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An isolated reaction center complex from the green sulfur bacterium *Chlorobium vibrioforme* can photoreduce ferredoxin at high rates

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Abstract

Chlorosome-depleted membranes and a reaction center complex with well-defined subunit composition were prepared from the green sulfur bacterium *Chlorobium vibrioforme* under anaerobic conditions. The reaction center complex contains a 15-kDa polypeptide with the N-terminal amino acid sequence MEPQLSRPETASNQVR/. This sequence is nearly identical to the N-terminus of the *pscD* gene product from *Chlorobium limicola* (Hager-Braun et al. (1995) Biochemistry 34: 9617–9624). In the presence of ferredoxin and ferredoxin:$NADP^+$ oxidoreductase, the membranes and the isolated reaction center complex photoreduced $NADP^+$ at rates of 333 and 110 μmol (mg bacteriochlorophyll *a*)$^{-1}$ h^{-1}, respectively. This shows that the isolated reaction center complex contains all the components essential for steady state electron transport. Midpoint potentials at pH 7.0 of 160 mV for cytochrome c_{551} and of 245 mV for P840 were determined by redox titration. Antibodies against cytochrome c_{551} inhibit $NADP^+$ reduction while antibodies against the bacteriochlorophyll *a*-binding Fenna-Matthews-Olson protein do not.

Abbreviations: FMO protein – Fenna-Matthews-Olson protein; TMBZ – 3,3′,5,5′-tetramethylbenzidine

Introduction

The photosynthetic reaction center complex from green sulfur bacteria has recently attracted increased attention. Already in 1969, Buchanan and Evans showed that membranes from *Chlorobium limicola* f. *thiosulfatophilum* were able to photoreduce ferredoxin, and this suggested that the green sulfur bacteria contained a reaction center complex related to Photosystem I as opposed to purple bacteria and green filamentous bacteria (Cloroflexaceae) with a reaction center related to Photosystem II. The relationship between the reaction center complex in green sulfur bacteria and Photosystem I has subsequently been documented by EPR studies and by the demonstrated sequence similarity between their reaction center polypeptides (Kjær et al. 1994; Kusumoto et al. 1994; Illinger et al. 1993; Oh-oka et al. 1993; Büttner et al. 1992; Feiler et al. 1992; Miller et al. 1992; Nitschke et al. 1990; Swarthoff et al. 1981a).

Isolated reaction center complexes from *Chlorobium* spp. contain 5 to 6 different subunits: two identical reaction center polypeptides of 82 kDa (encoded by *pscA*), a varying amount of a 40-kDa BChl *a* binding protein (the Fenna-Matthews-Olson (FMO) protein), an iron-sulfur protein of 24 kDa (encoded by *pscB*), a *c*-type cytochrome of 20 kDa (encoded by *pscC*), and one or two smaller subunits of 15 and 9 kDa with unknown function (Hager-Braun et al. 1995; Kjær et al. 1994; Kusumoto et al. 1994; Illinger et al. 1993; Oh-oka et al. 1993; Büttner et al. 1992; Feiler et al. 1992; Okkels et al. 1992; Nitschke et al. 1990; Swarthoff et al. 1981a). EPR spectroscopy has shown that the isolated complexes contain photoreducible iron-sulfur clusters similar to F_A and F_B in Photosystem I (Kjær et al. 1994; Kusumoto et al. 1994; Oh-oka et al. 1993;

Miller et al. 1992; Nitschke et al. 1990). However, an ability of the isolated reaction center complexes to sustain steady state reduction of ferredoxin has not been reported. A few studies have been carried out with membrane preparations but the measured activities were rather low (Miller et al. 1992; Buchanan and Evans 1969). In Photosystem I the ferredoxin docking PSI-D subunit mediates efficient electron transport from the terminal acceptors F_A and F_B bound to the iron-sulfur protein PSI-C to ferredoxin (Li et al. 1991; Chitnis et al. 1989). In addition, the presence of the PS I-E subunit is known to stimulate ferredoxin reduction (Rousseau et al. 1993). The isolated reaction center complexes from *Chlorobium* spp. do not contain subunits homologous to PS I-D and PS I-E, and therefore it is important to investigate whether the reaction center complexes are able to photoreduce soluble acceptors in the absence of such subunits or whether the reaction center complexes obtained are inactive due to loss of subunits during their isolation. In this work we show that membranes and a reaction center complex from *C. vibrioforme* are able to photoreduce $NADP^+$ at high rates when isolated under anaerobic conditions.

An important difference between Photosystem I and the green sulfur bacterial reaction center complex is the presence of a bound cytochrome *c* which appears to function as the donor to photooxidized P840 (Oh-oka et al. 1995; Kusumoto et al. 1994; Oh-oka et al. 1993; Miller et al. 1992; Okkels et al. 1992; Hurt and Hauska 1984; Swarthoff et al. 1981b; Swarthoff and Amesz 1979; Olson et al. 1976a; Prince and Olson 1976; Fowler et al. 1971). However, there are some unresolved discrepancies regarding this cytochrome, in particular with respect to the position of its α-band. Studies with membranes from *Chlorobium* strains indicate the presence of a photooxidizable Cyt c_{553} that is rapidly photooxidized (Miller et al. 1992; Prince and Olson 1976) whereas the isolated reaction center complexes contain a photooxidizable Cyt c_{551} (Oh-oka et al. 1995; Kusumoto et al. 1994; Oh-oka et al. 1993; Okkels et al. 1992, Hurt and Hauska 1984). Kinetic measurements have shown photooxidation of the cytochrome with halftimes of 50–110 μs (Oh-oka et al. 1995, 1993; Okkels et al. 1992) but the <5 μs phase observed in membranes from *C. limicola* (Prince and Olson 1976) has not been clearly seen in the isolated reaction center preparations. Interestingly, the <5 μs phase of cytochrome oxidation has not been observed in membranes from *Prostechochloris aestuarii* (Swarthoff et al. 1981b) or *C. vibrioforme* (Miller et al. 1992). Reasons for the discrepancy could be species differences or changes in the α-band position due to the use of detergents. Alternatively, the membranes could contain a Cyt c_{553} which is the real donor but which is lost upon isolation. Feiler et al. (1992) have speculated that the direct donor to P840 in *C. limicola* is a tetraheme Cyt c_{553} similar to the electron donor to the reaction center in *Rhodopseudomonas viridis*. In this work, we show that the Cyt c_{551} present in preparations from *C. vibrioforme* has a midpoint potential of 160 mV which is compatible with a function as donor to P840. Also we show that an antibody against this cytochrome inhibits the steady state electron transport.

Reaction center complexes from green sulfur bacteria usually contain some FMO protein. This protein can be removed from the remaining complex but this has not been accomplished without simultaneous loss of photochemical activity (Hager-Braun et al. 1995; Oh-oka et al. 1993; Vasmel et al. 1983; Olson et al. 1976a). An antibody against the FMO protein does not affect steady state electron transport, indicating that the protein does not play a role in for example docking of ferredoxin.

Materials and methods

Chlorobium vibrioforme strain NCIB 8327 (kindly provided by Professor J.G. Ormerod, Department of Biology, University of Oslo) was grown as previously described (Okkels et al. 1992). The chlorosome-depleted membranes and the reaction center complex were isolated anaerobically according to the previously reported procedure (Kjær et al. 1994; Feiler et al. 1992; Okkels et al. 1992).

FMO protein and cytochrome c_{551} were isolated from the reaction center complex by preparative SDS-PAGE and electroelution from the Coomassie-stained gels. Polyclonal antibodies against the electroeluted proteins were raised in rabbits.

N-terminal amino acid sequencing was carried out with the 15-kDa polypeptide after electroelution or transfer to polyvinylidene difluoride membranes. Sequencing was performed with an Applied Biosystems sequenator.

P840 was determined from the ferricyanide-oxidized minus ascorbate reduced spectrum using $\Delta\varepsilon_{830} = 90\ mM^{-1}\ cm^{-1}$ (Olson et al. 1976b).

$NADP^+$ reduction was measured in a 500 μl reaction mixture using an anaerobic cuvette to which the following components were added: 0.5 mM $NADP^+$, 20 mM Tricine (pH 7.5), 8 mM $MgCl_2$, 0.6 mg bovine

serum albumin, 0.04% decyl β-D-maltoside, 2 mM sodium ascorbate, 0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 6 μM ferredoxin, 0.05 μM barley ferredoxin:$NADP^+$ oxidoreductase, 2 units of glucose oxidase, 4 μg catalase, 6 mM glucose, 8 mM 2-mercaptoethanol, 0.1 or 0.4% dodecyl β-D-maltoside (for reaction center complex and for membranes, respectively) and reaction center complex equivalent to 3 μg BChl *a* or membranes equivalent to 1.3 μg BChl *a*. After 10 min incubation in the dark at 4 °C, the light-induced production of NADPH was measured at 340 nm in an Aminco DW 2000 spectrophotometer. The sample was kept at 25 °C and irradiated from the side with a Schott KL 1500 light source fitted with two red filters (Schott RG660 and Corning 2–58). The photomultiplier tube was protected from the actinic red light by a 340 nm interference filter (Schott UV-DAD-15–3).

The effect of antibodies on $NADP^+$ reduction was studied by anaerobic incubation of the reaction center complex or membranes with specific antibodies in a volume of 300 μl containing 70 mM MOPS (pH 7.5), 13 mM $MgCl_2$, 0.17 or 0.66% dodecyl β-D-maltoside (for the reaction center complex and for membranes, respectively). Bovine serum albumin was added to give a total protein content of 600 μg in all incubations. After incubation in the anaerobic chamber for 1 h at 4 °C in the dark, the remaining components of the reaction mixture for measuring $NADP^+$ reduction were added to a final volume of 500 μl. Tricine and decylmaltoside were excluded for these measurements.

Redox titration of P840 and Cyt c_{551} was performed at pH 7.0 essentially according to Dutton (1978). The cuvette was kept anaerobic during the measurement by flushing with argon. The following redox mediators were added: 100 μM 2,3,5,6-tetramethylphenylenediamine, 50 μM phenazine methosulfate, 100 μM 1,2-naphtoquinone, and 100 μM thymoquinone. Reductive and oxidative titrations were carried out with sodium dithionite and potassium ferricyanide, respectively. During the titrations, spectra were repeatedly aquired on an Aminco DW 2000 spectrophotometer operating in dual mode with a fixed reference wavelength. The initial spectra (fully oxidized for Cyt *c* and fully reduced for P840) were subtracted during aquisition. The absorbance changes were calculated from the spectra.

Clostridium pasteurianium ferredoxin, spinach ferredoxin, glucose oxidase, dodecyl β-D-maltoside and bovine serum albumin (highest grade available) were obtained from Sigma and catalase was obtained from Boehringer. Barley ferredoxin:$NADP^+$ oxidoreductase was isolated by affinity chromatography essentially according to Serrano and Rivas (1982). All other reagents were of analytical grade.

Results

The anaerobically isolated reaction center complex contains six different subunits of which the four largest have been identified previously (see 'Introduction'). The 15-kDa band was analyzed by N-terminal amino acid sequencing and the sequence MEPQLSRPETAS-NQVR/ was obtained. The N-terminal sequence is very similar to the recently published sequence of the *pscD* gene product from the reaction center complex from *Chlorobium limicola* (Hager-Braun et al. 1995). Thirteen residues are identical and the remaining three residues are conservative substitutions. The BChl *a*/P840 ratio was 35–45 in the isolated reaction center complex (Kjær et al. 1994) and about 80 in chlorosome-depleted membranes.

Staining of the electrophoresed proteins with TMBZ shows that the isolated reaction center complex contains Cyt c_{551} as the single heme containing subunit. Contrary to this, chlorosome-depleted membranes contain several *c*-type cytochromes. The antibodies raised against Cyt c_{551} and FMO protein were tested by western blotting. The cytochrome antibody is very specific and shows no cross-reactivity with other cytochromes in the membranes.

Photoreduction of $NADP^+$ was measured in chlorosome-depleted membranes and in the isolated reaction center complex using anaerobic conditions. In membranes, the activity (± SD) was 333 ± 11 μmol NADPH (mg BChl *a*)$^{-1}$ h^{-1}. In the isolated reaction center complex the activity was 110 ± 21 μmol NADPH (mg BChl *a*)$^{-1}$ h^{-1}. The highest value obtained with a preparation of isolated reaction center complex was 150 μmol NADPH (mg BChl *a*)$^{-1}$ h^{-1}. The absorbance changes observed at 340 nm were completely dependent on light, ferredoxin and ferredoxin:$NADP^+$ oxidoreductase. The high rates of photoreduction of $NADP^+$ reported in this study were obtained using *C. pasteurianum* ferredoxin as electron acceptor. With barley or spinach ferredoxin the rates were about one third (data not shown).

Incubation of the reaction center complex or chlorosome-depleted membranes from *C. vibrioforme* with antibodies raised against Cyt c_{551} inhibited $NADP^+$ photoreduction (Fig. 1). The extent of inhibi-

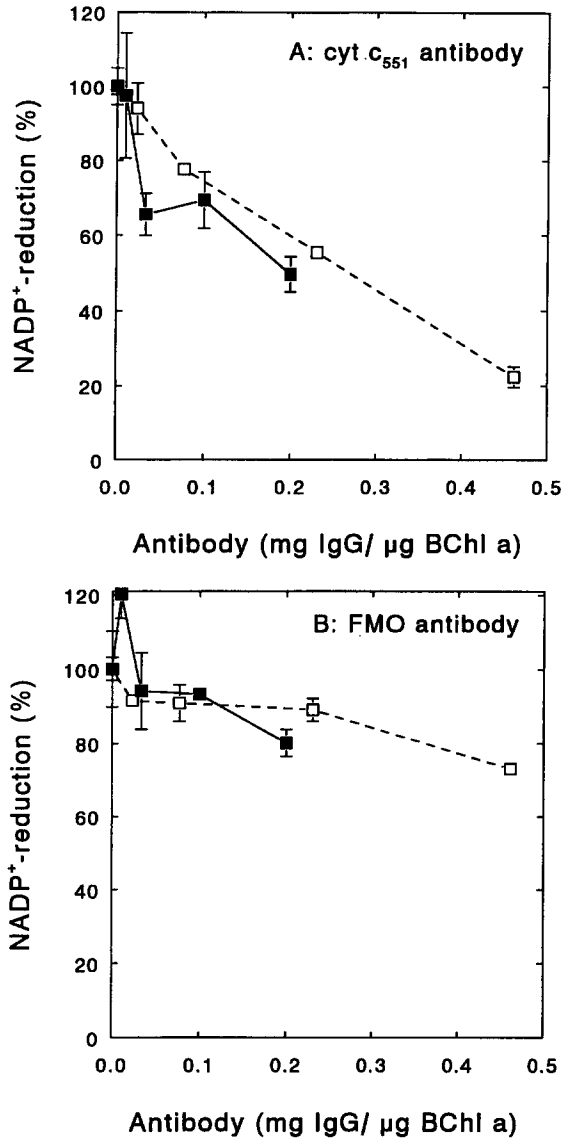

Fig. 1. Light-induced $NADP^+$ reduction activity in the reaction center complex (■) and the chlorosome depleted membranes (□) from *C. vibrioforme* after incubation with antibodies against Cyt c_{551} (A) and the FMO protein (B). Values are expressed as percent of control ± SD. In the anti-Cyt c_{551} experiment the control activity of the membranes and reaction center complex were 328 and 144 µmol NADPH (mg BChl *a*)$^{-1}$ h^{-1} . In the anti-FMO experiment the corresponding values were 337 and 102 µmol NADPH (mg BChl *a*)$^{-1}$ h^{-1}.

tion was similar in the two types of preparations. Contrary to this, incubation with antibodies raised against the FMO protein did not result in significant inhibition of $NADP^+$ photoreduction (Fig. 1). Incubation with a non-immune serum did not result in inhibition of activity (data not shown).

The redox titrations of P840 and Cyt c_{551} are shown in Fig. 2. For both curves, the best fit to the Nernst equation is obtained with n = 1. The E_m values are calculated to be 245 ± 10 mV for P840 and 160 ± 2 mV for Cyt c_{551}. The scattering of the points obtained for P840 is due to oxidative bleaching of pigment molecules in the sample.

Discussion

The anaerobically isolated reaction center complex from *C. vibrioforme* is able to photoreduce $NADP^+$ at a rate of up to 150 µmol NADPH (mg BChl *a*)$^{-1}h^{-1}$ corresponding to a P840 turnover of 3 s^{-1}. This is the first report of reduction of $NADP^+$ in an isolated reaction center complex from a green sulfur bacterium. The high activity shows that all the subunits necessary for reduction of soluble ferredoxin are present and active in the isolated complex. In the more native chlorosome-depleted membranes, a rate of 333 µmol NADPH (mg BChl *a*)$^{-1}h^{-1}$ can be measured. On a P840 basis this activity is about four to five times higher than in the reaction center complex. The lower activity in the isolated reaction center complex could be related to loss of an important component but it is conceivable that the lower activity is simply due to inactivation during the isolation procedure which involves detergent solubilization of the membranes. Photosystem I preparations containing all subunits important for $NADP^+$ reduction have for example been reported to exhibit an activity of 214 µmol NADPH (mg Chl)$^{-1}h^{-1}$ (Bengis and Nelson 1977); a value considerably lower than the 1800 µmol NADPH (mg Chl)$^{-1}h^{-1}$ obtained with a different detergent (Andersen et al. 1992). The rate of $NADP^+$ reduction obtained with chlorosome-depleted membranes is 15–30 times higher than the previously reported rates (Miller et al. 1992; Buchanan and Evans 1969). The higher rates of $NADP^+$ photoreduction obtained in this study are probably due to the anaerobic conditions used during isolation which result in a more native complex (Kjær et al. 1994). A second reason for the high activity is the use of *C. pasteurianum* ferredoxin as electron acceptor instead of plant ferredoxin. In previous studies, spinach ferredoxin (Miller et al. 1992) or *Chromatium* ferredoxin (Buchanan and Evans 1969) were used. The rate of $NADP^+$ photoreduction by the isolated reaction center complex could possibly be even higher if *Chlorobium* ferredoxin were used, but this was not attempted because the protein

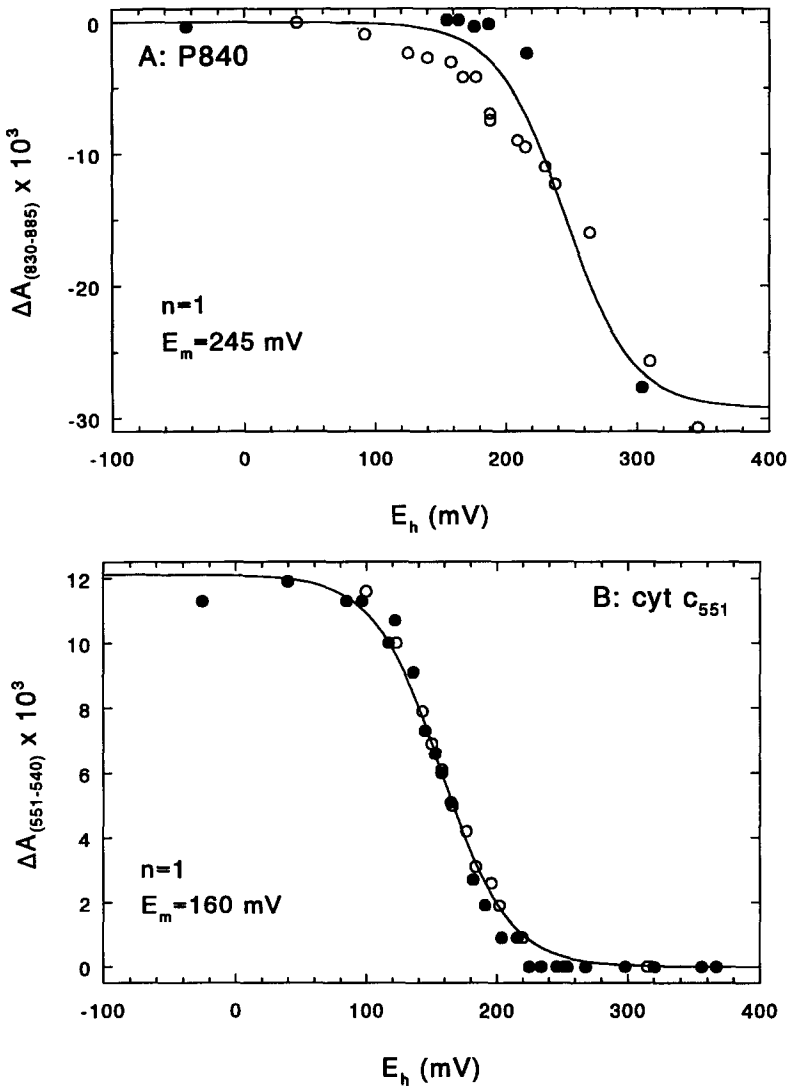

Fig. 2. (A) Redox titration of P840. The curve shows the absorption at 830–885 nm measured at different redox potentials (E_h) first obtained by oxidation with potassium ferricyanide (○) then by reduction with sodium dithionite (●). (B) Redox titration of Cyt c_{551}. The curve shows the absorption at 551–540 nm measured at different redox potentials (E_h) first obtained by reduction (●), then by oxidation (○). In both (A) and (B) the data were fitted to an n = 1 Nernst equation and the midpoint potentials (E_m) were estimated by a least-squares-fit program. A reaction center preparation with approximately 0.5 μM P840 was used in both titrations.

is reported to be very unstable (Evans and Buchanan 1965).

The present study has shown that photoreduction of $NADP^+$ is possible in an isolated reaction center complex from *C. vibrioforme*, where the only *c*-type cytochrome present is Cyt c_{551}. Furthermore the $NADP^+$ reduction is inhibited when the complex is incubated with antibodies raised against Cyt c_{551}. The fact that an immunoglobulin molecule bound to Cyt c_{551} results in inhibition of $NADP^+$ reduction strongly indicates that Cyt c_{551} has an important function in the $NADP^+$ photoreduction process.

The possibility that there are alternative pathways for reduction of P840 in vivo needs to be considered. In some purple bacteria alternative secondary donors have been found (Zanoni and Daldal 1993). The chlorosome-depleted membranes from *C. vibrioforme* contain several other *c*-type cytochromes in addition to Cyt c_{551}, among these one migrating around 32 kDa which could correspond to the putative tetra-

heme Cyt c_{553} in membranes from *C. limicola* (Feiler et al. 1992). However, $NADP^+$ reduction by the membranes is also strongly inhibited by the antibody raised against Cyt c_{551}. This does not exclude the involvement of other cytochromes but show that Cyt c_{551} is also important for $NADP^+$ reduction by membranes. One reason for scepticism regarding the function of Cyt c_{551} has been the fact that its absorbance characteristics are different from those reported for the secondary donor cytochrome in studies of more intact systems. With cells or membranes from several different strains of *Chlorobium*, the secondary donor has been reported to have an α-band of 553 nm (Okumura et al. 1994; Miller et al. 1992; Olson et al. 1976a; Prince and Olson 1976). An α-band of 553 nm was also reported for *P. aestuarii* (Swarthoff et al. 1981b; Swarthoff and Amesz 1979; Fowler et al. 1971). Contrary to this the *pscC* gene product in *C. vibrioforme* was found to absorb at 551.4 nm (Okkels et al. 1992). The same protein has been reported to absorb at 551 or 550.5 in *C. limicola* f. *thiosulfatophilum* (Oh-oka et al. 1993; Hurt and Hauska 1984) and at 552 or 551 nm in *C. tepidum* (Oh-oka et al. 1995; Kusumoto et al. 1994). In order to provide additional information on the Cyt c_{551} in our preparations we have determined its midpoint potential. This was found to be 160 mV at pH 7.0, a value very similar to the 170, 165 and 180 mV reported for Cyt c_{553} by Fowler et al. (1971), Prince and Olson (1976) and Okumura et al. (1994), respectively. Hurt and Hauska (1984) have reported a value of 220 mV for Cyt $c_{550.5}$. The reason for this discrepancy is not clear. For P840 we found E_m = 245 mV in good agreement with previous estimates of 230 mV (Okumura et al. 1994), 250 mV (Prince and Olson 1976) and 240 mV (Fowler et al. 1971). The Rieske iron-sulfur protein and soluble Cyt c_{555}, the presumed soluble carrier of electrons between the Cyt *bc* complex and the reaction center, both have E_m at pH 7.0 around 145 mV (Meyer 1994; Amesz and Knaff 1988). Thus an E_m value of 160 mV for Cyt c_{551} is in excellent agreement with a function of this protein as the donor to P840. We suggest that the membrane-bound Cyt c_{551} is identical to the Cyt c_{553} known from spectroscopical studies and that the discrepancy in α-band is due to detergent-introduced changes in the conformation of the protein. Another possibility could be that the membranes contain a cytochrome c_{553} which donates electrons to Cyt c_{551} in a very fast reaction.

Unlike the antibody raised against Cyt c_{551}, the antibody raised against the FMO protein does not result in any significant inhibition of $NADP^+$ reduction, neither in the isolated reaction center complex, nor in the chlorosome-depleted membranes. This indicates that the FMO protein is not directly involved in the $NADP^+$ reduction process, although it can not be excluded that the antibody binds to the relatively large protein at a place where it has no effect.

Photosystem I of plants and cyanobacteria contains the stromal subunits PSI-D and PSI-E which are important for the interaction with ferredoxin. PSI-D is essential for the docking of ferredoxin (Chitnis et al. 1989) while PSI-E is important for high rates of ferredoxin reduction (Rousseau et al. 1993). Homologous subunits have not been reported from green sulfur bacteria and clearly the docking and reduction of ferredoxin do not depend on such subunits in *C. vibrioforme*. The *pscB* iron-sulfur protein in *Chlorobium* is 24 kDa as compared to 9 kDa for the PSI-C iron-sulfur protein in Photosystem I. It can be speculated that the additional regions of the *pscB* protein functionally resemble the PSI-D and PSI-E subunits of Photosystem I. Hager-Braun et al. (1995) have speculated that the basic *pscD* protein could have a function similar to PSI-D in stabilizing the *pscB* iron-sulfur protein. Likewise, the *pscD* protein or the 9-kDa polypeptide could also be involved in ferredoxin docking.

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