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UNUSUAL OPTICAL PROPERTIES OF THE MONOMERIC CHLOROPHYLL *a* IN THE CYTOCHROME b_6f COMPLEX OF OXYGENIC PHOTOSYNTHESIS

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Keywords: Chlorophyll *a*, cytochrome b_6f complex, *Mastigocladus laminosus*, fluorescence quenching

INTRODUCTION

The cytochrome b_6f (cyt b_6f) complex in oxygenic photosynthesis mediates electron transfer between the reaction centers of photosystem I and photosystem II and facilitates coupled proton translocation across the membrane that forms an electrochemical proton gradient (Cramer et al 2004). High-resolution X-ray crystallographic structures (Kurisu et al 2003, Stroebel et al 2003) of the cyt b_6f complex unambiguously confirmed earlier findings (Bald et al 1992, Huang et al 1994, Pierre et al 1995) that a chlorophyll *a* (Chl *a*) molecule is an intrinsic component of the b_6f complex. The functional role of this Chl *a* is still unknown (Kühlbrandt 2003) – the b_6f complex does not participate in light harvesting, which is the usual function of a chlorophyll *a* molecule in photosynthetic complexes. The excited Chl *a* molecule is known to produce highly toxic singlet oxygen as the result of energy transfer from the excited triplet state of the Chl *a* to oxygen molecule. To prevent singlet oxygen formation, a carotenoid is typically positioned close to the Chl *a* molecule, effectively quenching the triplet excited state of the Chl *a*. However, according to the recently obtained structures of the cyt b_6f complex, the β-carotene is too far (14 Å) from the Chl *a* for effective direct quenching of the Chl *a* triplet excited state. In this study, optical properties of the Chl *a* molecule and their role in photostability of the complex were analyzed by transient absorption spectroscopy.

MATERIALS AND METHODS

The purification and crystallization procedures of the cyt b_6f complex from the thermophilic cyanobacterium *M. laminosus* have been described in detail elsewhere (Zhang et al 2003). Diffraction quality single crystals were dissolved in a buffer containing 30 mM Tris • HCl (pH 7.5), 50 mM NaCl and 0.05% undecyl-β-D-maltoside to ensure highest purity. Samples were housed in a cell with a 1 mm path length, and exhibited ~0.1 absorbance at an excitation wavelength of 660 nm. Transient absorption differences ΔA following optical excitation of a sample were recorded by the femtosecond pump-probe spectrometer described in (Savikhin et al 2000).

RESULTS

Samples where excited into the blue edge of the Chl *a* Q_y absorption band at 660 nm and the following transient absorption changes where probed in the range of 665–700 nm covering most of the Chl *a* Q_y absorption band. Figure 1 shows the representative absorption difference profile obtained at 680 nm. It could be described as a superposition of two exponential components: a major component with decay time of ~200 ps (91%), and a minor component with decay time of >1 ns (9%).

Simultaneous global fit to all measured absorption difference profiles required four decay components: 400 fs, 12 ps, 200 ps and >1 ns. The decay-associated spectra (DAS) for the 400 fs, 12 ps and >1 ns components were unstructured, while the DAS of the 200 ps component closely mimicked the photobleaching spectrum of a monomeric Chl *a* and constituted ~90% of the total signal. Peterman et al (Peterman et al 1998) measured the fluorescence lifetime of the Chl *a* in the monomeric cyt b_6f complex from the cyanobacterium *Synechocystis* PCC 6803 to be 250 ± 20 ps. Within the experimental error, this result agrees well with our findings, indicating that Chl *a* in the singlet excited state decays primarily to its ground state without forming the triplet state.

DISCUSSION

The 200 ps lifetime of the singlet excited state of the Chl *a* in the b_6f complex is in sharp contrast with the lifetime of 5–6 ns reported for

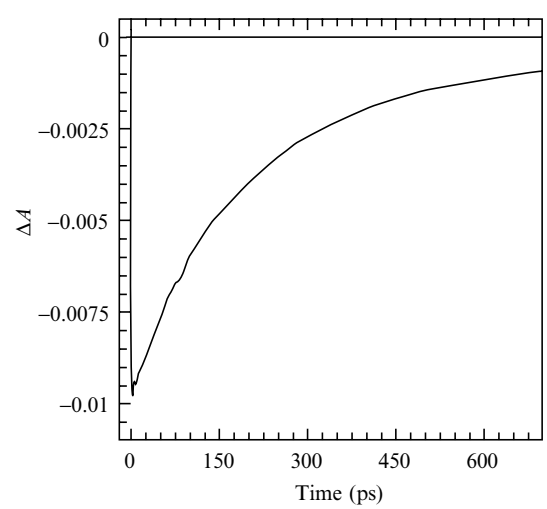

Figure 1: Time-resolved transient absorption difference profiles for the cyt b_6f complex obtained at 680 nm by exciting at 660 nm.

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monomeric Chl *a* molecules in organic solvents (Seely & Connolly 1986). In a typical pigment-protein complex where Chl serves as a light-harvesting molecule, singlet excitation is quenched within ~20–200 ps via rapid excitation transfer to a reaction center where its energy is used to initiate charge separation. The monomeric Chl *a* in the cytochrome b_6f does not serve a light-harvesting function and this quenching mechanism is not applicable. One of the ways to shorten the excited state lifetime is to increase the rate of intersystem crossing from the Chl *a* singlet excited state into the triplet excited state. However, according to our experiment the yield of the triplet state formation upon Chl *a* excitation in the cytochrome b_6f is very low, indicating that the rate of intersystem crossing for Chl *a* in this complex is similar to that in solvents. Alternatively, the quenching of the Chl *a* excited state may be caused by interaction with the nearby hemes – the edge-to-edge distance between the closest heme b_n and the Chl *a* is 8.6 Å. This scenario is also not supported by our experiments, which demonstrate that the excited state lifetime of the Chl *a* in the b_6f complex does depend on the redox states of the hemes.

We propose that the unusually short lifetime of the Chl *a* excited state in the cytochrome b_6f is caused by excitation-induced electron transfer interaction with the nearby aromatic amino acid residues. According to high-resolution structure of the cyt b_6f complex (Kurisu et al 2003, Stroebel et al 2003), the Chl *a* is surrounded by a large number of aromatic amino acid residues. Its oxidation potential and distance to the Chl *a* makes Tyr105 a likely residue responsible for rapid quenching of the Chl *a* singlet excited state.

When the Chl *a* is promoted to excited state its reduction potential changes by an amount approximately equal to the energy of the absorbed photon (Fig. 2), and it can efficiently reduce the nearby Tyr105 producing a radical pair $Tyr^{+}Chl^{-}$ (arrow "1"). In the next step (arrow "2"), an electron is transferred from Chl^{-} back to Tyr^{+} completing the cycle and bringing both molecules into neutral ground state. The experimental data implies that the first electron transfer step occurs in ~200 ps, and the second electron transfer step is faster than ~150 ps. The rate of an exothermic electron transfer k_{ET} can be estimated using the Moser-Dutton semi-empirical relationship:

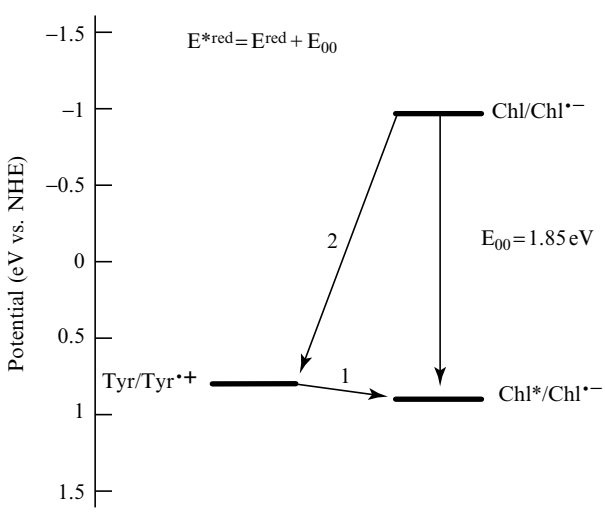

Figure 2: Redox potential level diagram demonstrating the quenching mechanism of the Chl *a* singlet excited state by electron transfer from and to tyrosine.

$$\log_{10} k_{ET} = 13 - 0.6R - 3.6(\Delta G^0 + \lambda)^2/\lambda \quad (1)$$

where R is edge-to-edge distance between electron donor and acceptor, ΔG^0 is the Gibbs free energy change of the electron transfer and λ is the reorganization energy. With a typical value of $\lambda = 0.7$ eV the Moser-Dutton equation predicts 234 ps for the first electron transfer step shown in Fig. 2. The latter value is in good agreement with our experimental data. However, Eq. 1 yields a $(0.8\,\mu s)^{-1}$ rate for the second electron transfer step (Fig. 2), which is inconsistent with the experiment. We suggest that this is the result of uncertainties in the reorganizational energy and redox potential values. If reorganizational energy for the second electron transfer step is 1.05 eV, the Eq. 1 results in 140 ps. Similar inconsistency can be observed in the case of riboflavine-binding protein where the excited state of riboflavine has been shown to be quenched by the same mechanism (Zhong & Zewail 2001). While the second electron transfer time was measured to be 8 ps for riboflavine, we calculated a value of 5.7 ns using Eq. 1 and $\lambda = 0.7$ eV. As in our case, the measured time of the first electron transfer step for riboflavine was in good agreement with the Moser-Dutton theory (100 fs versus 122 fs). The energetics of the second electron transfer step in riboflavine-binding protein is strikingly similar to our case. Assuming that the only difference between these two cases is the distance R between the chromophore and tyrosine the measured 8 ps time reported for riboflavine would increase to 190 ps for the distance between the Chl *a* and Tyr105 in the b_6f complex.

The unusually short lifetime of the singlet excited state of the Chl *a* in the cytochrome b_6f reduces the yield of Chl triplet state formation by a factor of ~25, partially protecting the complex from singlet oxygen formation. This protection mechanism is of special importance as there is no direct triplet-triplet energy transfer from the Chl to the β-carotene. We have compared the photostability of the cytochrome b_6f with that of the monomeric chlorophyll in organic solvent by observing photodegradation of Chl absorption band as a function of irradiation time. The Chl *a* in b_6f turned out to be ~130 times more stable than free monomeric Chl *a* in ethanol. This suggests that the quenching of the Chl excited state is not the only protection mechanism operating in the cytochrome b_6f complex.

ACKNOWLEDGMENTS

This work was supported by Purdue Research Foundation grant 6903680, and NIH GM-38323.

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IN VIVO SUPEROXIDE PRODUCTION BY THE CYTOCHROME bc_1 COMPLEX IN YEAST

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Keywords: bc_1 complex, superoxide, Q-cycle, *sod2*, yeast

INTRODUCTION

The cytochrome (cyt) bc_1 complex is a central component of photosynthetic and respiratory energy transduction machinery and is also believed to be a major source of deleterious superoxide. Superoxide production by the cytochrome bc_1 complex can be understood in the context of the normal catalytic mechanism of this enzyme, the modified Q-cycle (reviewed in Crofts 2004). The key reaction of the Q-cycle is the bifurcated oxidation of ubiquinol at the Q_o-site, where the first electron is transferred to the 'Rieske' 2Fe2S cluster, resulting in the formation of an unstable semiquinone, which then rapidly reduces cyt b_L.

This critical bifurcated electron transfer step also leads to the production of superoxide via a reactive semiquinone intermediate (Skulachev 1996, Muller 2000). The structure of Q_o-site is thus of central importance to our understanding of both energy conversion and reactive oxygen generation. The crystal structures of mitochondrial complexes (reviewed in Crofts 2004) confirm early studies indicating that there are two distinct pockets within the Q_o-site which have different functional roles (Link et al 1993, Crofts et al 1999). The 'proximal' Q_o-niche is located close to the cyt b_L heme, while the 'distal' niche is located close to the ISP (Crofts et al 1999). The two niches bind different classes of inhibitors (von Jagow & Link 1986, Link et al 1993, Crofts et al 1999). MOA-stilbene, mucidin, famoxadone and myxothiazol are proximal niche inhibitors (von Jagow & Link 1986, Link et al 1993) and bind close to cyt b_L. Stigmatellin and UHDBT, which strongly interact with the Rieske ISP and bind at the distal niche (von Jagow & Link 1986). Most Q-cycle models attribute functional significance to the two binding niches, either allowing for two quinones to bind simultaneously (Crofts et al 1999, Sharp et al 1999), or allowing the semiquinone intermediate to pass from the distal to the proximal niche (Crofts et al 1999).

Our recent work on yeast cyt bc_1 complex shows that proximal niche inhibitors also induce superoxide production in isolated cyt bc_1 complex (Muller et al 2002, 2003). Molecular modeling revealed that it was indeed possible for ubiquinol to bind at the distal Q_o-site concomitantly with a proximal Q_o-site inhibitor bound next to cyt b_L (Muller et al 2003). We concluded from these experiments that the distal Q_o-site could act as an independent quinol oxidase domain, even when the proximal Q_o-site was blocked. This finding gives strong support for cyt bc_1 models arguing for functional sub-compartmentalization of the Q_o-site.

In the present work, we extend our previous findings to living cells, by showing that proximal Q_o-site inhibitors also induce superoxide production at the distal Q_o-site of complex III *in vivo*, with marked physiological consequences. For this work, we chose a non-photosynthetic organism, *Saccharomyces cerevisiae*, as a model organism because it has been used in the past to assess the physiological effects of reactive oxygen species (Longo et al 1999, Fabrizio & Longo 2003).

MATERIALS AND METHODS

Superoxide dismutase knockout strains were kindly provided by Drs. E. B. Gralla and J. S. Valentine (Longo et al 1996). The strains used were EG103 (wild type) and EG110 (Manganese superoxide dismutase knockout; *sod2*). Yeast were grown on YEPD (1% Yeast extract 2% peptone 2% Dextrose) or SCD (synthetic complete 2% dextrose medium) as described by Longo et al (1996). Cultures were incubated at 24 °C (room temperature) if not otherwise stated and shaken at 220 rpm. Viability in stationary phase was determined as in Longo et al (1996, 1999). An inoculum was placed into a 50 ml vial containing 5 ml of SCD liquid medium and allowed to grow for 72 hours at room temperature to reach saturation, at which point various inhibitors were added. In all graphs, viability is expressed as fraction viable cells, with full viability being taken as the number of viable cells at entry into stationary phase (72 hours after inoculation, $\sim 4 \times 10^5$ viable cells per ml). Viability was determined by dilution and plating on YEPD solid medium as described by Longo et al (1999). Colonies were counted 3 days after plating and a viable cell was defined as one being able to form a colony on rich media after 3 days. *S. cerevisiae* was treated with antimycin A, myxothiazol (both from Sigma), stigmatellin (Fluka fine chemicals), MOA-stilbene, mucidin and famoxadone (gifts from Dr. Antony R. Crofts) and potassium cyanide (Sigma).

RESULTS

Antimycin A and Group II Inhibitors Decrease the Viability of the SOD2 Knockout Yeast by Promoting Superoxide Production. Antimycin A and group II inhibitors (Fig. 1) reduce viability of the *sod2* strain in stationary phase compared to untreated controls. In all

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