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Redox effects on the bacteriochlorophyll *a*-containing Fenna-Matthews-Olson protein from *Chlorobium tepidum*

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Abstract

The BChl *a*-containing Fenna-Matthews-Olson (FMO) protein from the green sulfur bacterium *Chlorobium tepidum* was purified and characterized. Fluorescence spectra indicate that efficient excited state quenching occurs at neutral or oxidizing redox potentials. The major fluorescence lifetime at room temperature is approximately 60 ps in samples that are in neutral or oxidizing conditions, and approximately 2 ns in samples where the strong reductant sodium dithionite has been added. A similar change is observed in pump-probe picosecond absorbance difference experiments, where the long life time component increases after dithionite addition. A 16 Gauss wide EPR signal with g factor = 2.005 is observed in samples without dithionite. This signal largely disappears upon addition of dithionite. Dithionite induces large reversibile changes in the 77 K absorbance spectra of the purified FMO protein and in whole cells. These results indicate that the FMO protein contains redox active groups, which may be involved in the regulation of energy transfer. Room temperature circular dichroism and low temperature absorption spectra show that dithionite also induces conformational or structural changes of the FMO protein complex.

Abbreviations: BChl – bacteriochlorophyll; FMO protein – Fenna-Matthews-Olson protein

Introduction

The green photosynthetic bacteria are anoxygenic phototrophs that contain specialized antenna complexes known as chlorosomes (Olson et al. 1988). The green sulfur bacteria differ from the filamentous green bacteria by having a BChl *a*-containing protein (known as the FMO protein after Fenna, Matthews and Olson (Olson 1980)) between the chlorosome and membrane-bound reaction center complex. The reaction centers of the two families of green bacteria are also very different (Blankenship 1985).

The X-ray structure of the FMO protein from *Prosthecochloris aestuarii* was determined and subsequently refined to 1.9 Å resolution (Matthews et al. 1980; Tronrud et al. 1986, 1993). The protein is a trimer of identical subunits, each consisting of seven bacteriochlorophyll *a* molecules wrapped in a polypeptide of 366 residues in *Prosthecochloris aestuarii* (Daurat-Larroque et al. 1986) and 365 residues in *Chlorobium tepidum* (Dracheva et al. 1992). As a structurally well-characterized antenna pigment-protein, the FMO protein has been studied intensely by both spectroscopic and theoretical methods (Johnson and Small 1991; Pearlstein 1992; Lu et al. 1993).

Earlier studies on antenna systems of green sulfur bacteria found dramatic effects of the strong reductant sodium dithionite on fluorescence intensity (Karapetyan et al. 1980; Vos et al. 1987). Previous work from our laboratory has shown that energy transfer efficiency in the green sulfur bacteria is modulated by redox potential (Blankenship et al. 1990; Wang et al. 1990; Cheng 1992; Blankenship et al. 1993). This effect appears to involve a direct chemical titration of

redox-active groups in the chlorosome antennas, and was observed in whole cells, isolated membranes with attached chlorosomes and purified chlorosomes. The efficiency of energy transfer ranges from nearly 100% in reducing conditions to 10% or less under oxidizing conditions (Wang et al. 1990; Blankenship et al. 1990, 1993). Redox titrations of fluorescence in isolated chlorosomes gave a pH dependent midpoint of −146 meV vs NHE at pH 7. A similar redox modulation was also found to be present in the isolated FMO protein (Cheng 1992; Blankenship et al. 1993). The fluorescence emission from the FMO protein can be increased more than five fold by lowering the redox potential.

A significant redox effect is not observed in the green gliding bacterium *Chloroflexus aurantiacus,* which contain chlorosomes but does not contain FMO proteins or low potential electron acceptors in its reaction center (Blankenship et al. 1990; Wang et al. 1990). Green sulfur bacteria have a completely different reaction center which has low potential iron sulfur centers and reduces ferredoxin directly. The reduced ferredoxin will readily react with oxygen to form superoxide, which leads to a variety of damaging photooxidative products (Orme-Johnson et al. 1969). Thus, it is reasonable for these bacteria to have a protective mechanism against oxygen-mediated damage.

We proposed previously that a redox regulation mechanism might be used by green sulfur bacteria to protect themselves under oxygenic conditions by quenching excitations in the antenna system before they reach the reaction center (Blankenship et al. 1993). This regulation may operate on at least two levels, within the chlorosome itself and within the FMO protein. The molecular mechanisms of these regulations are not yet clear. In this study, we have investigated redox effects on the FMO protein isolated from *Chlorobium tepidum.* Our results indicate that many of the spectroscopic parameters of this protein are reversibly modulated by redox potential, consistent with the view that it is part of the system that regulates energy transfer.

Materials and methods

Chlorobium tepidum was grown phototrophically and the FMO protein was purified by the method reported elsewhere (Savikhin et al. 1994). The buffer used in all measurements was 20 mM Tris, pH 8.0. A 1 M sodium dithionite solution freshly prepared by dissolving

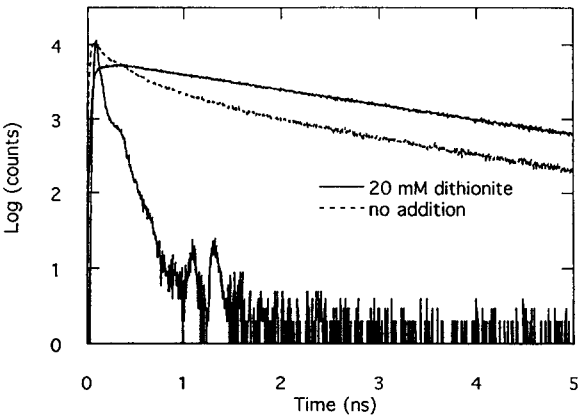

Fig. 1. Fluorescence decay kinetics of the FMO protein in the absence of dithionite (dashed line) and the presence of 20 mM dithionite (solid line) at room temperature. Excitation wavelength was at 602 nm and emission wavelength was at 810 nm. The lower curve is the instrument response function.

sodium dithionite into the 20 mM Tris/pH 8.0 buffer was used as reductant, and the final concentration of dithionite in all samples was 20 mM or else indicated specifically.

Samples for fluorescence emission measurements were diluted with buffer to OD = 0.1~0.2 at the absorbance peak of 809 nm. The excitation wavelength was 602 nm. Steady-state emission spectra were taken on a Jasco FP-777 spectrofluorometer. Fluorescence lifetimes were measured by the time-correlated single photon counting method in the instrument described by Causgrove et al. (1990). Data were collected at 5 ps/channel to 10,000 counts in the peak channel. Fluorescence decays measured at various emission wavelengths were fit with global analysis (Knutson et al. 1983).

For pump-probe picosecond transient absorption measurements, samples were loaded in a spinning cell with an optical pathlength of 2.5 mm and a diameter of 18 cm. The absorbance of the sample was 1.0 at 809 nm in this sample cell. A description of this apparatus is given by Lin et al. (1994). Decay-associated spectra were obtained using global analysis.

EPR spectra were obtained on a Bruker 300E spectrometer at 121 K using 1 mW microwave power and 3.9 G modulation amplitude. The data from 32 scans were collected and averaged.

Steady-state absorption spectra were measured on a Cary 5 spectrophotometer. For low temperature measurements, glycerol was added to the sample so that the final ratio of glycerol to buffer solution was

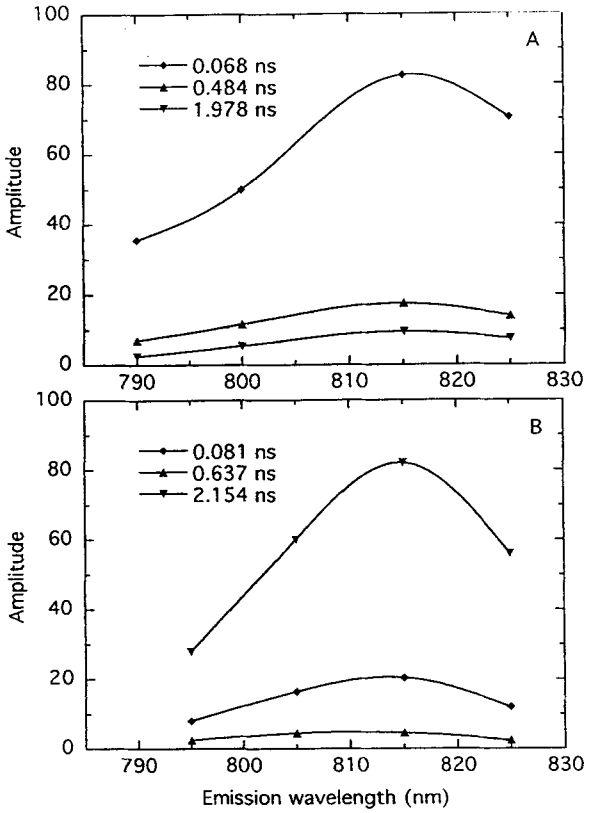

Fig. 2. Fluorescence decay-associated spectra obtained from a three-component global fit of the fluorescence decays upon excitation at 602 nm at room temperature. (A) In the absence of dithionite. (B) In the presence of 20 mM dithionite. Excitation wavelength was at 602 nm.

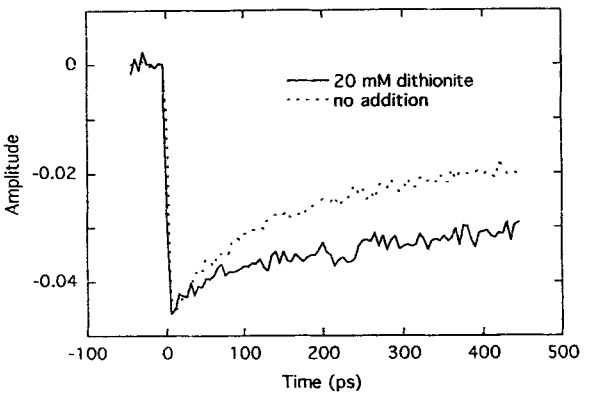

Fig. 3. Picosecond absorbance kinetics of the FMO protein in the absence of dithionite (dashed line) and the presence of 20 mM dithionite (solid line) at 812 nm with a time resolution of 5 ps per channel. Excitation wavelength was at 590 nm.

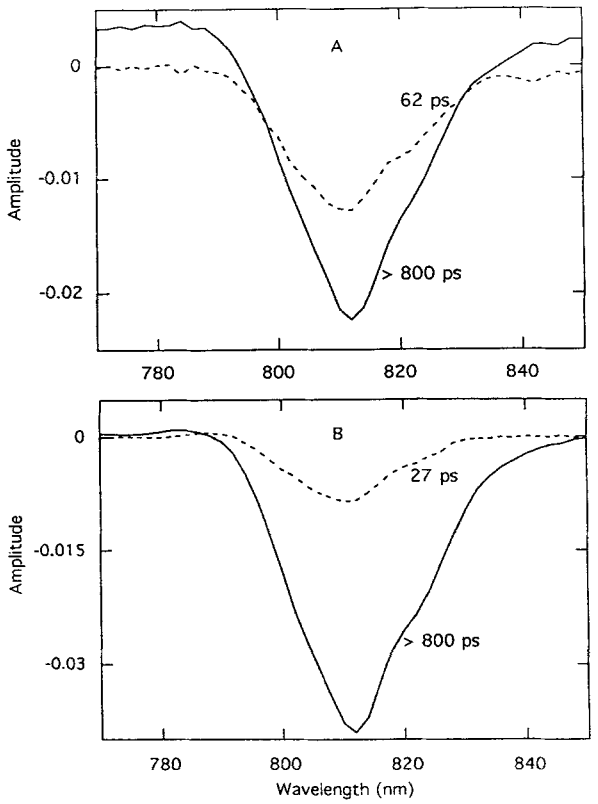

Fig. 4. Picosecond absorption decay-associated spectra from a two-component fit of the absorbance kinetics. (A) in the absence of dithionite. (B) in the presence of 20 mM dithionite. Excitation wavelength was at 590 nm.

7:3. For those experiments in which the reversibility of the dithionite effects on low temperature absorption spectra were investigated (Figs. 7 and 8), the dithionite-treated samples were measured, and the samples thawed. The isolated FMO protein samples were dialyzed against 20 mM Tris, pH 8.0 buffer, and the whole cells were washed extensively with the same buffer by centrifugation. The samples were then refrozen to 77K and the spectra measured again.

Circular dichroism spectra were taken on a Jasco J710 spectropolarimeter. Samples with OD 1.0 at 809 nm in the sample cell were used.

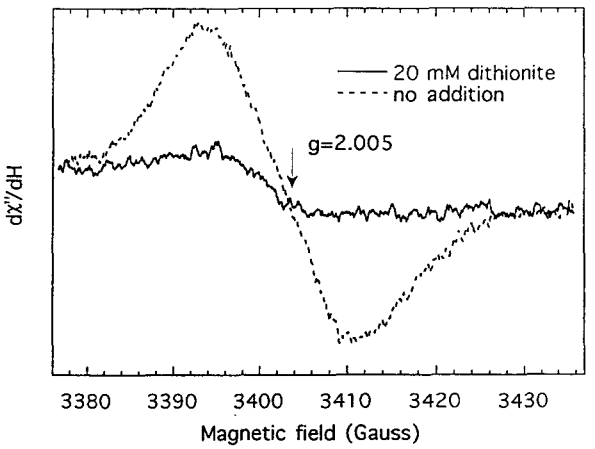

Fig. 5. EPR spectra of the FMO protein in the absence of dithionite (dashed line) and the presence of 20 mM dithionite (solid line) at 121 K.

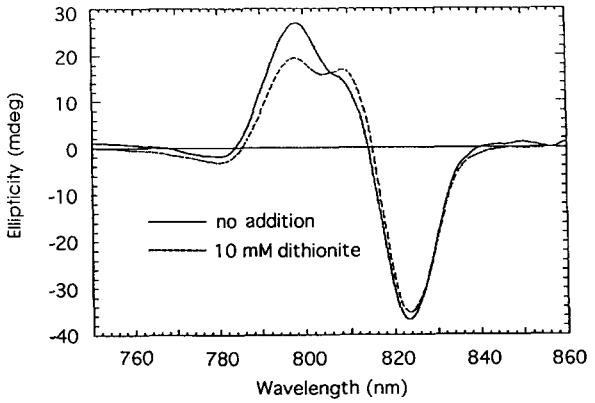

Fig. 6. Circular dichroism spectra of the FMO protein at room temperature. In the absence of dithionite (solid line), the peak positions are at 780 nm (−), 798 nm (+), 808 nm (+) and 823 nm (−). In the presence of 10 mM dithionite and 2 μM methyl viologen (dashed line), the peak positions are at 780 nm (−), 798 nm (+), 809 nm (+) and 823 nm (−).

Results

Investigation of redox effects

Figure 1 shows fluorescence decays of the FMO protein at 810 nm upon 602 nm excitation. A set of emission traces measured between 790–830 nm were fit with global analysis, giving the decay-associated fluorescence spectra shown in Fig. 2. The 68 ps component was dominant in neutral (Fig. 2A) or oxidizing conditions (in the presence of 20 mM ferricyanide) (data not shown). Upon addition of dithionite, the dominant component switched to 2 ns (Fig. 2B). When time-resolved or steady-state fluorescence emission were measured at 77 K, the emission in the absence of dithionite increased to such a extent that there was little difference between the samples with and without dithionite. The 2 ns lifetime component was dominant in both samples (data not shown).

Picosecond absorbance kinetics measured at 812 nm in the presence and absence of dithionite at room temperature are given in Fig. 3. A fast decay phase was observed in the absence of dithionite and significantly diminished after dithionite addition. Global analysis in the range of 700 nm to 850 nm gave the decay-associated spectra shown in Fig. 4. The fast decay phase in the absence of dithionite (Fig. 4A) has a lifetime of 62 ps, which is very close to that of the fast component found in the fluorescence decays (see Fig. 2A). The slow component was dominant in both cases. However, the ratio of slow to fast amplitude significantly increased upon dithionite addition. The lifetime of the slow component is beyond the time-scale of the picosecond measurement.

Other reductants such as sodium sulfide, ascorbate, sodium thiosulfate, dithiothreitol, sodium borohydride and 2-mercaptoethnol were tested, but none of them caused significant increase of fluorescence emission. That might indicate that either the redox active group has a very low redox potential or it is only accessible to dithionite, due to a special protein environment. We also found that methyl viologen dramatically sped up the increase of fluorescence emission upon addition of the dithionite. Without methyl viologen, the major kinetic phase of the increase of the dithionite-induced fluorescence took approximately 5 minutes, while in the presence of 1~2 μM methyl viologen, the rise took place within 1 minute (data not shown). Therefore, it is possible that the redox active group is buried inside the protein and methyl viologen somehow increases the accessibility of dithionite to it.

Fluorescence emission was also measured under anaerobic but not reducing conditions. The presence of oxygen did not significantly change the fluorescence emission. This result is different from the whole cell measurement where addition of oxygen inhibited excitation transfer and the removal of oxygen restored 100% excitation transfer efficiency, probably through the action of endogenous reductants (Wang et al. 1990). It is very likely that the redox active group in the FMO protein has been oxidized during the purification process.

An effort was made to determine the midpoint-potential of the purified FMO protein by redox titra-

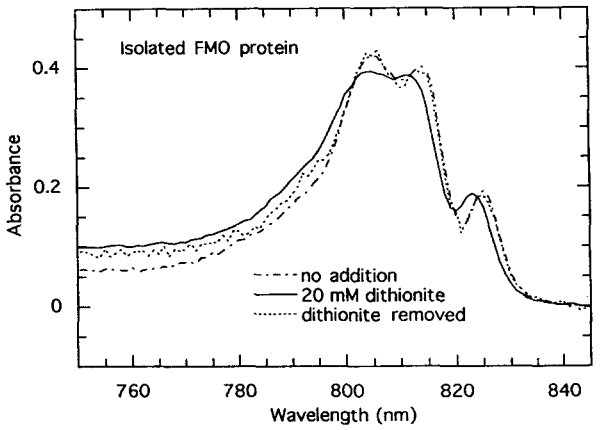

Fig. 7. Absorption spectra of the isolated FMO protein at 77 K. In the absence of dithionite (dot-dashed line), three peaks are at 805 nm, 814 nm and 825 nm. In the presence of dithionite (solid line), the peaks are shifted to 804, 812 and 823 nm. The changes are largely reversed upon removal of dithionite (dashed line).

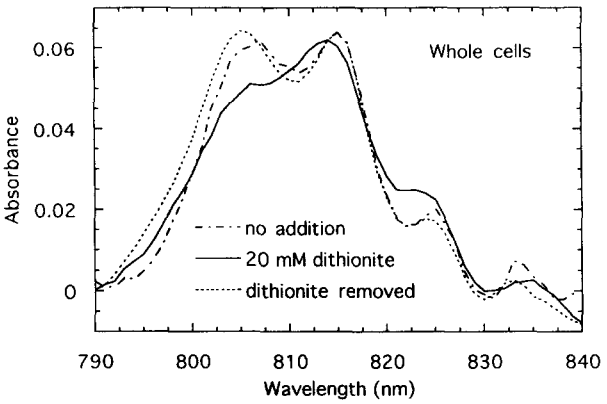

Fig. 8. Absorbance spectra of whole cells of *Chlorobium tepidum* at 77 K, with no additions (dot-dashed line), 20 mM dithionite (solid line) and after removal of dithionite (dashed line).

tion in which dithionite and ferricyanide were used as reductant and oxidant respectively, with fluorescence emission monitored at 814 nm. The titrations showed significant hysteresis, indicating that equilibrium conditions had not been achieved.

The nature of the redox active group

Only minor bacteriochlorophyll absorbance changes are apparent in the dithionite-treated protein compared to the untreated protein. This argues against an oxidation-reduction reaction directly involving the bacteriochlorophylls. One possibility is that the redox active group is a quinone, either tightly associated with the protein or covalently bound to it. Quinones are highly efficient quenchers of chlorophyll excited states (Kelly et al. 1970; Natarajan et al. 1983) and would be expected to be especially effective if located near the BChl *a* complex of the FMO protein. However, neither a redox-cycling staining assay (Paz et al. 1991) nor UV-visible spectroscopic studies of FMO protein after addition of hydrazine and phenylhydrazine (Davidson et al. 1992) gave any indication of existence of quinone groups. The mass of the purified FMO protein measured from laser desorption mass spectroscopy is very close to that deduced from the gene sequence (Dracheva et al. 1992), with 40, 160 ± 20 and 40, 165 respectively (data not shown). This does not suggest an extra quinone or its derivative covalently bound to the protein, unless other compensating mass changes are present.

In order to determine if there are any free radicals involved in the redox effects, EPR spectra of purified FMO protein were measured (Fig. 5). A 16 Gauss wide EPR signal with g factor = 2.005 was observed in the sample without dithionite. This signal largely disappeared in the presence of dithionite. The same samples were thawed and used to do room temperature fluorescence measurements with front-face geometry to minimize self absorption. The sample with dithionite had much higher fluorescence (data not shown), which is consistent with the right-angle fluorescence measurements and correlates with the disappearance of EPR signal. The chemical identity of this EPR signal is not yet clear, but the EPR parameters are distinct from those usually observed from chlorophyll-type pigments and are characteristic of aromatic free radicals such as semiquinone and tyrosine radicals (Borg et al. 1976; Barry et al. 1987; Whittaker et al. 1990; Babcock et al. 1992).

It is possible that a tyrosine residue adjacent to the BChl complex might be involved in the redox effect. The recent refined X-ray structure of the FMO protein shows that the oxygen of the ring 1 acetyl group of BChl *a* number 3 is H-bonded to a Tyr residue of the protein (Tronrud et al. 1993). It is also possible that a quinone-type group modified from amino acids gives rise to the EPR signal, although the mass spectroscopy, redox staining and derivatization results cited above argue against this explanation.

Implication of conformational or structural changes

Figure 6 shows room temperature CD spectra of the FMO protein. In the absence of dithionite, the peak positions were at 780 nm (−), 798 nm (+), 808 nm (+) and 823 nm (−). The addition of dithionite did not cause peak shifts with the exception of a 1 nm shift of the 808 nm peak to 809 nm, but caused an apparent decrease of the amplitude at 814 nm and 799 nm.

Both room temperature (Blankenship et al. 1993) and 77 K absorption spectra (Fig. 7) of the purified FMO protein are similar to those of analogous proteins from *Prosthecochloris aestuarii* and *Chlorobium limicola* f. *thiosulfatophilum* (Olson 1978). At room temperature, the Q_y bands are centered at 809 nm, and the Q_x bands at 602 nm. The addition of dithionite did not cause observable changes except a small decrease of absorption amplitude at 809 nm. At 77 K, the Q_y band is split into three sharp components, with peaks at 805 nm, 814 nm and 825 nm, and a broad component approximately centered at 795 nm. Dithionite addition induced a pronounced change at this temperature, as shown in Fig. 7. The 825 nm band shifted to 823 nm and the 814 and 805 nm bands broadened and shifted to 812 and 804 nm respectively. Both 805 and 814 bands are blue shifted and less resolved from each other. Figure 7 also shows that these changes were fully reversible when the dithionite-treated sample was thawed, dialyzed and refrozen. In some experiments, the absorbance changes upon dithionite treatment were even more dramatic than those shown in Fig. 7, and the 825 nm band was reduced to only a shoulder at 822 nm.

Figure 8 shows 77 K absorbance spectra of whole cells of *Chlorobium tepidum* in the 790 to 840 nm region. The characteristic absorbance bands due to the FMO protein are clearly observable. Dithionite treatment induced similar absorbance changes in the FMO absorbance bands in whole cells as it did in the purified protein, and the changes were also reversible upon thawing and washing of the cells.

Discussion

Our results further document the redox activity of the purified FMO protein. Dithionite induces a number of dramatic changes in the properties of the protein, and these changes are reversible upon removal of dithionite. The reversible spectral changes observed at 77 K in both the isolated protein and whole cells (Figs. 7 and 8) leave little doubt that this effect is a specific one and neither an artifact of preparation nor an irreversible disruption of the structure of the protein.

The 60∼70 ps lifetime component in both fluorescence emission decays and pump-probe absorbance decays does not appear to be a normal energy transfer component in the antenna system. The excited state lifetime of an isolated antenna species is typically 1–2 ns (Van Grondelle 1985), similar to the 2 ns phase observed in the reduced FMO protein. We propose that the 60 ps component is related to energy or electron transfer between excited BChl *a* and a redox active group or quencher.

A singlet excited BChl in the isolated FMO protein will have three possible fates: (1) fluorescence and recovery to the ground state on the nanosecond time scale; (2) triplet state formation and subsequent recovery to the ground state on the microsecond time scale; (3) interaction with quencher by either transferring its energy to a quencher in 60 ps or donating an electron to the quencher, forming a $BChl^+$ in 60 ps. This $BChl^+$ might then very rapidly recombine with the reduced quencher in a time shorter than 60 ps, so that $BChl^+$ never accumulates. When the interaction between excited BChl and quencher is interrupted in a low redox potential environment, the population of BChl *a* having fate (3) is expected to decrease and the populations with other two fates will increase. The diminution of the 60 ps component and the growth of the 2 ns component upon addition of dithionite (see Figs. 1–6) are in general agreement with the above predictions.

Dithionite also induced conformational or structural changes of the protein complex, as shown by CD and low temperature absorption spectra. There are two possible ways for dithionite to affect the structure of the protein: (1) dithionite directly interacts with the BChl *a*, which then changes the conformation of the BChl *a* complex. The recently refined X-ray structure suggests that there may be a chemically modified BChl *a* present (Tronrud et al. 1993). BChl *a* number 3 may be modified at ring II, with the ethyl group replaced by a carbonyl group. This may enhance the sensitivity of this BChl *a* to the strong reductant; (2) dithionite interacts with the protein and reduces some groups such as that inferred in EPR spectra, and the reduction of these groups causes a subsequent conformational rearrangement.

Earlier low temperature and circular dichroism studies suggested that seven BChls *a* of one subunit form an excitonic complex (Philipson et al. 1972; Olson et al. 1973; Olson et al. 1976; Whitten et al.

1980). Recently, more detailed analyses were carried out in several groups (Johnson et al. 1991; Pearlstein 1992; Van Mourik 1993; Van Mourik et al. 1993). Based on hole burning spectroscopy, Johnson et al. (1991) concluded that the 825 nm absorption band is composed of two components: 827.1 nm and 824.4 nm, and these two states are relatively localized states lying below the main exciton band which lies near 816 nm. By studying singlet-triplet transition difference spectra, Van Mourik (1993) and Van Mourik et al. (1993) proposed that the splitting of this 825 nm band is due to the trimeric interaction between the three individual subunit 825 nm transitions. Pearlstein (1991) reached the same conclusion from theoretical calculations and further pointed out that the BChl *a* number 7 in each monomer are major contributors to this intersubunit interaction.

At 77 K, the apparent change of the absorption spectrum after addition of dithionite did not correlate with the near independence of the fluorescence emission on the presence of dithionite at low temperature. The increase of low temperature fluorescence emission in the absence of dithionite could be explained by assuming that the rate of reaction between excited BChl *a* and the oxidized quencher is much slower at 77 K than that at room temperature, so that the quenching effect diminishes at low temperature.

At room temperature, the increase of steady-state fluorescence upon addition of dithionite always appears to be in two phases, a rapid increase in approximately 5 minutes or within 1 minute in the presence of 1~2 μM of methyl viologen followed by a slow additional increase taking approximately 40 minutes. The fast phase represented approximately 80% of the total increase (data not shown). The conformational or structural rearrangement might account for the slow phase, but the fast phase is likely to arise from the blocking of the quenching effect by dithionite.

It is still not completely clear how the conformational or structural changes are related to the regulation of energy transfer presumably mediated by the redox active group. Whether this is a series event or two simultaneous events is not clear. Additional experiments are underway to further investigate these effects.

The FMO protein has been studied intensively using a wide range of techniques. It is clearly the best understood photosynthetic antenna complex from a structural and spectroscopic point of view. However, essentially all previous studies appear to have been carried out on the oxidized form of the protein, based on the low temperature absorption spectra shown in Fig. 7. We report here some properties of the FMO protein under low redox potential. Our results clearly show that the spectroscopic properties change with the redox potential. Additional studies are underway to relate these redox-induced effects on spectroscopic properties to possible structural changes.

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