yielded E_a values of $42.0 \pm 2.4\,kJ\,mol^{-1}$ and $37.7 \pm 3.7\,kJ\,mol^{-1}$ for the uninhibited and AA treated reactions respectively.

When plastoquinol was used as a substrate, similar E_a values were measured for both the uninhibited complex and AA treated complex ($59.8 \pm 2.5\,kJ\,mol^{-1}$ and $50.1 \pm 3.4\,kJ\,mol^{-1}$ respectively). In the deuterated system, E_a values of $53.4 \pm 1.8\,kJ\,mol^{-1}$ and $57.3 \pm 6.2\,kJ\,mol^{-1}$ were measured in the uninhibited and AA treated complexes respectively.

DISCUSSION

In our hands, the steady state superoxide production rate of AA treated cyt cyt bc_1 complex in Yeast is about 8 mol $O_2^{\bullet -}$ mol cyt $bc_1{}^{-1}\,s^{-1}$ (data not shown). The auto-oxidation rates measured for cyt b_L were much too slow (by about 2–3 orders of magnitude) to account for the observed rate of superoxide production (see Fig. 1). We therefore rule out cyt b_L as a kinetically significant electron donor to oxygen during superoxide production. Eliminating all other possible candidates (Muller et al 2002), we conclude that the O_2 reductant is an intermediate of the Q_o pocket, most probably a semiquinone.

We measured essentially the same E_a values for uninhibited and AA resistant (leading to superoxide production) cyt bc_1 complex turnover, regardless of the substrate or isotope system used (protons or deuterons). In all cases, differences in rate could be attributed to changes in the pre-exponential factor. Since the oxidation of ubiquinol by the 2Fe2S center is almost certainly involved in formation of the transitions state for overall cyt bc_1 turnover (Snyder et al 1999, Guergova-Kuras et al 2000), these results are consistent with a shared transition state, most likely a semiquinone, for both normal and bypass reactions (see Kramer, DM, *these proceedings* for a model of the transition state for quinol oxidation).

We thus argue that (1) superoxide production can be used as a probe of the properties of the Q_o site intermediate; and (2) ubiquinol oxidation likely proceeds via sequential electron (plus proton – see Cape, JL, *these proceedings*) transfer reactions, and not via a *truly* concerted oxidation. This implies the existence of a distinct, and reactive, semiquinone species that must be 'tamed' by the Q_o pocket to prevent the production of deleterious reactive oxygen species.

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EVIDENCE FOR LONG RANGE TRIPLET STATE ENERGY TRANSFER FROM CHLOROPHYLL *a* TO β-CAROTENE IN THE CYTOCHROME b_6f COMPLEX OF OXYGENIC PHOTOSYNTHESIS

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Keywords: Chlorophyll *a*, cytochrome b_6f complex, β-carotene, singlet oxygen, *Mastigocladus laminosus*, triplet energy transfer

INTRODUCTION

The cytochrome b_6f complex of oxygenic photosynthesis mediates electron transfer between the reaction centers of photosystem I and photosystem II and facilitates coupled proton translocation across the membrane (Cramer et al 2004). High-resolution X-ray crystallographic structures (Kurisu et al 2003, Stroebel et al 2003) of the cytochrome b_6f complex unambiguously confirmed earlier findings of a chlorophyll *a* (Chl *a*) molecule as an intrinsic component of the b_6f complex. The functional role of the Chl *a* in the cytochrome b_6f complex is still unknown (Kühlbrandt 2003). 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 molecules. To prevent singlet oxygen formation, carotenoid is typically positioned close to the Chl *a* molecule, effectively quenching the triplet excited state of the Chl *a*. However, the high-resolution structures of the cytochrome b_6f complex show that the β-carotene (Car) is too far (14 Å) from the Chl *a* for effective direct quenching of the Chl *a* triplet excited state (^{3}Chl*). In this study, for the first time, we observed efficient formation of the triplet state of the β-carotene (^{3}Car*) upon excitation of the Chl *a* in the cytochrome b_6f complex. Based on experimental results, we propose that the singlet oxygen may act as a mediator in triplet energy transfer from Chl *a* to β-carotene and that the latter process also helps to reduce possible damage caused by the formation of singlet oxygen in this complex.

MATERIALS AND METHODS

The purification of the enzymatically active dimeric cytochrome b_6f complexes from the thermophilic cyanobacterium *M. laminosus* is described in detail elsewhere (Zhang et al 2003). Nanosecond pump pulses were generated by an excimer pumped dye laser (Lambda Physik EMG102 and Scanmate 2), and the transient absorption differences ΔA were probed by a broad-band xenon arc lamp driven

by an ultra-stable pulsed power supply (Applied Photophysics, LKS .60). Home-built solid state probe light detectors were optimized for detection of small absorption changes ($<10^{-3}$) with temporal resolution of ~20 ns. Experiments were performed in aerobic and anaerobic conditions. The latter were achieved by keeping the sample under a nitrogen atmosphere.

RESULTS

The cytochrome b_6f complex was excited in the blue edge of the Chl *a* Q_y absorption band at 660 nm and the transient absorbance difference signal ΔA was probed in the range 465–700 nm. Figure 1 shows typical ΔA kinetics probed at 520 nm.

The signal is dominated by the 3–8 μs decay component. Its lifetime and decay-associated spectrum (DAS) shown in Fig. 2 are consistent with the decay of the excited triplet state of β-carotene that is created within less than 20 ns after the excitation. The DAS of the 300 ns and 520 μs components measured under anaerobic conditions are consistent with the decay of the Chl *a* triplet state. The latter two components also exhibit strong photobleaching bands centered at ~670 nm following Q_y absorption band of Chl *a* (not shown).

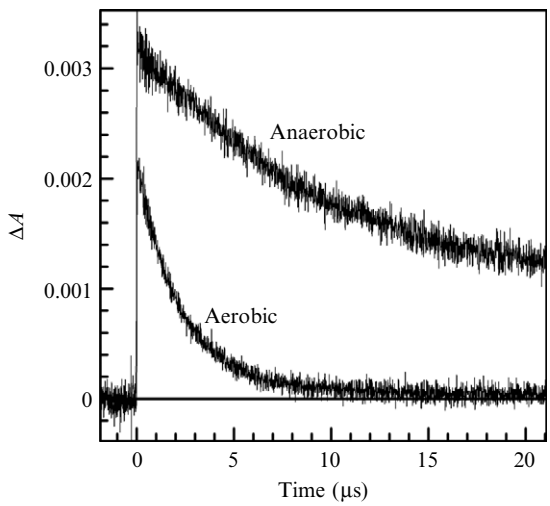

Figure 1: Absorbance difference kinetics measured at 520 nm.

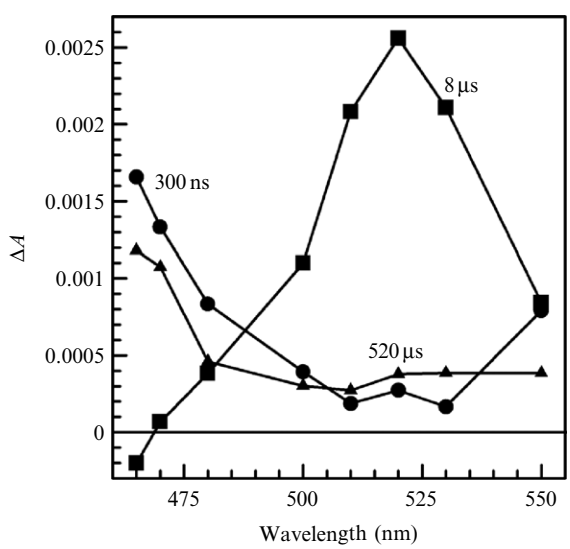

Figure 2: Decay-associated spectra (DAS) of three exponential components obtained by global analysis of wavelength-dependent ΔA profiles measured for the cytochrome b_6f complexes from *M. laminosus* after excitation at 660 nm.

The excitation wavelength dependence of the amplitude of the 3–8 μs component confirmed that the formation of the triplet state of the β-carotene is a consequence of the optical excitation of the Chl *a* molecule. Similar data were obtained for cytochrome b_6f complexes isolated from spinach. In addition, preliminary experiments on the spinach system indicated that the 3–8 μs component characteristic for β-carotene exhibits progressively lower amplitude when samples are kept in oxygen-free conditions for longer times, and disappears when complexes are cooled down to ~50 K.

DISCUSSION

The rate of direct triplet-triplet energy transfer k_{TT} from ^{3}Chl* to Car can be described by Dexter equation:

$$k_{TT}(r) = k_{TT}(0)\ \exp\left(-\frac{2r}{L}\right) \tag{1}$$

where r is the edge-to-edge distance between excitation donor and acceptor, $k_{TT}(0)$ is the excitation transfer rate for zero distance between the cofactors and L is the effective average Bohr radius in the context of exchange energy transfer. High-resolution crystallographic structures of the cytochrome b_6f show that the Chl *a* and β-carotene molecules are at least 14 Å apart. Using Eq. 1 and the triplet-triplet energy transfer data presented in (Bodunov & Berberan-Santos 2004, Schödel et al 1998), we estimated the rate of triplet-triplet energy transfer from ^{3}Chl* to Car in the b_6f complex to be $\sim(0.3\ \text{ms})^{-1}$, which is at least 4 orders of magnitude slower than the formation time of ^{3}Car* observed in the experiment and cannot explain the observed data. Moreover, the calculated value of 0.3 ms is ~2 orders of magnitude longer than the measured triplet energy transfer from ^{3}Chl* to O_2 in aerobic conditions and therefore offers no protection against singlet oxygen formation.

To reproduce the observed instantaneous rise of ^{3}Car* state excitation transfer should occur within less than 20 ns, which would be possible if the excited Chl *a* molecule was positioned within not more than ~7 Å away from β-carotene. In principle, typical cytochrome b_6f samples can contain a small amount of stray Chl *a* molecules that are not bound to the Chl pocket shown in X-ray structures, and it is possible that these extra Chl molecules are responsible for the rapid transfer of excitation to the β-carotene. After careful analysis of the cytochrome b_6f structure we inferred that in this case the extra Chl *a* molecule must be bound to the exterior of the complex in the immediate vicinity of the β-carotene end that protrudes to the surface. However, we found no obvious structural reason that could cause such a specific binding of Chl *a*.

We propose that the rapid formation of ^{3}Car* occurs due to an indirect triplet energy transfer from the excited ^{3}Chl* molecule to the β-carotene via a third, bridging molecule, which mediates otherwise slow triplet energy transfer. While such indirect triplet energy transfer process to carotenoid has not been reported for any Chl-containing pigment-protein complex, the possibility of indirect

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transfer mechanism has been demonstrated in mixed solutions of monomeric Chls and carotenoids (Siefermann-Harms 1987). In the latter case the energy of the Chl triplet excited state was first transferred to an oxygen molecule naturally present in solvent in aerobic conditions. The resulting singlet oxygen then diffused and collided with a carotenoid molecule that efficiently quenched the singlet state of the oxygen forming triplet excited state:

$$^3\text{Chl}^* + \text{O}_2 + \text{Car} \rightarrow \text{Chl} + {}^1\text{O}_2^* + \text{Car} \rightarrow \text{Chl} + \text{O}_2 + {}^3\text{Car}^* \quad (2)$$

In analogy with the above scheme, an oxygen molecule could be mediating triplet energy transfer in the cytochrome b_6f complex as well. To investigate this possibility we measured the ^{3}Chl* and ^{3}Car* dynamics in the complexes that were kept in nitrogen-saturated environment to reduce the concentration of oxygen by up to four orders of magnitude. The dynamics of the signal showed a clear dependence on oxygen concentration. In the case of the cytochrome b_6f complexes purified from spinach, the yield of ^{3}Car* noticeably decreased after the nitrogen treatment, though this effect occurred only under the lowest oxygen concentrations reachable in our experiment and required a daylong exposure to low-oxygen environment. In a separate experiment on the solution of monomeric Chl *a* in ethanol, the exposure of the sample to nitrogen-saturated atmosphere had an almost immediate effect on the extended Chl *a* triplet state lifetime, indicating that the oxygen concentration in solvent follows closely the surrounding atmospheric conditions. These data suggests that, if oxygen does mediate triplet energy transfer within the cytochrome b_6f complex, the O_2 molecule must be captured inside of an isolated protein pocket with a very low probability of escape to the surrounding solvent. The fact that the substantial amount of triplet excitation is transferred from ^{3}Chl* within less than 20 ns supports the presence of a special oxygen channel connecting the Chl *a* and β-carotene molecules. We also found that in the case of the b_6f complexes from spinach formation of ^{3}Car* state is completely blocked at 50 K, which could be explained by a significantly lower mobility of oxygen at low temperatures. Within the oxygen-mediated energy transfer scenario, the main energy transfer component is faster than 20 ns. The 300 ns ^{3}Chl* decay may be caused by O_2 molecules that are initially remote from the Chl, but still trapped inside the oxygen channel, and the longest 500 μs component shown in Fig. 2 represents stray Chls that are quenched by oxygen since this lifetime depends strongly on the oxygen concentration in a way that is similar to the monomeric Chl *a* in organic solvents. So far, we were unable to observe the reduction in ^{3}Car* formation under low oxygen conditions in cytochrome b_6f complexes from *M. laminosus*, which may indicate that O_2 molecules in these complexes are better bound to the interior of the protein.

In conclusion, we have observed rapid and efficient triplet excitation energy transfer from the Chl *a* molecule to the β-carotene incytochrome b_6f complexes and propose that a third molecule, possibly oxygen, serves as a mediator in this transfer.

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MUTATIONAL ANALYSIS OF THE CYTOCHROME *bf* RIESKE IRON-SULFUR PROTEIN AND QUINONE-REDUCTASE (Q_I) SITE

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Keywords: cytochrome *bf* complex, Rieske protein, Q_i site, oxygen radicals

INTRODUCTION

The cytochrome *bf* complex catalyzes the rate-limiting quinol-oxidation step of oxygenic photosynthesis, establishes a transmembrane proton gradient, and has been implicated in redox signaling and oxygen radical production. Four major subunits (cytochrome b_6, subunit IV, the Rieske iron-sulfur protein (ISP), and cytochrome *f*), four well-known prosthetic groups (two *b*-hemes, the Rieske 2Fe−2S cluster, and a *c*-type cytochrome), and four small subunits (PetG, PetL, PetM, PetN) comprise the *bf* complex. Recent X–ray crystal structures reveal an additional, unique heme designated heme *x* in the cyanobacterium, *Mastigocladus laminonsus* (Kurisu et al 2003) or heme $c'(c_i)$ in the alga, *Chlamydomonas reinhardtii* (Stroebel et al 2003). The extra heme, a chlorophyll, and a carotenoid are unique features that distinguish cytochrome *bf* from bacterial/mitochondrial *bc* complexes. We have investigated: (i) the *Chlamydomonas* chloroplast ISP disulfide bridge, flexible hinge, and N-terminal domains in catalysis, assembly, and signaling, and (ii) the enigmatic quinone-reductase (Q_i) site in catalysis and oxygen radical production in the cyanobacterium *Synechococcus* PCC 7002.

MATERIALS AND METHODS

Rieske ISPs were expressed as thioredoxin fusion proteins, reconstituted with iron-sulfur clusters *in vitro*, and analyzed by EPR