ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 1/2012
61–64
on-line at: www.actabp.pl

Regular paper

Antioxidant effects of carotenoids in a model pigment–protein complex*

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The effect of carotenoids on stability of model photosynthetic pigment-protein complexes subjected to chemical oxidation with hydrogen peroxide or potassium ferricyanide was investigated. The oxidation of carotenoid-less and carotenoid-containing complexes was conducted in the presence or absence of ascorbic acid. The progress of the reactions was monitored by use of absorption and fluorescence spectroscopy. Our results show that carotenoids may significantly enhance the stability of photosynthetic complexes against oxidation and their protective (antioxidant) effect depends on the type of the oxidant.

Key words: carotenoid, chemical oxidation, antioxidant, stability

Received: 24 October, 2011; accepted: 01 March, 2012; available on-line: 17 March, 2012

INTRODUCTION

The photosynthetic apparatus in purple photosynthetic bacteria comprises reaction centers and light harvesting pigment protein complexes LH1 and LH2. The latter, i.e. the peripheral antenna complex, is not produced by all bacterial species. The crystal structures of LH2 complexes from several species of phototrophic bacteria were solved with a high resolution (McDermott *et al.*, 1995; Koepke *et al.*, 1996) while the structure of LH1 is known in less detail. As shown by Ghosh *et al.* (1988) each complex is composed of identical blocks of low molecular weight and very hydrophobic α- an β-apoproteins to which bacteriochlorophylls (BChls) and carotenoids (Crts) are non-covalently attached (Cogdell *et al.*, 1996). In the absence of Crts, the pigment-polypeptide units spontaneously oligomerize to form the complex whose main absorption maximum is located near 870 nm. In the presence of Crts this band shifts to 880 nm (in the case of *Rhodospirillum rubrum*). In micellar media, the Crt-less LH1 (B870) reversibly dissociates into monomeric subunits (Loach *et al.*, 1995; Fiedor *et al.*, 2009). The ability of B870 to undergo reversible dissociation renders the complex a unique model system that allows for investigation of interactions occurring between its components. The oligomerization and reconstitution of LH1 from its native or chemically modified components has been studied extensively (Davidson *et al.*, 1981; Miller *et al.*, 1987; Strugis *et al.*, 1994; Frank, 1999; Pandit *et al.*, 2001). Also a full reconstitution, including all components of the LH1 antenna was achieved (Davis *et al.*, 1995; Fiedor *et al.*, 2001b; Fiedor *et al.*, 2004; Fiedor *et al.*, 2009).

It has been shown that BChl components of LH1 complexes undergo oxidation upon treatment with potassium ferricyanide. Such treatment leads to changes in the absorption, circular dichroism (CD), fluorescence emission, and electron paramagnetic resonance spectral signals of the complex (Gomez *et al.*, 1982; Picorel *et al.*, 1984; Law *et al.*, 1998). We have previously studied the effects of Crts on stability of BChla and its derivatives in a model system (Fiedor *et al.*, 2001a; Fiedor *et al.*, 2002), however, so far, the effect of Crts on stability of LH1 complexes exposed to chemical oxidation has not been demonstrated. Crts are structurally and functionally a very diverse group of isoprenoid pigments (Britton, 1995). They occur in all photosynthetic organisms in which they carry out various functions, with light harvesting and (photo)protection considered as the most important. Crts act both as physical quenchers of (B)Chl excited states and efficient scavengers of reactive oxygen species (ROS). They also act as chemical quenchers of ROS undergoing irreversible modifications that may be responsible for a change from the antioxidant to prooxidant activity (Fiedor *et al.*, 2001a; Fiedor *et al.*, 2005).

In the present paper, we demonstrate our results on the role of Crts in stabilization of pigment-protein complexes against chemical oxidation. The model Crt-containing and Crt-less LH1 complexes were subjected to chemical oxidation with hydrogen peroxide or potassium ferricyanide. The antioxidant and stabilizing activity of Crt was monitored by following the changes in the electronic absorption and fluorescence emission spectra of the oxidized complexes.

MATERIALS AND METHODS

Isolation of spheroidene. Spheroidene (Sph) was isolated from the cells of *Rhodobacter sphaeroides* 2.4.1 as described in Fujii *et al.* (1998) with minor modifications. The purity of the isolated Sph was checked spectrophotometrically and by HPLC (Fiedor, 2007).

Preparation of B870. Crt-depleted LH1 was prepared as described by Fiedor *et al.* (2004; 2009) and used immediately after preparation.

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*Presented at the 16th International Symposium on Carotenoids, 17–22 July, 2011, Kraków, Poland

#Work performed in partial fulfillment for the M.Sc. degree

Abbreviations: AA, ascorbic acid; BChl, bacteriochlorophyll; B870, carotenoid-devoid light harvesting complex 1 with the main absorption band near 870 nm; CD, circular dichroism; Crt, carotenoid; LH, light harvesting complex; ROS, reactive oxygen species; Sph, spheroidene; Sph-LH1, light harvesting complex 1 reconstituted with spheroidene.

Reconstitution assay. The incorporation of Sph into LH complexes was carried out as described previously (Fiedor *et al.*, 2004). Briefly, for the reconstitution, a portion of bacterial Crt-less LH1 in 0.033% LDAO was titrated with a solution of Sph in acetone (OD_{457nm} ~1.2). The progress of the reconstitution was monitored by absorption spectroscopy. The reconstitution was considered complete when a red shift of the LH1 Q_Y band from 873 nm to 880 nm was observed. The freshly prepared Sph-binding LH1 (Sph-LH1) was kept overnight at 4°C and used without further purification.

Chemical stability of B870 and Sph-LH1. The resistance of Crt-less and Sph-containing LH1 complexes toward chemical oxidation was tested in reactions with hydrogen peroxide (70 mM, 150 mM and 290 mM) or potassium ferricyanide (25 mM, 50 mM, and 100 mM) in the absence or presence of 1 mM ascorbic acid (AA). The progress of the oxidation was monitored by following the changes in the absorption (Cary50 Bio, Varian) and fluorescence emission spectra (FluoroMax-P, Horiba Jobin Yvon). The spectra were recorded just before addition of an oxidant, and then after being kept in the dark for 5, 15, 30, 45, 60, 75 and 90 min at room temperature. Simultaneously, control (no oxidant) experiments were performed.

RESULTS AND DISCUSSION

The model Crt-less B870 and Crt-containing LH1 complexes were prepared following the published procedures (Fiedor *et al.*, 2004; Fiedor, 2006; Fiedor *et al.*, 2009). The absorption spectrum of B870 exhibits a characteristic maximum at 875 nm assigned to the BChl Q_Y transition (Fig. 1A). The excitation at the BChl Q_X band (590 nm) results in the emission of fluorescence with a maximum at 890 nm. A strong conservative signal, with a minimum at 849 nm, a maximum at 884 nm and a crossover point at ~872 nm are characteristic features of the CD spectrum of B870. In the Q_X range, a prominent positive signal is also present (Fig. 1A, inset). The spectral properties of B870 are consistent with those described in the literature (Picorel *et al.*, 1983; Chang *et al.*, 1990; Fiedor *et al.*, 2009).

A recently developed method of the LH1 reconstitution was applied to prepare model Crt-containing complex. Crts are known to have a high affinity to Crt-less LH1 subunits, resulting in changes of thermodynamic parameters of the LH1 formation (Fiedor *et al.*, 2009). Sph, a bacterial acyclic Crt, with one metoxy group and 10 conjugated double bonds, was incorporated to the BChl-protein matrix. The absorption spectrum of reconstituted Sph-LH1 (Fig. 1B) resembles the spectrum of native LH1 (Fiedor *et al.*, 2004). It exhibits a strong transitions in the NIR range (around 880 nm) and Crt bands with characteristic for Sph maxima at 442, 470 and 502 nm. The excitation of Sph-LH1 at 470 nm results in an emission spectrum with a maximum at 898 nm. The correctness of the reconstitution process was evidenced by the efficient Crt-to-BChl singlet energy transfer. As estimated from the excitation spectrum of the Sph-LH1, it occurs with the efficiency of about 85%. This is in agreement with the value previously reported for LH1 complexes containing Sph (Noguchi *et al.*, 1990; Akahane *et al.*, 2004).

The Sph-LH1 complex was also characterized by the use of the CD technique. The CD spectrum shows a weak conservative signal in the NIR region with the crossover point at ~881 nm (Fig. 1B, inset). Other signals appear in the regions of the BChl Q_X and Sph absorption bands. The CD spectrum provides a further evidence that the environment of BChl is affected by the presence of Sph.

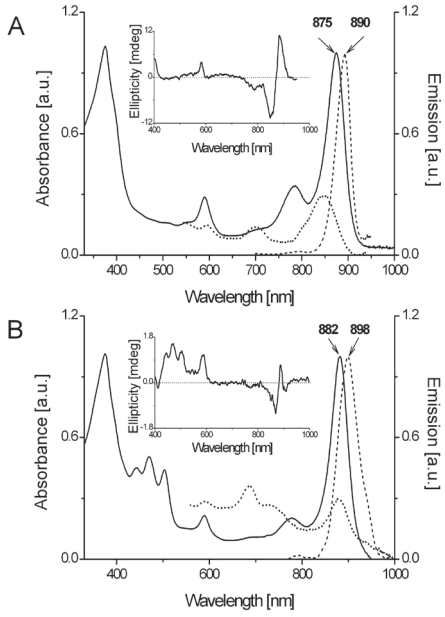

Figure 1. Normalized absorption (solid line), emission (dash line) and circular dichroism (insets) spectra of (A) the B870 complex, and (B) the Sph-binding LH1 complex.
The steady-state emission spectra were recorded upon excitation at 590 nm (B870) and 470 nm (Sph-LH1), respectively. The CD spectra were measured using a Jasco J-815 spectropolarimeter. Additionally, normalized (to 0.3) absorption spectra of the complexes treated with 25 mM $K_3Fe(CN)_6$ for 90 min are shown (dotted line).

In order to determine the effect of Crt on the stability of LH1 against chemical oxidants a series of experiments with hydrogen peroxide and potassium ferricyanide was performed. In all cases, a gradual decrease of the LH1 Q_Y band was observed, accompanied by an increase of absorption at ~690 nm (Fig. 1). The control experiments performed at room temperature in darkness showed that B870 and Sph-LH1 are stable. The decrease of the respective Q_Y bands did not exceed 7%.

B870 and Sph-LH1 were exposed to 70 mM, 150 mM or 290 mM H_2O_2. For example, after 5 min treatment of B870 with 70 mM H_2O_2 the intensity of the BChl Q_Y band decreased by 10% (Fig. 2A). A prolonged incubation resulted in a further decrease of the absorption band by about 20%, 35% and 63% after 15, 30 and 90 min, respectively. A decrease of the Q_Y intensity was accompanied by a shift of about 1-2 nm toward longer wavelength in the B870. An increase in H_2O_2 concentration to 290 mM caused a larger drop of the Q_y band, by about 50% and 90% after 15 and 90 min, respectively. A very similar B880 degradation kinetics was observed during the initial 15 min of the treatment of Sph-LH1 with 70 mM H_2O_2. At longer times, a very clear protective effect of Crt on LH1 is seen, as after 90 min the concentration of Sph-LH1 is twice as high as that of B870 (Fig. 2B). At higher concentrations of H_2O_2 only a weak effect of Sph was observed (Fig. 2B). The kinetics of emission decrease (not shown) was much faster than the one seen in the absorption but after 90 min a similar 60–70% drop occurred.

The stability of B870 and Sph-LH1 was also monitored in the presence of $K_3Fe(CN)_6$. The samples were

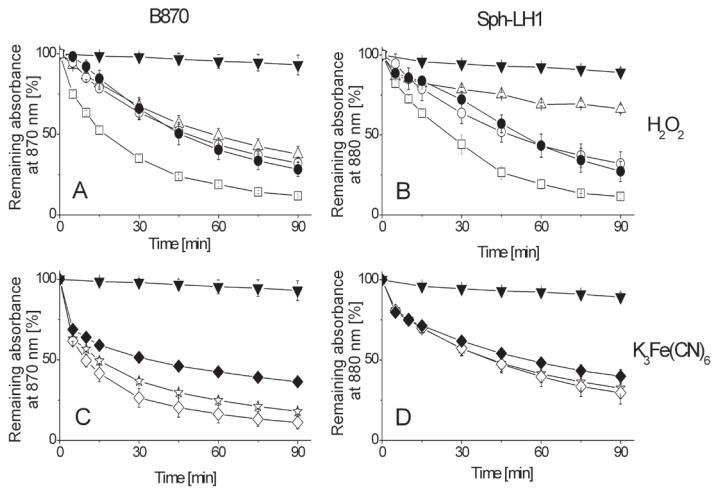

Figure 2. The stability of B870 (A, C) and Sph-LH1 (B, D) exposed to oxidants: H_2O_2 (A, B) or $K_3Fe(CN)_6$ (C, D), respectively, as judged from decrease of the absorbance at the BChl Q_Y band.
Labels: dark control (▼), 70 mM H_2O_2 (△), 150 mM H_2O_2 (○); 290 mM H_2O_2 (□); 150 mM H_2O_2 in the presence of ascorbic acid (●); 25 mM $K_3Fe(CN)_6$ (☆), 50 mM $K_3Fe(CN)_6$ (◇), 50 mM $K_3Fe(CN)_6$ in the presence of ascorbic acid (◆). The data represent a mean value of three independent experiments.

exposed to the following concentrations of $K_3Fe(CN)_6$: 25 mM, 50 mM and 100 mM. For example, after 5, 30 and 90 min treatment of B870 with 25 mM $K_3Fe(CN)_6$ the intensity of the BChl Q_Y band decreased by about 40%, 65%, and 82%, respectively (Fig. 1A, 2C). At the higher concentrations of $K_3Fe(CN)_6$ the effect was stronger. The decrease of the absorption intensity was accompanied by a shift of the B870 Q_Y band toward shorter wavelengths (by about 25 nm). No such a shift was observed for Sph-LH1 but the kinetics of the Q_Y band quenching slowed down by a factor of about 2 (Fig. 2D). Again, the effect of $K_3Fe(CN)_6$ was much more pronounced in the emission from B870, causing a 90% drop after a 5 min treatment, while in Sph-LH1, the quenching reached 65% of the initial value (not shown).

Additionally, the effect of ascorbate, a well-known scavenger of ROS (Bodannes *et al.*, 1979), on the kinetics of oxidant-induced degradation of B870 and Sph-LH1 was tested. In the reactions with $K_3Fe(CN)_6$, the addition of ascorbate enhanced the stability of all LH1 complexes while in the case of H_2O_2 it seemed to be effective only at the early stages of the reaction (Fig. 2A, B). As was already demonstrated by Picorel *et al.* (1984) in various LH1 systems, the effect of chemical oxidation with $K_3Fe(CN)_6$ can be substantially reversed (~85%) upon immediate addition of an excess of ascorbate. Since under our experimental condition the excess of ascorbate was present from the beginning we may expect that a direct reduction of ferricyanide takes place as the main antioxidant reaction.

Ascorbic acid readily undergoes a one- or two-electron reduction, yielding ascorbyl radical and dehydroascorbic acid, respectively. Ascorbate and ascorbyl radical have low reduction potentials and therefore can effectively interact with other radicals and oxidants. Moreover, ascorbate can be regenerated from both ascorbyl radical and dehydroascorbic acid (Buettner *et al.*, 1996).

On the other hand, the antioxidant effect of ascorbate in the H_2O_2 treated complexes in not unequivocal. One possible explanation of this effect might be the formation of some reactive intermediates, which could further act as prooxidants, promoting pigment degradation. The formation of unstable and highly reactive hydroperoxide derivatives of dehydroascorbic acid has indeed been reported (Kwon *et al.*, 1988).

In conclusion, potassium ferricyanide turned out to be a stronger oxidant than hydrogen peroxide. Both oxidants cause similar changes in the absorption spectra of the complexes, pointing out to the formation of analogous degradation products such as e.g. 2-desvinyl-2-acetylchlorophyll a, as judged from the appearance of a band at ~690 nm in the absorption spectra (Smith *et al.*, 1966). Picorel *et al.* (1984) or Law & Cogdell (1998) have already demonstrated that oxidation of native LH1 complexes with $K_3Fe(CN)_6$ leads to the changes in their spectral properties. Similar changes in the absorption and emission spectra of the reconstituted Sph-LH1 complexes were seen in the present work. The comparison of the degradation kinetics clearly indicates that Crt significantly influences the stability of the complexes, however it does not prevent them from a gradual irreversible decomposition. The changes in the absorption spectra of LH1 caused by oxidants are slower than the ones in the emission signals. A more rapid drop in the emission intensity can be explained by the fact that the presence of even a small number of excitation traps (oxidized BChl) may completely inhibit the intracomplex energy transfer (Law *et al.*, 1998; Fiedor *et al.*, 2000; Fiedor *et al.*, 2001b).

Acknowledgments

The project was partially supported by the grant N302 195035 from Polish Ministry of Science and Higher Education and its grant for Scientific Research (AGH), and by a grant from the Foundation for Polish Science (TEAM/2010-5/3 to L.F.). The groups cooperate within the BIONAN project.

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