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ORIGINAL ARTICLE

Effects of exogenous β-carotene, a chemical scavenger of singlet oxygen, on the millisecond rise of chlorophyll *a* fluorescence of cyanobacterium *Synechococcus* sp. PCC 7942

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Abstract Singlet-excited oxygen ($^1O_2^*$) has been recognized as the most destructive member of the reactive oxygen species (ROS) which are formed during oxygenic photosynthesis by plants, algae, and cyanobacteria. ROS and $^1O_2^*$ are known to damage protein and phospholipid structures and to impair photosynthetic electron transport and de novo protein synthesis. Partial protection is afforded to photosynthetic organism by the β-carotene (β-Car) molecules which accompany chlorophyll (Chl) *a* in the pigment-protein complexes of Photosystem II (PS II). In this paper, we studied the effects of exogenously added β-Car on the initial kinetic rise of Chl *a* fluorescence (10–1000 μs, the OJ segment) from the unicellular cyanobacterium *Synechococcus* sp. PCC7942. We show that the added β-Car enhances Chl *a* fluorescence when it is excited at an intensity of 3000 μmol photons m^{-2} s^{-1} but not when excited at 1000 μmol photons m^{-2} s^{-1}. Since β-Car is an efficient scavenger of $^1O_2^*$, as well as a quencher of ^{3}Chl *a** (precursor of $^1O_2^*$), both of which are more abundant at higher excitations, we assume that the higher Chl *a* fluorescence in its presence signifies a protective effect against photo-oxidative damages of Chl proteins. The protective effect of added β-Car is not observed in O_2-depleted cell suspensions. Lastly, in contrast to β-Car, a water-insoluble molecule, a water-soluble scavenger of $^1O_2^*$, histidine, provides no protection to Chl proteins during the same time period (10–1000 μs).

Keywords β-Carotene · Chlorophyll fluorescence · Cyanobacteria · Singlet oxygen · *Synechococcus* sp. PCC7942

Abbreviations

β-Car	β-Carotene
Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethyl sulfoxide
GO	Glucose oxidase
PS I, PS II	Photosystem I, photosystem II
PQ-pool	Plastoquinones shuttling electrons between PS II and PS I
ROS	Reactive oxygen species

Introduction

Singlet-excited molecular oxygen ($^1\Delta_g$; $^1O_2^*$), a high energy form of oxygen and a byproduct of light capture and conversion in photosynthesis, is known to damage oxygenic photosynthetic organisms (Krieger-Liszkay et al. 2008; Triantaphyllidès and Havaux 2009; Vass 2012; Kreslavski et al. 2013; Tyystjaervi 2013; Pospišil and Prasad 2014; Telfer 2014; Demmig-Adams et al. 2014). In particular, it initiates the degradation of the D1 protein of the photosystem II reaction center (PSII-RC) complex (Edelman and Mattoo 2008) and inhibits de novo proteins synthesis, which then leads to the photoinactivation of PS II (Allakhverdiev and Murata 2004; Nishiyama et al. 2004;

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Murata et al. 2012). $^1O_2^*$ is formed upon transfer of triplet electronic excitation from chlorophyll *a* (^{3}Chl *a**) to ground-state triplet oxygen ($^3O_2$). Excited Chl *a* triplets are formed in two ways: (a) by spontaneous intersystem crossing (spin inversion) of singlet-excited Chl *a* (^{1}Chl *a**) to the triplet state, a transition which is favored when photochemical de-excitations are prevented (Asada 1999; Vass 2012; Telfer 2014) and (b) by charge recombination between the primary electron donor cation (P_{680}^+) and the primary electron acceptor anion ($Pheo^-$) of PSII-RC to a virtual triplet state $^3(P_{680}^+Pheo^-)$, followed by excitation transfer to $^3O_2$ (van Mieghem et al. 1989; Vass et al. 1992; Keren et al. 1997; Mamedov et al. 2015).

^{3}Chls *a** have been detected also in whole photosystem I (PS I; Blankenship et al. 1975; Shuvalov 1976; Frank et al. 1979), in peripheral PS I antenna proteins (Carbonera et al. 2005; Croce et al. 2007), as well as in its reaction center (PSI-RC; Schlodder et al. 2005, 2007). However, $^1O_2^*$ could not be detected in isolated PS I particles (Hideg and Vass 1995). On the other hand, a mutant of *Arabidopsis thaliana* with a low *β*-Car content in PSI was found to be more susceptible to photo-oxidative damage at chilling temperatures than wild-type plants (Cazzaniga et al. 2012); also, a carotenoidless *Synechocystis* mutant has been described which is unable to synthesize PS II complexes although it can form and assemble PS I complexes (Domonkos et al. 2013). All these may suggest that $^1O_2^*$ is formed in PS I but it is effectively scavenged by the *β*-Cars and xanthophylls of pigment-protein complexes.

In the trimeric (LHCII) and the monomeric (CP29, CP26, CP24) peripheral antenna proteins of PSII, $^1O_2^*$ is trapped by xanthophylls, while in the core antenna proteins (CP43, CP47) it is by *β*-Cars (e.g., Ramel et al. 2013; Pospišil and Prasad 2014). In general, ground-state *β*-Cars and carotenoids quench ^{1}Chl *a** and ^{3}Chl *a** and scavenge chemically $^1O_2^*$, thereby decreasing $^1O_2^*$ levels directly, as well as indirectly (Edge and Truscott 1999; Ostrumov et al. 2014; Telfer 2014). In cyanobacteria, which have no Chl *a*-binding peripheral antenna proteins, $^1O_2^*$ is formed only in the core antenna (CP43, CP47) and in the PSII-RC (D_1, D_2) complexes. In these photosynthetic prokaryotes, the homodimeric core antenna complex binds, per monomer, 35 Chls *a* and 11 *β*-Car. Each of the reaction center complexes (i.e., D_1 and D_2), binds 3 Chls *a* and 1 *β*-Car, while CP43 binds 13 Chls *a* and 4 *β*-Cars and CP47 binds 16 Chls *a* and 5 *β*-Cars (see Broser et al. 2010).

$^1O_2^*$ forms within the hydrophobic domains of the thylakoid membrane, where Chls *a* is located. During its long lifetime (25–100 μs in non-polar solvents, 2–4 μs in water; Knox and Dodge 1985) it diffuses to aqueous membrane domains where it is usually detected by means of water-soluble compounds that act as chemical traps (e.g., imidazole, histidine, sodium azide; see: Telfer et al. 1994; Rehman et al. 2013) or as fluorescence sensors (Hideg et al. 2002; Fryer et al. 2002; Flors et al. 2006; Sinha et al. 2011). In this research, we investigated the possibility of detecting $^1O_2^*$ within hydrophobic membrane domains by means of the water-insoluble scavenger *β*-Car. Our expectation was that this scavenger would report the presence of $^1O_2^*$ by eliminating its destructive effects on Chl *a* and on the fluorescence signal it emits.

To this end, we compared initial rise kinetics of Chl *a* fluorescence (the OJ phase; lasting from 10 to 1000 μs), upon transition from darkness to light, recorded in cell suspensions with and without added *β*-Car. For this comparison, to OJ traces are normalized at J, namely at equal concentrations of Q_A, the primary quinone electron acceptor of PSII. This normalization is justified by the fact that, upon dark adaptation, cyanobacteria shift to state II because their intersystem plastoquinones (the PQ-pool) become fully reduced by respiratory substrates (Dominy and Williams 1987; Tsimilli-Michael et al. 2009). This is reflected in the fact that, in cyanobacteria, fluorescence levels at J, I, and P of the fluorescence induction trace are nearly equal (see Tsimilli-Michael et al. 2009; Fig. 1b, insert). In contrast, in eukaryotic photosynthetic cells (plants, algae) it is as follows: $J < I < P$. Practically, the single independent variable that determines the rise of Chl *a* fluorescence along OJ in cyanobacteria is the reduction level of Q_A. By normalizing the OJ traces to J, we compare *Synechococcus* samples with equal Q_A contents.

Our results show enhanced Chl *a* fluorescence along the OJ kinetic phase (see Stirbet and Govindjee 2011) when plus-*β*-Car *Synechococcus* suspensions are compared with minus-*β*-Car ones. Since the 650 nm light, used in these experiments, does not excite *β*-Car to higher electronic states, and since ground-state *β*-Car is an efficient chemical scavenger of $^1O_2^*$ (Di Mascio et al. 1989), but has no other action except light harvesting (Ramel et al. 2013) we take this result to indicate that exogenous *β*-Car does indeed afford protection to Chls *a* in situ. We show, also, that the fluorescence difference $\Delta F_{\pm} = F_{+\beta\text{-Car}} - F_{-\beta\text{-Car}}$ is higher at higher excitation intensities and close to zero in O_2-depleted (anoxic) cell suspension. Further, we show that $^1O_2^*$ does depress the very first recorded Chl *a* fluorescence signal (F_0, or point O, at 10 μs). With exogenously added *β*-Car, therefore, $^1O_2^*$ can be detected directly within hydrophobic membrane domains where it is formed and before it diffuses to hydrophilic membrane domains.

Materials and methods

Cell cultures and preparations

The single-cell cyanobacterium *Synechococcus* sp. PCC7942 was cultured photo-autotrophically in the BG11

medium (Rippka et al. 1979) as described by Stamatakis et al. (2014). Cells were harvested after 4 days (during exponential growth) and were resuspended in fresh BG11 medium at 1 μg Chl *a* ml^{-1} to be used in fluorimetric assays. Depending on the assay, cell samples further contained: 20 μM of the PS II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), added from a stock solution in ethanol; 5 μg ml^{-1} *β*-Car, from a stock solution in the water miscible aprotic solvent dimethyl sulfoxide (DMSO); and 5 mM histidine.

Anoxic cells were prepared by adding to cell suspensions 5 μg ml^{-1} glucose oxidase (GO) and 20 mM glucose, followed either by a 5-min dark incubation without stirring (low level anoxia), or a 30-min dark incubation (high-level anoxia).

All used chemicals were obtained from Sigma-Aldrich.

Kinetic assays of Chl *a* fluorescence

Time courses of the intensity of Chl *a* fluorescence, which is emitted after turning on continuous exciting light on dark-adapted cell suspensions (fluorescence induction, FI) were recorded with a Plant Efficiency Analyser fluorometer (Handy-PEA, Hansatech, King's Lynn, Norfolk, UK). Continuous excitation was provided to cell suspensions at 650 nm, $\Delta\lambda = 22$ nm, 3000 μmol photons m^{-2} s^{-1} (strong light, SL), or at 1000 μmol photons m^{-2} s^{-1} (weak light, WL). Chl *a* fluorescence was detected above 700 nm and was recorded continuously from 10 μs to 2 min with data acquisition every 10 μs for the first 300 μs, then every 100 μs (300 μs–3 ms), then every 1 ms (3–30 ms). Each displayed OJ trace is the average of 3 independent traces.

Results and discussion

Effects of exogenous *β*-Car on the OJ segment of the Chl *a* fluorescence time trace

The time trace of Chl *a* fluorescence, or Chl *a* fluorescence induction, as recorded after illuminating dark-adapted photosynthetic samples with continuous and steady exciting light, reflects the interplay of various photochemical and non-photochemical processes that impact on the population of singlet-excited Chl *a* (^{1}Chl *a*; reviewed by Papageorgiou et al. 2007; Papageorgiou 2012; Papageorgiou and Govindjee 2014). In cyanobacteria, the fluorescence induction trace takes the typical form shown in the insert of Fig. 1b, whose extrema and inflections are labeled as OJIPSMT ("O" is for the origin, the minimum; "J" & "I" are inflections; "P" is for peak; "S" is for semi-steady-state; "M" is for maximum; and "T" is for the terminal steady-state; also see Govindjee 1995). In this research, we focus on the OJ fluorescence rise segment (cf. Fig. 1b, inset) which lasts for ~1 ms and its main cause in cyanobacteria is the photochemical reduction of Q_A, the primary plastoquinone electron acceptor of PSII. The reason for this is that in these prokaryotes photosynthesis and respiration are both located in the thylakoid membrane and this allows the dark reduction of the PQ-pool by respiratory substrates. Thus, after dark adaptation, cyanobacteria shift to the low fluorescence state 2, so upon illumination fluorescence signal is not subject to changes by state 1 to state 2 transitions or by direct quenching by oxidized PQ-pool plastoquinones. This justifies the normalization of the OJ traces to J (i.e., to equal concentrations of Q_A), and allows us to compare only two independent variables affecting Chl

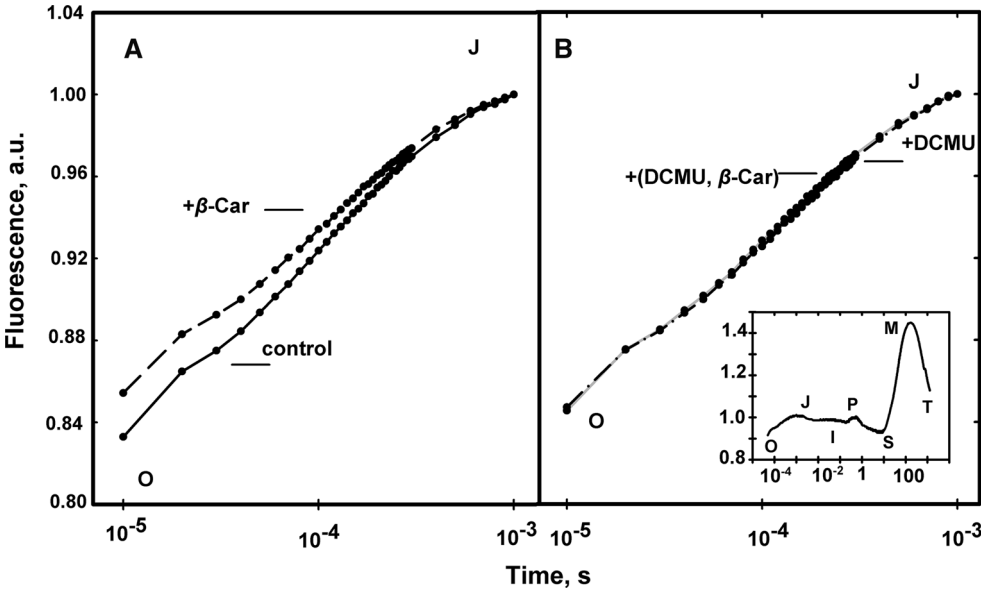

Fig. 1 Effects of strong light excitation (3000 μmol photons m^{-2} s^{-1}; $\lambda_{exc} = 650$ nm, $\Delta\lambda = 22$ nm) on the OJ segment of the Chl *a* fluorescence induction trace (see *inset* in **b**) of *Synechococcus* sp. 7932 cells. **a** OJ traces of −DCMU cells, in the absence (*black line*, control), or in the presence of exogenous *β*-carotene (*dashed black line*). **b** OJ traces of +DCMU cells, in the absence (*gray line*), or in the presence of exogenous *β*-carotene (*gray dashed line*). All OJ traces displayed are normalized at equal J, namely to equal concentrations of fully reduced Q_A

a fluorescence, the reduction level of Q_A and the presence of exogenous β-Car.

In Fig. 1a experiment, we compare normalized OJ traces of Chl *a* fluorescence of cell suspensions with and without added β-Car. In Fig. 1b, we do the same experiments, but with DCMU-containing cell suspensions. Very characteristically, in Fig. 1a the OJ trace of the $+\beta$-Car sample lies above that of the $-\beta$-Car one. Since β-Car has no effect on the redox level of Q_A (see Concluding Remarks) while, on the other hand, it is a very effective scavenger of $^1O_2^*$ (Di Mascio et al. 1989) the fluorescence difference magnitude $\Delta F_{\pm} = F_{(+\beta\text{-Car})} - F_{(-\beta\text{-Car})}$ must reflect a protective effect of the added β-Car against photo-oxidative damage by $^1O_2^*$ that occurs within 1 ms of exciting light illumination. In contrast, in Fig. 1b, the $-\beta$-Car trace and the $+\beta$-Car trace coincide, $\Delta F_{\pm} = 0$ and therefore no apparent protective effect is expressed by the added β-Car. Since the −DCMU and +DCMU samples differed not in the dissolved O_2 content but only in the inability of the +DCMU sample to evolve oxygen (Velthuys 1981), we conclude that the protective effect of added β-Car must relate to the photosynthetically evolved O_2, and more specifically to the PSII-RC. Therefore, it appears that only the $^1O_2^*$ formed in PSII-RC by charge recombination causes the photo-oxidative damages against which the protective effect of the exogenous β-Car is exerted.

Effects of low light excitation intensity on F_0 and OJ

Since ^{3}Chls a^*, the precursors of $^1O_2$, are products of photonic reactions, it is expected that higher excitation intensities will lead to higher $^1O_2^*$ populations. In Fig. 2 experiment, we asked if the protective effect of exogenous β-Car against the $^1O_2^*$-induced suppression of Chl *a* fluorescence, clearly evident in Fig. 1a upon exciting *Synechococcus* with 3000 µmol photons m^{-2} s^{-1}, will also materialize at the lower excitation intensity of 1000 µmol photons m^{-2} s^{-1}. In spite of the more noisy OJ traces of Chl *a* fluorescence, due to the lower excitation intensity, no difference can be discerned in Fig. 2 between the minus-β-Car trace and the plus-β-Car one.

Effects of cell suspension anoxia on F_0 and OJ

If the protective effect of exogenous β-carotene on Chl *a* fluorescence is due to the removal of $^1O_2^*$, then it should not materialize in O_2-depleted (anoxic) cell suspensions. In such suspensions, the light-induced formation of $^1O_2^*$ and the extent of the attendant suppression of Chl *a* fluorescence are expected to be reduced, compared to O_2-replete suspensions. To test this expectation, we prepared anoxic cyanobacteria by incubating cell suspension with glucose oxidase (GO) and glucose. This reaction consumes O_2 by

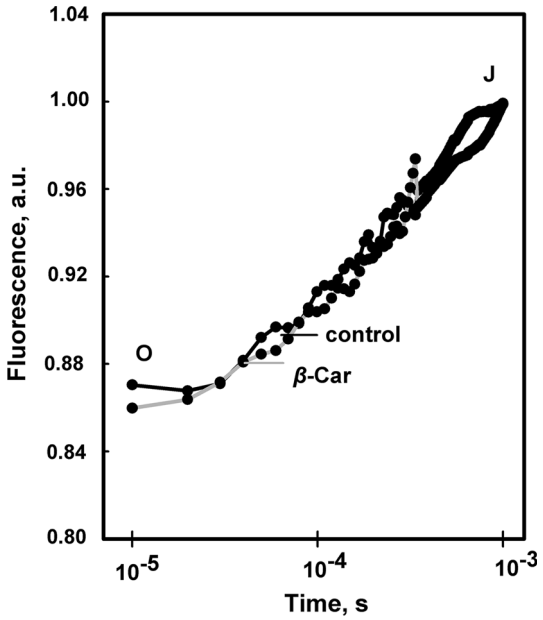

Fig. 2 Effects of weak light excitation (1000 µmol photons m^{-2} s^{-1}; $\lambda_{exc} = 650$ nm, $\Delta\lambda = 22$ nm) in the absence (control, *black line*), or in the presence of exogenous β-carotene (*gray line*)

oxidizing glucose to gluconic acid and H_2O to H_2O_2. Since the cell membrane is impermeable to the enzyme, the de-oxygenation reaction operates only on the dissolved O_2 in the suspension medium. Cells, therefore, become anoxic by the natural diffusion of the cytoplasmic O_2 to the O_2-poor suspension medium, and this may require longer incubations of the reaction mixture.

Figure 3 shows data from experiments in which we tested whether de-oxygenation of the call suspension can afford a similar protection to *Synechococcus* cells as the addition of β-Car. Cells were dark incubated with GO and glucose for 5 min in (a) or for 30 min in (b) and the OJ traces of Chl *a* fluorescence were recorded. According to Fig. 3a, the 5-min dark incubation with GO and glucose caused nearly no effect since $\Delta F_{\pm GO} \approx 0$. We interpret this result to reflect the incomplete de-oxygenation of samples due to the short incubation time. Indeed, when the dark incubation time was increased to 30 min (Fig. 3b), the OJ fluorescence trace of the deoxygenated cells was clearly above that of the control cells (i.e., $\Delta F_{\pm GO} > 0$). The enhanced fluorescence of the anoxic cells may signify a suppressed generation of $^1O_2^*$ and, in addition, a suppressed excitation quenching by ground-state O_2 (Papageorgiou et al. 1972). Further, the addition β-Car to the 30-min dark incubated cells revealed a supplementary protective effect, on top of that of the GO-effected anoxia (i.e., $\Delta F_{+\beta\text{-Car},+GO} > \Delta F_{+GO}, > \Delta F_{-GO}$). This proves that $^1O_2^*$ was involved in the suppression of Chl *a* fluorescence of anoxic cells.

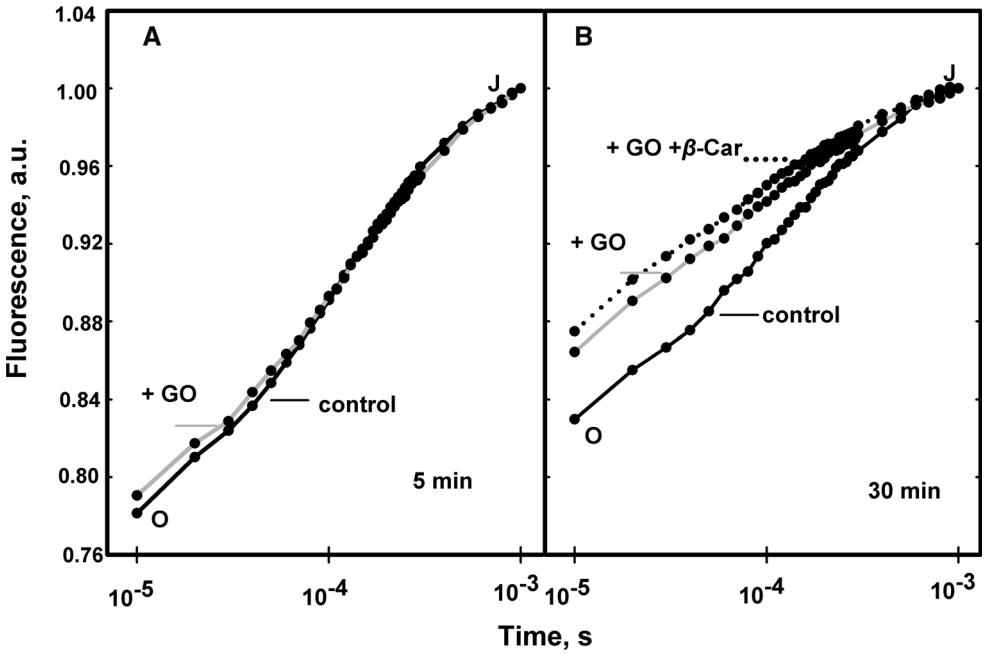

Fig. 3 Effects of cell suspension anoxia on the OJ traces of Chl *a* fluorescence induction of *Synechococcus* cells. **a** Lower level of anoxia, after 5 min dark incubation of the cells with (GO) and glucose. **b** Higher level of anoxia, after 30 min dark incubation of the cells with GO and glucose

Effects of histidine, a water-soluble scavenger of $^1O_2^*$ on the millisecond rise of Chl *a* fluorescence of *Synechococcus*

Singlet oxygen formed during photosynthesis has been detected and determined by methods employing chemical trapping, spin trapping, dye bleaching, and fluorescent traps (Rehman et al. 2013; Telfer 2014). All these probes are water-soluble compounds, so they respond to $^1O_2^*$ that diffuses from hydrophobic to hydrophilic regions of the thylakoid membrane. A method that has been applied by several laboratories is the chemical trapping of $^1O_2^*$ by histidine (see e.g., Telfer et al. 1994; Rehman et al. 2013). In the experiment shown in Fig. 4, we asked whether this water-soluble chemical trap of $^1O_2^*$ would afford protection to Chls *a* during the OJ rise of Chl *a* fluorescence.

According to the figure, the OJ traces recorded with *Synechococcus* cells suspended in the absence and in the presence of histidine are very nearly the same. Therefore, as detected by means of OJ rise of Chl *a* fluorescence, water-soluble histidine affords no protection to chlorophyll structures against photo-oxidative damage within approx. 1 ms from the onset of the exciting illumination.

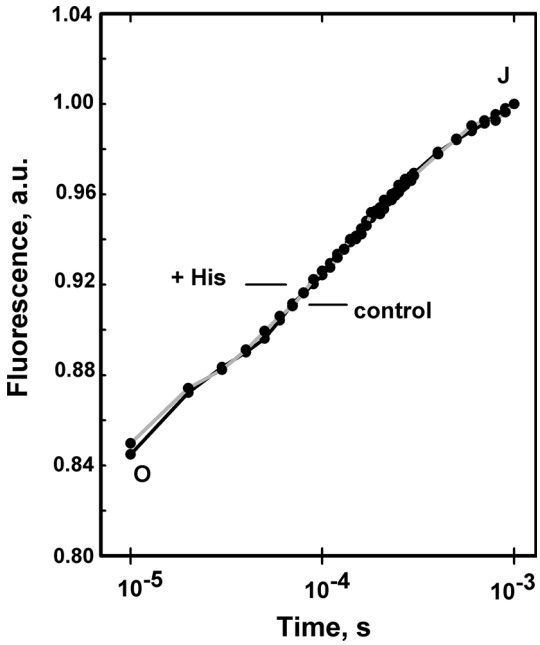

Fig. 4 Effects of histidine on the OJ segment of the Chl *a* fluorescence induction trace

Concluding remarks

The time course of the Chl *a* fluorescence in vivo, following a transition from darkness to light, particularly the OJIP of the total OJIPSMT pattern (see Fig. 1b, insert) has been simulated quite successfully by several authors (see, e.g., Strasser et al. 2004; Belyaeva et al. 2008, 2011, 2015; Lazar and Jablonsky 2009; Stirbet and Govindjee 2011; Schansker et al. 2014; Vredenberg 2015). None of these simulations, however, did include $^1O_2^*$ among the factors that determine the early kinetic rise of Chl *a* fluorescence. As shown in the present paper, $^1O_2^*$ is indeed involved, particularly at high light excitation intensities).

In the Results and Discussion section, we interpreted the higher Chl a fluorescence along OJ of +β-Car cell suspensions only in terms of scavenging of $^1O_2^*$ and quenching of ^{3}Chls a^* by the exogenous β-Car. This interpretation would be valid only if the added β-Car is not involved in other processes that may impact on Chl *a* fluorescence. One such process could be the direct reduction of Q_A and of PQ-pool quinones by the exogenous β-Car. This would be possible if the 1-electron redox potential of the added β-Car is a more negative than that of the Q_A/Q_A^- couple ($E_m^0 \approx 0$ V). However, β-Cars with ($E_m^0 \approx 1$ V; Edge et al. 2000; Ishikita and Knapp 2005) have been reported to transfer electrons only to $P680^+$, the strongest oxidant of PS II ($E_m^0 \approx 1.1$ V; Hanley et al. 1999; Vrettos et al. 1999) and not, of course, to plastoquinones. A direct reduction, therefore, of Q_A and of intersystem plastoquinones by exogenously added β-Car seems highly unlikely. Further, the involvement of triplet-excited β-Car as a non-photochemical modulator of F_0 (Belyaeva et al. 2015) also appears unlikely in our case, since the 650-nm exciting light we used is not absorbed by carotenes.

In conclusion, it appears that the enhancement of Chl a fluorescence in cyanobacteria by exogenously added β-Car is due only to the scavenging of $^1O_2^*$, and therefore it is a unique way to detect its presence in non-polar domains of the thylakoid membrane.

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