Function-Directed Mutagenesis of the Cytochrome b_6f Complex in *Chlamydomonas reinhardtii*: Involvement of the cd Loop of Cytochrome b_6 in Quinol Binding to the Q_o Site[†]

Giovanni Finazzi,[‡] Sylvie Büschlen,[§] Catherine de Vitry, Fabrice Rappaport, Pierre Joliot, and Francis-André Wollman*

CNRS UPR 9072, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

Received October 30, 1996; Revised Manuscript Received January 15, 1997[⊗]

ABSTRACT: The FUD2 mutant from the green alga *Chlamydomonas reinhardtii* expresses a cytochrome b_6 variant of higher apparent molecular mass [Lemaire et al. (1986) *Biochim. Biophys. Acta 851*, 239−248]. Here, we show that the mutation corresponds to a 36 base pair duplication in the chloroplast *petB* gene, which corresponds to a 12 amino acid duplication in the cd loop of cytochrome b_6. The resulting protein still binds its heme cofactors and assembles into cytochrome b_6f complexes, which accumulate in wild type amounts in exponentially growing cells of FUD2. However, these cytochrome b_6f complexes show loosened binding of the Rieske protein and are more prone to degradation in aging cells. Electron transfer through the cytochrome b_6f complexes is about 8 times slower in FUD2 than in wild type cells. This is due to a slower oxidation of plastoquinol at the Q_o site, the folding of which is most likely altered by the duplication. By varying the redox state of the plastoquinone pool *in vivo*, we show that there is a dramatic decrease in the affinity of the Q_o site for plastoquinols, which is about 100 times lower in FUD2 than in wild type cells. Our results show that the value of the binding constant of plastoquinol to the Q_o site (2×10^4 M^{-1}) derived in [Kramer et al. (1994) *Biochim. Biophys. Acta 1184*, 251−262] may be extrapolated to *in vivo* conditions.

Most structure/function correlations are nowadays drawn from site-directed mutagenesis studies which are heavily dependent on pre-existing structural knowledge for the choice of target residues. As an alternative, function-directed mutagenesis does not require pre-existing structural knowledge but relies on selection or screening procedures based on an altered function as the major criterion of identification of the mutant strain to be studied. The chloroplast FUD2 mutant of the green unicellular alga *Chlamydomonas reinhardtii*, which has been isolated upon random mutagenesis with fluorodeoxyuridine and screened as a strain displaying altered photosynthesis function at the level of the cytochrome b_6f complex (Bennoun et al., 1978), accumulates near wild type levels of the cytochrome b_6f complex and produces a cytochrome b_6 variant of slightly higher apparent molecular mass (Lemaire et al., 1986). Thus, FUD2 offers a rather unique opportunity to look for a mutational event which causes both some structural and functional alterations of a protein which still accumulates to physiological levels in a mutant strain.

The purified cytochrome b_6f complex from *C. reinhardtii* (Pierre et al., 1995) is similar to that of higher plants. It comprises four high molecular mass subunits participating in electron transfer, cytochrome *f*, cytochrome b_6, the Rieske iron−sulfur protein and subunit IV, and three small transmembrane subunits of unknown function, molecular mass (MM) ≈ 4 kDa, each displaying a single hydrophobic α-helix and a short extramembrane segment. Cytochrome *f*, which binds one *c*-heme, is predicted to contain a single transmembrane α-helix at its C-terminus whereas cytochrome b_6, which binds two *b* hemes (b_l (low potential) and b_h (high potential)), and subunit IV have, respectively, four and three transmembrane α-helices. The Rieske protein, which binds a 2Fe-2S center, has a hydrophobic region at its N-terminus that could form a transmembrane α-helix, but extraction experiments and hydrophobicity analysis in *C. reinhardtii* strongly suggest that it does not span the membrane (de Vitry, 1994; Breyton et al., 1994). Crystallographic data are available for the hydrophilic domains of cytochrome *f* (Martinez et al., 1994) and the mitochondrial Rieske protein (Iwata et al., 1996).

The cytochrome b_6f complex mediates electron transfer in the thylakoid membranes, from a two-electron carrier, plastoquinol, to a one-electron carrier, plastocyanin. Electron transfer within the complex induces proton pumping and contributes to the formation of a membrane potential in the millisecond time range [see Hope (1993) for a review]. The plastoquinones bind on two sites of the cytochrome b_6f complex, the Q_o and Q_i sites, located, respectively, close to the lumenal and stromal sides of the membrane. The Rieske protein contributes to the formation of the Q_o site through its interaction with cytochrome b_6 and suIV on the lumenal face of the membrane. Its iron−sulfur cluster provides the

[†] This work was supported by the CNRS UPR 9072 and the E. C. Biotech program BIO2-CT93-0076. G. F. was recipient of a doctoral fellowship from the Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica. F. R. was supported by La Société de Secours des Amis des Sciences.

* Corresponding author. Tel: 33 1 43 25 26 09. Fax: 33 1 40 46 83 31. E mail: Wollman@IBPC.fr.

[‡] Present address: Centro di Studio del CNR sulla Biologia Cellulare e Molecolare delle Piante, Universita' degli Studi di Milano. Dipartimento di Biologia, Milano, Italy.

[§] Present address: Institut Curie, UMR 177, Bâtiment 110, Centre Universitaire d'Orsay, Orsay ville, France.

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1997.

S0006-2960(96)02717-1 CCC: $14.00

primary electron acceptor during quinol oxidation, and it interacts with inhibitors of the Q_o pocket (Link & Iwata, 1996 and references therein). According to the mechanism of the Q cycle the oxidation of plastoquinol at the Q_o site is associated with the reduction of both cytochromes *f* and b_l (Crofts et al., 1983; Mitchell, 1975). Oxidation of the *b* hemes then occurs through the reduction of a plastoquinone molecule at the Q_i site.

In this study we examined how the primary mutation in FUD2, that we describe as a 12 amino acid duplication in the cd loop connecting α-helices C and D of cytochrome b_6 on the lumen side of the thylakoid membranes, alters electron transfer through the cytochrome b_6f complex. We show that it lowers drastically the affinity for quinols of the Q_o site.

MATERIAL AND METHODS

Growth Conditions. The wild type and FUD2 mutant strains of *C. reinhardtii* were grown in Tris-Acetate Phosphate (TAP) medium (Gorman & Levine, 1965) at room temperature under 300 lx of continuous illumination.

Nucleic Acid Analyses. Comparative analysis of the *petB* gene from FUD2 and wild type strains was performed by PCR amplification of fragments from total DNA, using the following oligonucleotide primers: cod0 (5′-AGCCTAATGGTCATGTCACA-3′), cod1 (5′-ACCAAGTTGGTTACTGGGCGGT-3′), rev1 (5′-GTGCATTAACATGAAAACAGCTGT-3′), and rev3 (5′-TCAATGGCCAACTGCCTTGGA-3′).

The PCR conditions were 40 cycles (90 °C 45 s, 55 °C 60 s, 74 °C 90 s) terminated by 74 and 37 °C steps for 5 and 1 min, respectively. The cod0/rev3 fragment encompassing the whole *petB* gene from FUD2 was sequenced on both strands following the same strategy and the same set of oligonucleotide primers as in (Buschlen et al., 1991). Sequencing was performed by the dideoxy chain termination method.

Protein Analysis. Cytochrome b_6f complexes were purified by Hecameg[1] solubilization of thylakoid membranes and sucrose gradient centrifugation of the supernatant (Pierre et al., 1995). Hecameg was used at a 28 mM concentration for solubilization of FUD2 thylakoid membranes, after having checked that it did not detach the Rieske protein from cytochrome b_6f complexes purified from the wild type. For immunoblotting, antibodies against the Rieske protein (Breyton et al., 1994), cytochrome *f*, suIV and cytochrome b_6 (Kuras & Wollman, 1994), PetG (Pierre & Popot, 1993), PetL (Takahashi et al., 1996), PetM (Pierre & Popot, 1993) were used at 1/200 dilution when detected by iodinated protein A or at 1/4000 when using enhanced chemiluminescence (ECL kit; Amersham) except for petL and petM where it was used at 1/200. Urea/SDS−PAGE and TMBZ staining were performed as previously described (Kuras & Wollman, 1994).

Spectroscopic Analysis. When used without pretreatment, exponentially growing cells (2×10^6 cells/mL) were concentrated 25 times in 50 mM phosphate-KOH buffer pH 7.2 in the presence of 10% Ficoll to avoid cell sedimentation. When treated with pBQ before spectroscopic analysis, the algae were harvested at a density of 2×10^6 cells/mL, concentrated to 10^7 cells/mL in 50 mM HEPES pH 7.2, vigorously aerated by shaking for 1 h, which ensures plastoquinol oxidation, and then incubated for 5 min with pBQ 500 μM. Algae were then centrifuged, washed twice in the same medium without pBQ, and finally resuspended at 5×10^7 cells/mL in 50 mM phosphate-KOH buffer pH 7.2 in the presence of 10% Ficoll.

Spectroscopic measurements were made at room temperature with a home built spectrophotometer described in (Joliot et al., 1980) and modified as in (Joliot & Joliot, 1994). When used in anaerobic conditions, algae were kept under an argon flux in a large reservoir, connected to the measuring cuvette. When used in aerobic conditions, algae incubated under argon atmosphere were subsequently flowed with air, to reoxidize the plastoquinone pool.

Actinic flashes were provided by a xenon flash (3 μs duration at half-height) filtered through red filter (Schott RG 8). Flashes were fired at a frequency of 0.15 Hz. In anaerobiosis, under these illumination conditions, cytochromes b_h and *f* are rereduced in the dark time between flashes (Joliot & Joliot, 1988).

The transmembrane potential was estimated by the amplitude of the electrochromic shift at 515 nm, which yields a linear response with respect to membrane potential (Junge & Witt, 1968). Under our experimental conditions, the kinetics of the electrochromic signal displays two sequential phases (Joliot & Delosme, 1974), a fast phase completed in less than 1 μs, associated with PS1 and PS2 charge separations (phase a) and a slow phase in the millisecond time range, associated with the turnover of the b_6f complex (phase b). PS2 absorption changes were prevented by preilluminating the samples in the presence of DCMU (10 μM) and HA (1 mM), (Bennoun, 1970). In this case, phase a is a measure of PS1 charge separations.

Kinetic analysis of phase b requires deconvolution of the membrane potential decay. We assumed the latter process has first-order kinetics. Kinetics of phase b were then corrected assuming that the rate of decay of the membrane potential was linearly related to the actual value of the membrane potential.

Cytochrome *f* redox changes were evaluated as the difference between absorption at 554 nm and a baseline drawn between 545 and 573 nm. The signal was corrected for a contribution of the electrochromic shift (5% of the signal measured at 515 nm).

Estimation of the amount of cytochrome *f* photooxidizable *in vivo* was performed by illumination of pBQ-treated cells with a series of 15 saturating flashes in the presence of DCMU and HA. Under these conditions the quinone pool is fully oxidized, PS II activity is inhibited and 15 flashes induce the complete oxidation of cytochrome *f*. The ratio of the amplitude of this signal to that of phase a is proportional to the ratio of cyt b_6f to PS1.

Cytochrome *b* redox changes were measured as the difference between the absorption changes at 564 and 573 nm.

NQNO was kindly provided by Professor J. Whitmarsh (Urbana University, IL, U.S.A.).

[1] Abbreviations: Cyt, cytochrome; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimetylurea; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HA, hydroxylamine; Hecameg, 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glycopyranoside; HEPES, (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate)); NQNO,2-*N*-4-hydroxyquinoline-*N*-oxide; pBQ, *p*-benzoquinone; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Su, subunit; TMBZ, 3,3′,5,5′-tetramethylbenzidine; Tris, tris-(hydroxymethyl)aminomethane.

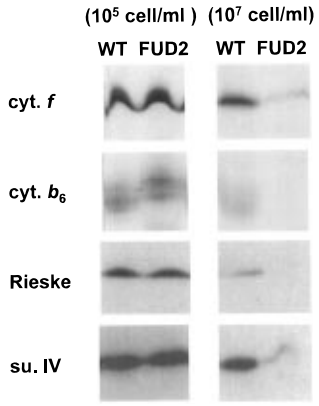

FIGURE 1: Accumulation of the cytochrome b_6f subunits in wild type and FUD2 from cultures in exponential and late stationary growing phases. Cells were analyzed by SDS−PAGE (12−18% acrylamide, 8 M urea). Cytochrome *f* and cytochrome b_6 were heme stained by TMBZ and H_2O_2. The Rieske protein and subunit IV were detected by immunoblotting.

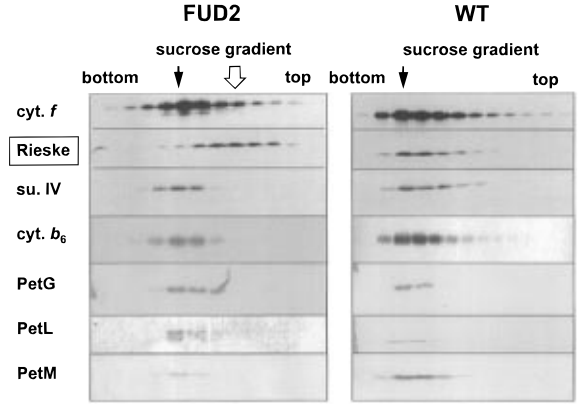

FIGURE 2: Binding of the Rieske protein to the b_6f complex is loosened in FUD2. Thylakoid membranes were solubilized with Hecameg. The supernatant was centrifuged on a 10−30% sucrose gradient. Fractions of the gradient were collected, analyzed by SDS−PAGE (12−18% acrylamide, 8 M urea), and immunodetected.

RESULTS

Biochemical Characterization of Cytochrome b_6f Complexes in the FUD2 Mutant. Previous studies have shown that FUD2 has a fluorescence phenotype typical of mutants blocked in electron transfer at the level of cytochrome b_6f complexes (Bennoun et al., 1978). In addition, it synthesizes a cytochrome b_6 variant of higher apparent molecular mass upon electrophoresis which accumulates as a heme-binding polypeptide larger than wild type cytochrome b_6 (Lemaire et al., 1986). We compared the accumulation of the various cytochrome b_6f complex subunits in FUD2 with that in the wild type at two stages of a cell culture, in exponentially growing cells and in cells in their late stationary phase (Figure 1). The apparently larger size of cytochrome b_6 in FUD2 is visible in Figure 1, left. The content in cytochrome b_6 in the FUD2 and wild type strains was similar on a chlorophyll basis at an early stage of the cell culture, as was the content in the six other subunits of the cytochrome b_6f complex (Figure 1, left). Similar results were obtained from an estimation of cytochrome *f* photooxidizable *in vivo* with respect to the PS1 reaction centers (see Materials and Methods, data not shown). In contrast, the whole set of subunits of the cytochrome b_6f complex was barely detectable in FUD2 cells from the late stationary phase (Figure 1, right). Thus, cytochrome b_6f complexes in FUD2, which contain a modified form of cytochrome b_6, are less stable than their wild type counterparts.

Cytochrome b_6f complexes were then purified from exponentially growing cells of FUD2 using an Hecameg solubilization procedure described in (Pierre et al., 1995). Figure 2 shows an immunoblot analysis of the distribution of their subunits upon sucrose gradient centrifugation in comparison to that in the wild type: cyt *f*, cyt b_6, suIV, PetG, PetL, and PetM subunits comigrated both in the FUD2 and wild type gradients, which is indicative of their stable assembly in the two strains. In addition their distribution peaked in fractions of similar sucrose density, 21.5% for the wild type and 20.2% for FUD2 at a position known to be that of the dimeric form of cytochrome b_6f complexes in the wild type (Breyton et al., 1995). However, at variance with those from the wild type, cytochrome b_6f complexes from FUD2 were depleted in most of the Rieske protein. This subunit was now found in upper gradient fractions, peaking at a sucrose density of 16.9%.

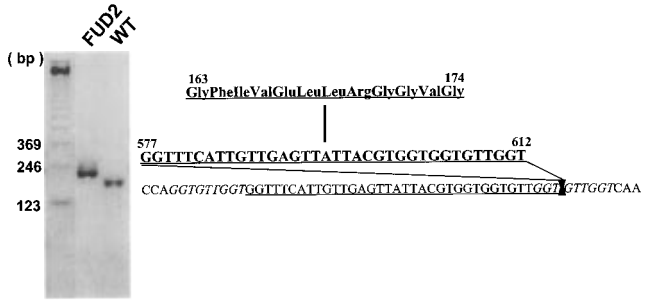

FIGURE 3: The mutation in FUD2 is a 36 bp duplication in the *petB* gene. Size fractionation of PCR-amplified products using cod0 and rev3 primers from FUD2 and wild type DNA. Sequence of 36 bp duplicated in the wild type *petB* gene is underlined. Nine base pairs direct repeats responsible for duplication are shown in italics. Nucleotides and residues are numbered according to (Buschlen et al., 1991).

Identification of the petB Mutation in Mutant FUD2. The slower electrophoretic migration of the protein in FUD2, illustrated on Figure 1, could originate from a genuine change in the molecular mass of apocytochrome b_6 as well as from variations in the state of denaturation of the protein since heme-binding to cytochrome b_6 is preserved upon urea/SDS denaturing electrophoresis. Therefore, we looked for possible changes in the sequence of the chloroplast *petB* gene by performing a PCR amplification of various *petB* fragments. Using cod0 and rev3 primers (see Materials and Methods) which amplified the whole *petB* gene, we observed fragments of somewhat larger size in FUD2 than in the wild type (not shown). We still observed a size difference between DNA fragments from the wild type and FUD2 when we narrowed them down using primers cod1/rev1, which amplified a *petB* fragment of ~200 bp, 250 bp upstream from the *petB* stop codon (Figure 3). This fragment was estimated to be ~40 bp larger in the FUD2 mutant. Accordingly, mRNA filter hybridization analysis showed that the petB mRNA was slightly larger in FUD2 relative to wild type (experiments not shown).

We then sequenced the cod1/rev1 fragments from both the wild type and FUD2 mutant and found, in the latter, a duplication of a 36 base pair sequence which is flanked by two 9 bp direct repeats in the wild type (Figure 3). This

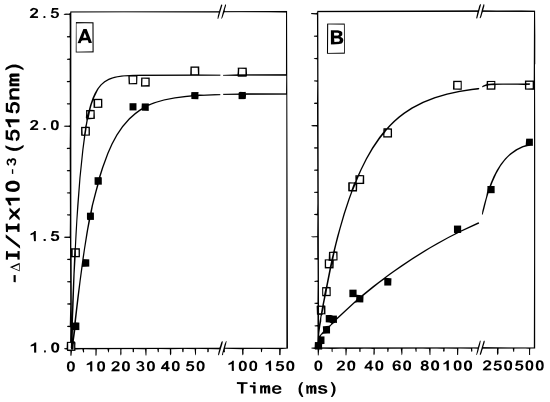

FIGURE 4: Slow electrochromic reaction (b phase) in wild type and FUD2 mutant. Algae (5×10^7 cells mL^{-1}) under anaerobic conditions, was illuminated with nonsaturating red flashes hitting 30% of centers at a frequency of 0.15 Hz. The incubation medium consisted of phosphate-KOH, 50 mM, pH 7.2, Ficoll 10%. DCMU and HA were added at the concentration of 10 μM and 1 mM to block PS2 activity. Kinetics were corrected for membrane potential decay as described in the Materials and Methods. Measurements were performed in the absence (closed square) and presence (open square) of 1 μM FCCP. (A) Wild type cells; (B) FUD2 cells. The curves are the best fits of the data using a single exponential.

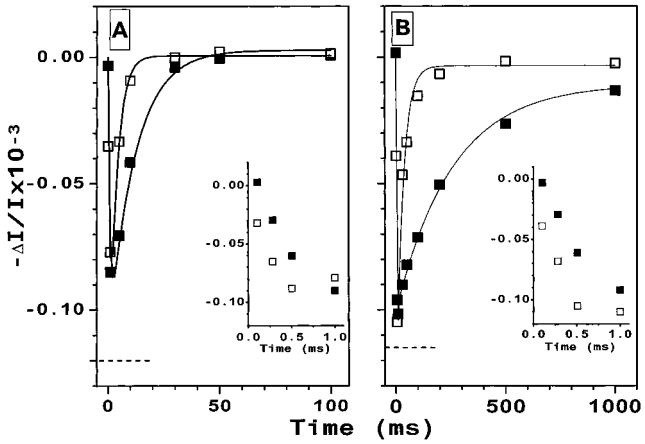

FIGURE 5: Cytochrome *f* redox changes in wild type and FUD2 cells. Cytochrome *f* redox changes were measured as described in the Materials and Methods. Same conditions as in Figure 4. Actinic flashes were saturating: no addition (open square); FCCP 1 μM (open square). (A) Wild type cells; (B) FUD2 cells. Curves are best fits to data points. The dashed lines indicated the maximal extent of cyt *f* oxidation that can be calculated from data fit. The inset shows the oxidation kinetics on a smaller time scale.

duplication at the gene level resulted in a duplication at the protein level of 12 residues, ^{163}GFIVELLRGGVG^{174}, in position 175 between helices C and D of apocytochrome b_6. Sequencing of the whole *petB* gene from FUD2, born within the cod0/rev3 amplified PCR fragment, showed no other difference with the sequence of the wild type allele of the *petB* gene.

Kinetic Analysis of the Electrochromic Shift in Wild Type and FUD2 Cells. We performed a detailed spectroscopic analysis of the functional characteristics of the modified cytochrome b_6f complex *in vivo*. The kinetics of the b phase of the 515 nm electrochromic shift in FUD2 and in wild type algae are shown in Figure 4. Both cell samples were placed in anaerobic conditions, preilluminated in the presence of DCMU and HA in order to inactivate PS2, and treated, or not, with FCCP. This latter chemical was used to collapse the transmembrane potential built in darkness. It has been shown that, after dark adaptation of algae to anaerobic conditions, the fermentation pathway is sufficient to produce ATP, the hydrolysis of which generates a large electrochemical gradient across the thylakoid membrane (Bennoun, 1982).

In wild type cells, the amplitude of the b phase was about 1.2 times that of the fast phase (Figure 4A). The half-time of this reaction was of ~7 ms under saturating light illumination. The addition of the ionophore FCCP at a concentration of 1 μM enhanced the rate of the reaction by a factor of ~3 ($t_{1/2}$, ~2.4 ms), in agreement with previous observations (Joliot & Joliot, 1994).

In FUD2 cells, the electrochromic signal had still a nearly exponential character, with a half-time of ~105 ms, indicating a pseudo-first-order reaction for electron transfer in the complex (Figure 4B). The phase b/phase a ratio was the same as in the wild type. In the presence of FCCP, the kinetics had a half-time of ~19 ms. Thus, the decrease in the rate of phase b in FUD2 was more marked in the absence of the ionophore, ~15 times, than in its presence, ~8 times.

Cytochrome f Redox Changes in Wild Type and FUD2 Cells. Figure 5 shows a comparison of the kinetics of cyt *f* redox changes in the wild type and FUD2 cells in the same experimental conditions as described above. Data are normalized to the amplitude of the fast electrochromic signal, that is to the same amount of PS1 charge separations. In the two strains, a rapid increase in absorbance corresponding to a flash induced oxidation of cytochrome *f* was followed by a slower rereduction which requires electron transfer from the reduced plastoquinones. In wild type cells (Figure 5A), the half-time of cytochrome *f* oxidation was ~500 μs in the absence of FCCP and ~250 μs in its presence (see inset in Figure 5), in agreement with previous reports (Zhou et al., 1996; Kuras et al., 1995; Delosme, 1991). Half-times of ~6 and ~2.7 ms were obtained for cyt *f* reduction in the absence and presence of FCCP, respectively. In FUD2 cells the oxidation of cyt *f* had nearly the same half-time as in wild type cells (Figure 5B). In addition, we found that the same amount of cyt *f* was oxidized by plastocyanin (as indicated by the dashed lines in Figure 5) in wild type and FUD2 cells. These observations clearly show that the plastocyanin-mediated interaction between cytochrome b_6f complex and photosystem1 is unaltered in FUD2 cells. In contrast, the reduction of cyt *f* was markedly slowed down in FUD2 mutant cells with half-times of ~140 and ~20 ms in the absence and presence of FCCP, respectively. It should be noted that the half-times of phase b, which reflect a transmembrane reaction, were similar to those of cytochrome *f* reduction both in the wild type and in FUD2 cells, when treated with FCCP. This is in agreement with the hypothesis of a concerted oxidation of the plastoquinol by the cytochrome *f* and b_6 [Cramer et al. (1996) for a review]. However, in FUD2 without FCCP treatment, the half-time for cytochrome *f* reduction was slightly larger than what we calculated for phase b (140 vs 105 ms). We attribute this poor correlation to an increased inaccuracy of the deconvolution procedure for the kinetics of the b phase when it slows down to rates close to those of the membrane potential decay (Joliot & Joliot, 1988).

Cytochrome b_6 Redox Changes in Wild Type and FUD2 Cells. In algae, the two hemes of cytochrome b_6 have very similar spectra (Joliot & Joliot, 1988). This prevented us from discriminating spectrally between redox changes of

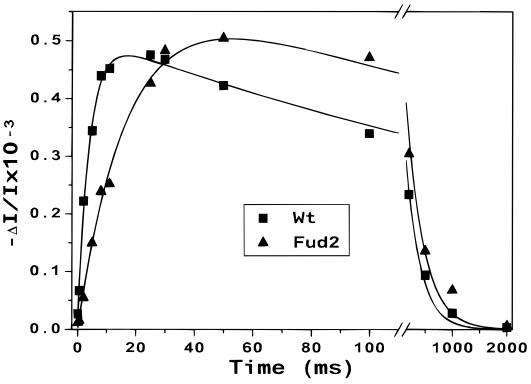

FIGURE 6: Cytochrome b_6 reduction in wild type and FUD2 cells. Same conditions as in Figure 4. NQNO 4 μM was added to slow down cytochrome b_6 oxidation through the Q_i site. FCCP 1 μM was present in both samples to collapse permanent membrane potential.

hemes b_h and b_l. However, the two hemes can be distinguished on kinetic basis, taking advantage of their different rates of rereduction in the dark in anaerobic conditions (Joliot & Joliot, 1988). Under these conditions, the rereduction of cytochrome b_h occurs within a few seconds whereas rereduction of cytochrome b_l takes more than 10 min. Thus, under the conditions of illumination that we used in this study (repetitive flashes at a frequency of 0.15 Hz) cyt b_h is rereduced during the dark time interval between two flashes, while cyt b_l is not. The electron transfer reactions sequence is then reduction of cyt b_l by the plastoquinol followed by its oxidation by cyt b_h, which is itself triggered by the oxidation of cyt b_h at the Q_i site. In both the wild type and FUD2 cells, we observed a small absorption decrease, due to cyt b_l reduction, followed by a larger oxidation signal. This oxidation signal showed the same decreased rate as the b phase of the electrochromic shift in FUD2 (not shown). One cannot draw unequivocal conclusions from these observations because the oxidation process can only take place after cytochrome b_l has been reduced at the expense of PQH_2. Therefore, a decrease in the oxidation rate may arise either from an alteration of the electron transfer between the two *b* hemes or from a decreased rate of cytochrome b_l reduction by PQH_2. To test which reaction was slowed down in FUD2, we studied cytochrome b_6 absorption changes in the presence of NQNO and under the same illumination conditions as in the preceding experiment. This compound slows down cytochrome b_h oxidation at the Q_i site (Selak & Whitmarsh, 1982), thereby allowing the measure of the full extent of the reduction kinetics of cyt b_l. At 4 μM NQNO we obtained maximal inhibition of cytochrome b_6 oxidation, without any effects on the kinetics of PQH_2 oxidation (data not shown). The kinetics of cytochrome b_l reduction in the presence of NQNO and FCCP are shown on Figure 6. This reaction is much slower in the mutant than in the wild type. After correction for the oxidation kinetics, we obtained half-times for cytochrome b_l reduction of ~17 ms in FUD2 vs ~2.5 ms in wild type cells. These values are very close to those observed for cytochrome *f* reduction (Figure 5). Thus, we observed a concerted decrease in the reduction rates of cytochrome *f* and cytochrome b_l in FUD2. The major consequence of the 12 amino acid duplication in cytochrome b_6 is to slow down drastically the concerted oxidation of the plastoquinol at the Q_o site.

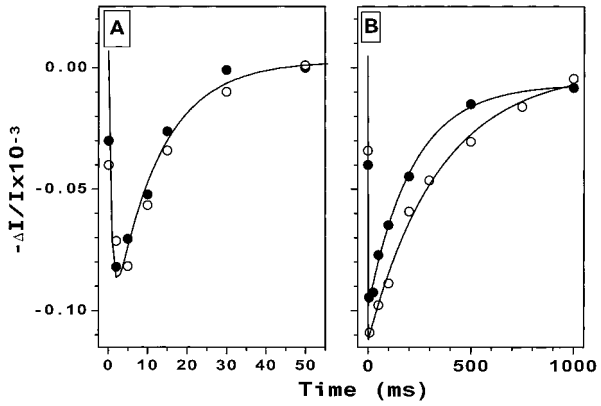

FIGURE 7: Cytochrome *f* reduction under anaerobic and partial aerobic conditions in wild type and FUD2 cells. Cells were incubated under anaerobic conditions (closed circle) or partial aerobic conditions (open circle) as described in the Materials and Methods. The fraction of reduced plastoquinone pool was estimated to be 100% and 50%, respectively, from fluorescence induction measurements. (A) Wild type cells; (B) FUD2 cells. Other conditions as in Figure 4.

Effect of the Concentration of PQH_2 on Cytochrome b_6f Turnover. Since reduction of cytochromes b_l or *f* involves a PQH_2 bound at the Q_o site, two hypotheses may be considered to account for the functional alteration observed in FUD2: a decrease in the rate of the concerted electron transfer reactions from bound plastoquinol to the cytochromes or a decreased affinity of the Q_o site for its substrate. Obviously, the two possibilities are not mutually exclusive. We addressed this question by changing the concentration of PQH_2 *in vivo* (Figure 7). The experiments described below were performed in the absence of FCCP, since its addition in the dark induces a strong reduction of the plastoquinone pool (Bulté et al., 1990).

In a first experiment, the cells were incubated under argon, to achieve a complete reduction of the plastoquinone pool. This was verified by evaluating the area bound by the fluorescence induction curve and its F_{max} asymptote. This parameter is a relative measure of the size of the plastoquinone pool (Murata et al., 1966). In another experiment, the same algae were flowed with air in order to induce a reoxidation of the plastoquinol pool. We estimated that 50% of the plastoquinol pool was reoxidized by such a treatment of both the wild type and FUD2 cells.

Cytochrome *f* reduction was measured under these two conditions (Figure 7). In anaerobic conditions the results are identical to those presented in Figure 5. After oxygenation, wild type cells retained the same kinetics of cytochrome *f* reduction, although they contained a lower concentration of PQH_2 (Figure 7A). This is indicative of a saturation of the occupancy at the Q_o site by the plastoquinol present in the membrane. By contrast, there was a marked decrease in the rate of cytochrome *f* reduction upon lowering the concentration of PQH_2 in FUD2: it displayed a half-time of ~270 ms (Figure 7B).

Effect of a pBQ Treatment on Cytochrome f Reduction in Wild Type and FUD2 Cells. We further analyzed the kinetics of cytochrome *f* reduction as a function of PQH_2 concentration in another series of experiments using benzoquinone-treated cells. It has been shown that this treatment inhibits the reducing pathway of the plastoquinone pool, which is activated by anaerobiosis. Thus, the pool is maintained in its oxidized state (Diner & Joliot, 1976). After two saturating

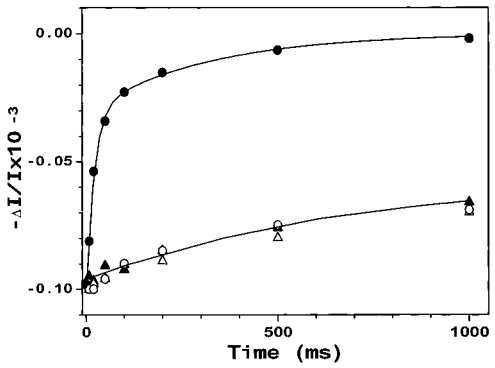

FIGURE 8: Effect of *p*-benzoquinone treatment on cytochrome *f* reduction. Algae was treated with pBQ as described in the Materials and Methods. Same conditions as in Figure 4. Kinetics were measured after the second of two saturating red flashes 20 ms apart: wild type cells, no addition (closed circle); wild type cells, with DCMU 10 μM and HA 1 mM (open circle); FUD2 cells, no addition (closed triangle); FUD2 cells, with DCMU 10 μM and HA 1 mM (open triangle).

flashes, the only PQH_2 available for the reduction of cytochrome b_6f is the one formed at the acceptor side of PS2. The kinetics of cytochrome *f* obtained under these conditions are shown in Figure 8. The data are normalized to the amplitude of the fast phase of the 515 nm absorbance change, measured after addition of DCMU and HA to the algae.

We observed a biphasic reduction of cytochrome *f* in the wild type cells, with a fast phase of half-time ~14 ms and a very slow phase in the time range of seconds, probably due to equilibration with the ambient potential. The fast phase was abolished by the addition of DCMU and HA, indicating that it originated from the oxidation of the single PQH_2 formed at the acceptor side of PS2. In contrast to the wild type behavior, we observed no fast phase in the reduction of cytochrome *f* in the pBQ treated cells from FUD2. They displayed only the DCMU-insensitive slow phase (Figure 8). Thus, the production of about 1 PQH_2/cyt b_6f in FUD2 was not sufficient to allow significant electron transfer through the Q_o site. These results further confirmed the decreased affinity of the Q_o site for PQH_2 in FUD2 cells.

DISCUSSION

Effect of the Mutation in FUD2 on the Assembly of Cytochrome b_6f Complexes. The duplication of 12 amino acids in the cd loop of cytochrome b_6 in FUD2 occurred in a region of the protein which has been implicated in the folding of the Q_o site in the case of bacterial and mitochondrial cytochrome *b* (Brasseur et al., 1996). This region has also been identified as participating in the binding of the Rieske protein to cytochrome *b* in *Saccharomyces cerevisiae* (Giessler et al., 1994) and *Rhodobacter capsulatus* (Saribas et al., 1996). In this study, we observed that, indeed, the mutation in cytochrome b_6 loosens the binding of the Rieske protein to the rest of the complex as evidenced by its release from the cytochrome b_6f fractions upon detergent solubilization of the membranes and sucrose gradient centrifugation. The interaction of the Rieske protein with the other subunits of the cytochrome b_6f complex is, however, sufficiently preserved to allow their stoichiometric accumulation *in vivo*. Were the Rieske protein partly dissociated from the cytochrome b_6f complex *in vivo*, it would have been rapidly degraded, as is the case in other mutants defective in cytochrome b_6f assembly (Kuras & Wollman, 1994).

The altered interaction between the Rieske protein and cytochrome b_6 is most likely responsible for the loss of the whole complex in aging cells of FUD2. Indeed, *Chlamydomonas* mutants lacking the Rieske protein become depleted in cytochrome b_6f complexes in aging cells (de Vitry & Wollman, unpublished observation). Similarly, a *Chlamydomonas* strain deleted for the gene encoding the *PetL* product, in which the binding of the Rieske protein to the cytochrome b_6f complex is also loosened, displayed a drastic decrease in cytochrome b_6f content upon aging (Takahashi et al., 1996).

In yeast, the cytochrome bc_1 complex, when purified from a deletion strain lacking a small hydrophobic subunit of 8.5 kDa, looses the Rieske protein with which it was associated in the starting membranes (Brandt et al., 1994). On the other hand, the cytochrome bc_1 complex purified from a yeast mutant of cytochrome *b*, G137E, lacked both the Rieske protein and the small 8.5 kDa subunit, which were associated in the starting membranes (Giessler et al., 1994). We observed no such correlation between the behavior of the Rieske protein and that of either of the three small hydrophobic subunits of the cytochrome b_6f complex, PetG, PetL, and PetM. These ones, which show no significant homology with the yeast 8.5 kDa subunit, remained associated with the purified cytochrome b_6f complex in FUD2 even though the Rieske protein was lost.

Mitochondrial and chloroplast *bc/bf* complexes are purified in an active form as dimers (Huang et al., 1994 and references therein). During purification, loss of the Rieske protein and monomerization often, but not always, occur simultaneously (Breyton et al., 1995). Here, we observed that the dimeric form was retained although the Rieske protein was detached from the cytochrome b_6f complex in mutant FUD2. This illustrates that the loss of the Rieske protein does not lead necessarily to monomerization of the complex. We conclude that the Rieske protein does not control the state of oligomerization of the cytochrome b_6f complex.

The Mutation in FUD2 Lowers the Affinity of the Q_o Site for Plastoquinols. The present study shows that the mutation in FUD2 altered specifically the Q_o site of the cytochrome b_6f complex. We observed an 8-fold decrease in the rate of the concerted reduction of cytochrome *f* and cytochrome b_l. We found no evidence for significant changes in other aspects of the activity of the cytochrome b_6f complex. In particular, the rates of cytochrome *f* oxidation remained similar to those in the wild type, which indicates a preservation of the interactions between cytochrome *f* and plastocyanin.

Decreasing the concentration of PQH_2 in FUD2 cells led to a further decrease in the rates of reduction of cytochrome *f*. This is a strong indication that the major effect of the present duplication in the cd loop was to decrease the affinity of the mutated cytochrome b_6f complex for PQH_2. In bacterial and mitochondrial bc_1 complexes several cytochrome *b* mutations were previously reported to decrease the affinity of the Q_o site for the quinone substrates (Ding et al., 1995; Crofts et al., 1995; Giessler et al., 1994).

We observed that the kinetics of cytochrome *f* reduction in the wild type remained identical whether the plastoquinone pool was fully reduced or half-reduced. This is an indication that the Q_o site was fully occupied in both circumstances.

In contrast, we observed a 2-fold decrease in the reduction rates of cytochrome *f* when the plastoquinol concentration dropped by a factor of 2 in FUD2. This means that even under conditions in which the PQ pool was fully reduced, the occupancy of the Q_o site was far from saturation. Therefore, binding of PQH_2 is rate-limiting in the process of electron transfer in FUD2. The affinity constant for PQH_2 is thus dramatically decreased by the mutation.

From competition experiments based on the measure of duroquinone/plastoquinone cytochrome b_6f turnovers, Kramer et al. reported a value of 2×10^4 M^{-1} for the binding constant of the plastoquinol at the Q_o site of cytochrome b_6f in isolated spinach thylakoids (Kramer et al., 1994). Given this value and assuming that, in the thylakoids membranes, the plastoquinone concentration is 1 mM (Rich, 1982) and the stoichiometry of plastoquinone per cytochrome b_6f complex is 10 (P. Joliot & A. Joliot, unpublished results), it is possible to compute[2] the expected decrease in the rate of plastoquinol oxidation at the Q_o site upon decreasing the concentration of plastoquinol: the oxidation rate at Q_o should be slowed down by factors of 1.06 and 1.90, respectively, when reduction of the PQ pool is only 50% or 10% of its maximal value. These should be compared with the decrease factors that we observed experimentally, 1.08 and 2 when the pool was respectively half-reduced or 10% reduced, that is after pBQ treatment and two flashes generating only 1 plastoquinol/cytochrome b_6f. The remarkable agreement between expected and observed decreases of the rate of oxidation of the plastoquinol at the Q_o site leads us to conclude that the value of the binding constant of plastoquinol at the Q_o site (2×10^4 M^{-1}) drawn from isolated spinach thylakoids using exogenous quinones (Kramer et al., 1994) applies to intact system with native quinones.

Thus, we conclude that, in an unaltered photosynthetic apparatus, the rate of electron transfer from PS2 to PS1 is not limited by the diffusion of PQH_2, but by the intrinsic properties of the cytochrome complex, as previously suggested (Mitchell et al., 1990; Joliot & Joliot, 1992).

The same calculation applied to the FUD2 led us to estimate a decreased affinity of the Q_o site of about 2 orders of magnitude, yielding a 2.5×10^2 $M^{-1} < K_a < 8 \times 10^2$ M^{-1}.

Effects of the Resting Proton Electrochemical Gradient. An intriguing observation emerging from this study is the differential sensitivity of the mutant and wild type membranes to the addition of ionophores. Uncoupling thylakoid membranes in FUD2 had a stronger effect on PQH_2 oxidation kinetics than uncoupling wild type membranes. A similar observation has been recently reported for a PetL mutant of *C. reinhardtii* (Takahashi et al., 1996).

A possible explanation for this effect is that the magnitude of the resting electrochemical gradient is not the same in wild type and FUD2 strains, being larger in the latter cells. This hypothesis can be assessed through the effect of this resting proton electrochemical gradient on cyt *f* oxidation kinetics. Delosme (1991) has shown that the rate of cyt *f* oxidation is dramatically slowed down upon incubation of algae under anaerobic conditions and that addition of ionophores restored a fast kinetics. This effect has been interpreted as reflecting either a reversible association between cyt b_6f and PS1 or changes in plastocyanin mobility induced by the building of the resting proton gradient. Whatever is the actual control mechanism, the rate of cyt *f* oxidation can be considered as an indicator of the magnitude of the resting proton electrochemical potential *in vivo*, independent of the intrinsic kinetic properties of the cytochrome b_6f complex.

As reported in Figure 5, we observed very similar rates of cyt *f* oxidation in wild type and FUD2 cells both in the absence or presence of FCCP. Therefore, the resting proton electrochemical gradient are similar in the mutant and wild type cells.

As an alternative hypothesis, we favor a larger sensitivity toward the resting proton electrochemical gradient of the electron transfer at the Q_o site in FUD2 and PetL mutants as compared to that in the wild type. The molecular basis for this larger sensitivity would stem from the loosened binding of the Rieske protein, which has been observed in both mutants. In the presence of an electrochemical proton gradient, the interaction of the Rieske protein with the rest of the protein complex would be further weakened, thereby further altering the conformation of the Q_o site and its electron transfer ability.

[2] We consider the ratio of the maximum rate (k_{max}) of oxidation of a plastoquinol at the Q_o site (observed when the pool is fully reduced) versus this rate when the pool is partially reduced (k). The model assumes that the observed rate is proportional to the fraction of bound substrate and to the reaction rate. Thus, k_{max}/k is equal to the ratio of bound PQH_2 when the pool is fully reduced versus the amount of bound PQH_2 for a given concentration of quinol that is

$$\frac{k_{max}}{k} = \frac{(C + PQH_2^{max} + K_D) - \sqrt{(C + PQH_2^{max} + K_D)^2 - 4 \cdot PQH_2^{max} \cdot C}}{(C + PQH_2 + K_D) - \sqrt{(C + PQH_2 + K_D)^2 - 4 \cdot PQH_2 \cdot C}}$$

where C is the concentration of cytochrome b_6f complex and PQH_2 the concentration of plastoquinol.

ACKNOWLEDGMENT

We thank J.-L. Popot and R. Kuras for stimulating discussions and J. Girard-Bascou for the care taken of the mutant strains of *Chlamydomonas*.

REFERENCES

Bennoun, P. (1970) *Biochim. Biophys. Acta 216*, 357−363.
Bennoun, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4352−4356.
Bennoun, P., Masson, A., Piccioni, R., & Chua, N.-H. (1978) in *Photosynthesis* (Akoyunoglou, G., Ed.) pp 721−726, Elsevier-North/Holland Biomedical Press, Amsterdam.
Brandt, U., Uribe, S., Schagger, H., & Trumpower, B. L. (1994) *J. Biol. Chem. 269*, 12947−12953.
Brasseur, G., Sami Saribas, A., & Daldal, F. (1996) *Biochim. Biophys. Acta 1275*, 61−69.
Breyton, C., de Vitry, C., & Popot, J.-L. (1994) *J. Biol. Chem. 269*, 7597−7602.
Breyton, C., Tribet, C., Olive, J., Recouvreur, M., & Popot, J.-L. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) pp 591−594, Kluwer Academic, Netherlands.
Bulté, L., Gans, P., Rebeille, F., & Wollman, F.-A. (1990) *Biochim. Biophys. Acta 1020*, 72−80.
Buschlen, S., Choquet, Y., Kuras, R., & Wollman, F. A. (1991) *FEBS Lett. 284*, 257−262.
Cramer, W. A., Soriano, G. M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S. E., & Smith, J. L. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 477−508.
Crofts, A. R., Meinahrdt, S. W., Jones, K. R., & Snozzi, M. (1983) *Biochim. Biophys. Acta 723*, 202−218.

Crofts, A. R., Barquera, B., Bechmann, G., Guergova, M., Salcedo-Hernandez, R., Hacker, B., Hong, S., & Gennis, R. B. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) pp 493−500, Kluwer Acad. Pub., Dordrecht, Netherlands.
Delosme, R. (1991) *Photosynth. Res. 29*, 45−54.
de Vitry, C. (1994) *J. Biol. Chem. 269*, 7603−7609.
Diner, B. A., & Joliot, P. (1976) *Biochim. Biophys. Acta 423*, 479−498.
Ding, H., Moser, C. C., Robertson, D. E., Tokito, M. K., Daldal, F., & Dutton, P. L. (1995) *Biochemistry 34*, 15979−15996.
Giessler, A., Geier, B. M., de Rago, J. P., Slonimski, P. P., & von Jagow, G. (1994) *Eur. J. Biochem. 222*, 147−154.
Gorman, D. S., & Levine, R. P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1665−1669.
Hope, A. B. (1993) *Biochim. Biophys. Acta 1143*, 1−22.
Huang, D., Everly, R. M., Cheng, R. H., Heymann, J. B., Schagger, H., Sled, V., Ohnishi, T., Baker, T. S., & Cramer, W. A. (1994) *Biochemistry 33*, 4401−4409.
Iwata, S., Saynovits, M., Link, T. A., & Michel, H. (1996) *Structure 4*, 567−579.
Joliot, P., & Delosme, R. (1974) *Biochim. Biophys. Acta 357*, 267−284.
Joliot, P., & Joliot, A. (1988) *Biochim. Biophys. Acta 933*, 319−333.
Joliot, P., & Joliot, A. (1992) *Biochim. Biophys. Acta 1102*, 53−61.
Joliot, P., & Joliot, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1034−1038.
Joliot, P., Beal, D., & Frilley, B. (1980) *J. Chim. Phys. 77*, 209−216.
Junge, W., & Witt, H. T. (1968) *Z. Naturforsch. 24b*, 1038−1041.
Kramer, D. M., Joliot, A., Joliot, P., & Crofts, A. R. (1994) *Biochim. Biophys. Acta 1184*, 251−262.
Kuras, R., & Wollman, F.-A. (1994) *EMBO J. 13*, 1019−1027.
Kuras, R., Wollman, F. A., & Joliot, P. (1995) *Biochemistry 34*, 7468−7475.
Lemaire, C., Girard-Bascou, J., Wollman, F.-A., & Bennoun, P. (1986) *Biochim. Biophys. Acta 851*, 239−248.
Link, T. A., & Iwata, S. (1996) *Biochim. Biophys. Acta 1275*, 54−60.
Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A., & Smith, J. L. (1994) *Structure 2*, 95−110.
Mitchell, P. (1975) *FEBS Lett. 59*, 137−199.
Mitchell, R., Spillmann, A., & Haehnel, W. (1990) *Biophys. J. 58*, 1011−1024.
Murata, N., Nishimura, M., & Takamiya, A. (1966) *Biochim. Biophys. Acta 120*, 23−33.
Pierre, Y., & Popot, J.-L. (1993) *C. R. Acad. Sci. Paris 316*, 1404−1409.
Pierre, Y., Breyton, C., Kramer, D., & Popot, J.-L. (1995) *J. Biol. Chem. 49*, 29342−29349.
Rich, P. R. (1982) in *Function of quinones in energy conserving systems* (Trumpower, B. L., Ed.) pp 73−83, Acad. Press Inc., New York.
Saribas, A. S., Tokito, M., & Daldal, F. (1996) *Biophys. J. 70*, 356
Selak, M. A., & Whitmarsh, J. (1982) *FEBS Lett. 150*, 286−292.
Takahashi, Y., Rahire, M., Breyton, C., Popot, J.-L., Joliot, P., & Rochaix, J.-D. (1996) *EMBO J. 15*, 3498−3506.
Zhou, J., Fernandez-Velasco, J. G., & Malkin, R. (1996) *J. Biol. Chem. 271*, 6225−6232.

BI962717Y