [18] Bald, D., Kruip, J., Boekema, E.J. and Rögner, M. (1992) in: *Research in Photosynthesis* (Murata, N., Ed.) Vol. I, pp. 629–632, Kluwer, Dordrecht.
- [19] Kallas, T. (1994) in: *Molecular Biology of Cyanobacteria* (Bryant, D.A., Ed.), pp. 259–317, Kluwer, Dordrecht.
- [20] Cramer, W.A., Soriano, G.M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S.E. and Smith, J.L. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 477–508.
- [21] Joliot, A. and Joliot, P. (1995) in: *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp. 615–618, Kluwer, Dordrecht.
- [22] Barbato, R., Polverino de Laureto, P., Rigoni, F., De Martini, E. and Giacometti, G.M. (1995) *Eur. J. Biochem.* 234, 459–465.
- [23] Peter, G.F. and Thornber, J.P. (1991) *J. Biol. Chem.* 266, 16745–16754.
- [24] Williams, J.G.K. (1988) *Methods Enzymol.* 167, 766–778.
- [25] Mayes, S.R., Dubbs, J.M., Vass, I., Hideg, E., Nagy, L. and Barber, J. (1993) *Biochemistry* 32, 1454–1465.
- [26] Bassi, R., Høyer-Hansen, G., Barbato, R., Giacometti, G.M. and Simpson, D.J. (1987) *J. Biol. Chem.* 262, 13333–13341.
- [27] Thomas, P.E., Ryan, D. and Levin, W. (1976) *Anal. Biochem.* 75, 168–176.
- [28] Ferguson, K.A. (1964) *Metabolism* 13, 985–990.
- [29] Cleveland, D.W., Fisher, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) *J. Biol. Chem.* 252, 1102–1106.
- [30] Dunn, S.D. (1986) *Anal. Biochem.* 157, 144–153.
- [31] MacKinney, G. (1941) *J. Biol. Chem.* 140, 315–322.
- [32] Hauska, G. (1986) *Methods Enzymol.* 126, 271–285.
- [33] Barbato, R., Race, H.L., Friso, G. and Barber, J. (1991) *FEBS Lett.* 286, 86–90.
- [34] Ting, C.S., Wollman, F.-A. and Popot, J.-L. (1995) in: *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp. 519–522, Kluwer, Dordrecht.
- [35] Takahashi, Y., Rahire, M., Breyton, C., Popot, J.-L., Joliot, P. and Rochaix, J.-D. (1996) *EMBO J.* 15, 3498–3506.
- [36] Popot, J.-L., Pierre, Y., Breyton, C., Lemoine, Y., Takahashi, Y. and Rochaix, J.-D. (1995) in: *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp. 507–512, Kluwer, Dordrecht.