Reconstitution of carotenoids in the light-harvesting 1 complex of *Rhodospirillum rubrum* for excited state dynamics studies

Joseph D. Shih*, Seth I. Breitbart, and Robert A. Niederman

Department of Molecular Biology and Biochemistry,
Rutgers, The State University of New Jersey,
Piscataway, New Jersey 08854

*Rutgers Undergraduate Research Fellow and Federal Aviation Administration/NASA Fellow

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Abstract

During the primary events of photosynthesis, carotenoids function in important accessory light harvesting and photoprotective roles. In both oxygen evolving and anoxygenic phototrophs, these highly unsaturated polyenes are found in light-harvesting (LH) proteins where their energy transfer and excited state deactivation pathways have become active areas of investigation. Foremost among unresolved questions is the role played by the protein scaffolding in conferring functionally crucial conformational constraints that modulate these unique photophysical properties. Their LH activity is mediated by two singlet-excited states: S_2, accounting for their strong blue-green light absorption, and S_1, accessible only through decay of S_2. In LH complexes from anoxygenic phototrophic bacteria, a unique S_2 decay channel, designated as S^*, that promotes direct, ultrafast carotenoid triplet formation, has been discovered. In carotenoids of sufficiently high S_1 energy, S^* also functions in a new energy transfer pathway to bacteriochlorophyll α (BChl). In the *Rhodospirillum rubrum* LH1 complex, containing spirilloxanthin in a twisted configuration, the energy of the S_1 state is too low to transfer energy to BChl, and the triplet yield from the S^* state is unusually high (~30%). The triplet state is thought to be generated directly by fission of the S^* state into a pair of triplets localized on separate halves of the polyene chain, as governed by protein-induced polyene chain distortion. To test whether this protein binds other carotenoids in a twisted conformation and alters their excited state dynamics, the *Rs. rubrum* LH1 protein in a carotenoidless form was reconstituted with neurosporene, a carotenoid of shorter conjugation length. The reconstituted carotenoid was found to occupy most of the potential carotenoid binding sites and showed an absorption redshift characteristic of protein-bound polyenes. The strength of the carotenoid association was confirmed in a photoprotection assay ($t_{½}$ of BChl Q_y absorption band of neurosporene reconstituted LH1 = 203 min, vs. 238 min in wild-type, and 17.5 min in carotenoidless LH1 proteins). Preliminary ultrafast transient absorption measurements showed that the reconstituted complex transferred some energy from the S_1 state to BChl and also generated a neurosporene triplet state. Future studies will involve a more complete characterization of excited state dynamics, determination of the conformation

assumed by the incorporated carotenoid, and reconstitution of carotenoids of other conjugation lengths.

Introduction

Carotenoids are highly unsaturated long-chain polyenes that play two important roles in the light reactions of photosynthesis: (i) they act as accessory light-harvesting (LH) pigments by absorbing light energy in the visible spectrum unavailable to chlorophylls; and (ii) they function in a photoprotective capacity by directly quenching the chlorophyll excited triplet state, that could otherwise sensitize formation of singlet oxygen, a highly reactive and potentially destructive species (Figure 1). In both oxygenic and anoxygenic phototrophs, carotenoids exist mainly in LH pigment-protein complexes, where they also play structural and assembly roles (1).

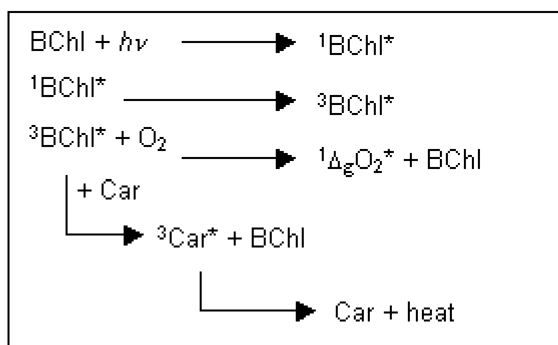


Figure 1. Photoprotective reaction mediated by carotenoids in anoxygenic phototrophic bacteria (1). $^1BChl^*$, bacteriochlorophyll (BChl) singlet excited state; $^3BChl^*$, BChl triplet state formed by intersystem crossing; $\Delta_gO_2^*$, singlet oxygen; $^3Car^*$, carotenoid triplet state. Since the energy level of the $^3Car^*$ state is significantly below that of $\Delta_gO_2^*$, carotenoids outcompete molecular O_2 by ~3 orders of magnitude, completely preventing $\Delta g_gO_2^*$ formation during illumination. For carotenoids with <9 conjugated double bonds, the triplet energy is too high to become populated from the BChl triplet state.

With the availability of high-resolution structures for intramembrane LH complexes of purple anoxygenic phototrophic proteobacteria (2-4), together with recent advances in time-resolved femtosecond spectroscopy (5), carotenoid energy transfer and deactivation mechanisms have become a focus of active investigation (1). Foremost among the important unresolved questions is the role that the protein scaffold plays in conferring functionally crucial conformational constraints on the carotenoid chains that govern their unique photophysical properties.

The bacterial LH complexes are major proteins of the intracytoplasmic membrane (ICM), and are organized into photosynthetic units in which the LH1 complex, present in all species of anoxygenic phototrophs, forms a ring-like structure that surrounds the photochemical reaction center. In those species that contain the independently regulated, peripheral LH2 complex, radiant energy collected by this annular structure is rapidly transferred to LH1, which directs these excitations to the reaction center, where they are transduced into a transmembrane charge separation.

In both LH complexes, carotenoid and BChl chromophores are bound noncovalently to apoproteins that consist of heterodimers of α-and &bgr-polypeptides containing ~50-60 amino acid residues. LH1 binds two molecules of BChl and one (6) or two (7) carotenoids, while LH2 binds two B850 BChls, one B800 BChl and two carotenoids (4). Fully oligomerized LH2 rings consist of 8 (ref. 3) to 9 (ref. 2) αβ-heterodimers, while LH1 rings contain 16 αβ-heterodimers (8), with 32 BChls and 16-32 carotenoids.

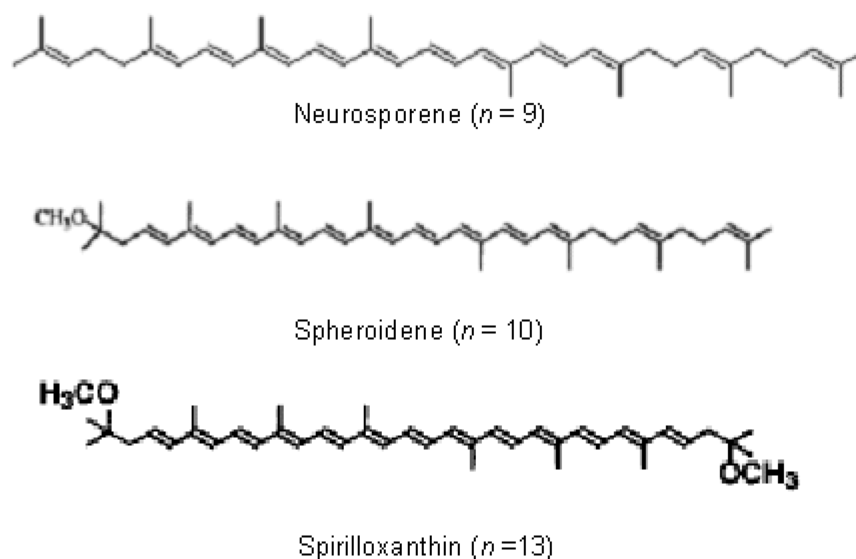


Figure 2. Structures of relevant carotenoids.

As a result of their polyene nature, carotenoids have a unique electronic excitation structure. At least two carotenoid singlet states are important in their functional photochemistry; the S_2 ($1B_u^+$ in the C_{2h} polyene symmetry group notation) state, accounting for the strong absorption of blue-green light, and the S_1 ($2A_g^-$) state which is inaccessible from the ground S_0 ($1A_g^-$) state, by one-photon processes. In spirilloxanthin, a carotenoid with 13 conjugated double bonds (n = 13) (Figure 2), a new singlet state, designated as S^*, has recently been identified in a transient absorption analysis of the LH1 complex of *Rhodospirillum rubrum*, and shown to promote ultrafast, direct carotenoid triplet formation (9). In the spheroidene (n = 10) containing LH2 complex of *Rhodobacter sphaeroides* (10), the S* state was subsequently shown to also function in a new pathway for mediating energy transfer to BChl. The new carotenoid triplet state is thought to be generated by excited-state fission of the S^* state into a pair of triplets localized on separate halves of the polyene chain, with apparent protein-induced conformational distortion of the carotenoid backbone governing the probability of this localization (11). Since the LH1 protein of *Rs. rubrum* binds its native carotenoid in an unusual twisted configuration (11,12), it was of considerable interest to incorporate other carotenoids into this protein and to assess how this new protein environment might alter their conformational and photophysical properties.

Materials and Methods

LH1 Isolation. For carotenoid reconstitution, the LH1 complex was purified from the carotenoidless *Rs. rubrum* G9 strain essentially as described by Miller et al. (13). After growth and cell disruption as described previously (14), ICM preparations were washed with 1% Triton X-100/10 mM EDTA to remove peripheral proteins, followed by three washes in deionized H_2O. The washed pellet was titrated with *n*-octyl β-D-glucopyranoside (β-OG) until a 777-nm peak appeared, usually at a concentration of 90 mM. This was followed by centrifugation and purification from the supernatant by isopycnic ultracentrifugation on a 5-25% (w/v) sucrose gradient containing 30 mM β-OG. The pigmented bands were recovered, held on ice until the 873-nm Q_y peak reappeared, concentrated by ultracentrifugation and dialyzed overnight.

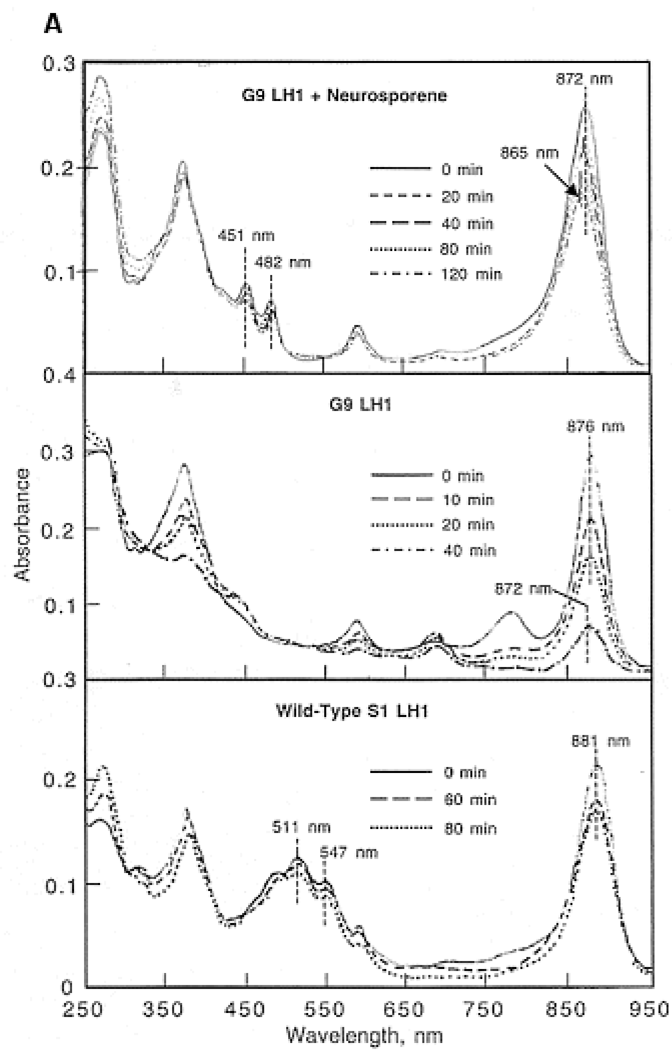
Carotenoid Isolation. For the isolation of neurosporene ($n = 9$)(15), the neurosporene-only *R. sphaeroides* G1C strain was grown as described previously (16). Cells were extracted with methanol to remove BChl, followed by acetone to extract carotenoid, which was transferred to *n*-hexane and chromatographed on a 1.5 x 45 cm alumina column. The column was eluted with one column volume of *n*-hexane, followed by equal quantities of 10 and 20 % diethyl ether in *n*-hexane for removal of all of the pigment. Fractions (4 ml) containing pure neurosporene were pooled, evaporated under nitrogen, and dissolved in a minimal volume of petroleum ether.

Carotenoid Reconstitution. Neurosporene was incorporated into the purified carotenoidless LH1 preparations as in (17). This involved exchanging the detergent in the LH1 preparation with 2 % sodium deoxycholate by dialysis, layering the carotenoid dissolved in petroleum ether on the surface of the protein-detergent complex (a 4:1 (mol/mol) neurosporene to BChl ratio gave the best results), and evaporating the petroleum ether with a stream of N_2. After sonication for 30-45 min at 4°C, excess carotenoid was removed by isopycnic ultracentrifugation on a discontinuous sucrose density gradient prepared with 0.75, 1.5, and 2.0 M sucrose. Reconstituted LH1 banded between the 1.5 M and 2.0 M layers, while excess carotenoids did not enter the gradient. The reconstituted LH1 was dialyzed overnight against 15 mM Tris-HCl (pH 8.0) containing 0.02% deoxycholate.

Results

Although direct generation of an ultrafast triplet state in the *Rs. rubrum* LH1 complex is primarily determined by the electronic structure of spirilloxanthin, perturbing interactions with the protein environment also appear to play an important role. Because the bound carotenoid is twisted from its symmetric configuration, dissociation of S^* into constituent triplets is thought to be favored, instead of the direct ~5 ps decay of S^* to the ground state, as seen with this carotenoid in organic solvent (9).

To determine whether carotenoids other than spirilloxanthin can mediate a significant direct singlet-to-triplet carotenoid conversion when present in the putative polyene "twisting" environment of the *Rs. rubrum* LH1 protein, the effects of carotenoid conformation and unsaturation were examined by incorporating neurosporene into the carotenoidless protein. By using a 4:1 molar ratio of neurosporene/LH1, a preparation was obtained in ~50% yield, in which the red-most neurosporene absorption (0-0 vibrational) band was red-shifted by 12 nm to 482 nm (Figure 3) from its position in organic solvent, and a BChl:neurosporene molar ratio of 3.3 was obtained, as compared to the BChl:spirilloxanthin ratio of 2 for wild-type LH1 (6). Thus, at least 60% of the potential carotenoid binding sites were occupied with neurosporene after reconstitution. Importantly, in a photoprotection assay (Figure 3), the half-life of neurosporene-reconstituted LH1 ($t_{½} = 203$ min) was comparable to that of the wild-type LH1 ($t_{½} = 238$ min), and much longer than in the carotenoidless strain G9 LH1 ($t_{½} = 17.5$ min). Therefore, the bound neuro-sporene is highly active in quenching the $^3BChl^*$ state that could otherwise sensitize formation of potentially damaging singlet oxygen (Figure 1). When a 10:1 molar ratio of neurosporene/LH1 was used in the reconstitution, a considerable quantity of loosely bound neurosporene remained with the protein which was rapidly degraded in the photoprotection assay, leaving fully red-shifted carotenoid that was also active in protection against the build up of the potentially damaging $^3BChl^*$ species ($t_{½} = 152$ min)(Figure 3B).


A
G9 LH1 + Neurosporene
872 nm
865 nm
0 min
20 min
40 min
80 min
120 min
451 nm
482 nm
G9 LH1
876 nm
0 min
10 min
20 min
40 min
872 nm
Absorbance
Wild-Type S1 LH1
881 nm
0 min
60 min
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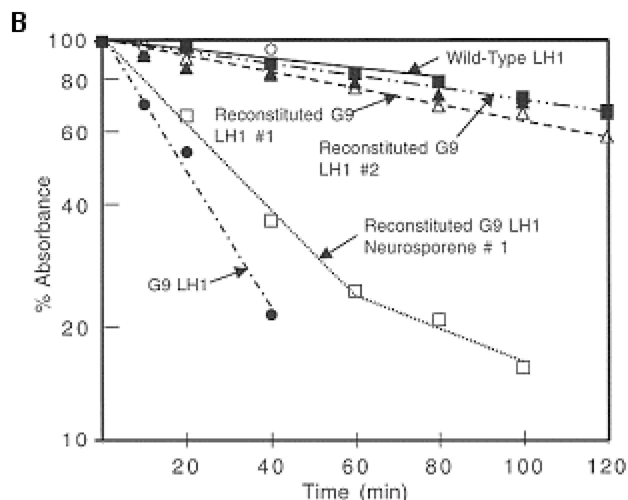

Figure 3. Photoprotection of LH1 complexes by carotenoid. A) Absorption spectra recorded at time = 0, and at various intervals during the assay: top, carotenoidless *Rs. rubrum* G9 strain reconstituted with 4:1 (mol/mol) ratio of neurosporene to LH1; middle, LH1 complex of carotenoidless *Rs. rubrum* G9 strain; bottom, LH1 complex of *Rs. rubrum* wild-type S1 strain. LH1 samples in a quartz cuvette (l = 1 cm) were exposed to white light from a 150-W flood lamp at a light intensity of 1200 W m^{-2} (18); radiated heat was dissipated with a fan. B) Plot of remaining absorbance vs. time. For reconstituted LH1 #1, a 10:1 molar ratio of neurosporene to LH1 was used, while for reconstituted LH1 #2, the ratio was 4:1. The line plotted for the later preparation represents the disappearance of both the LH1 Q_y band and the neurosporene 0-0 vibrational band. Photoprotection half-lives are presented in the text and were calculated using the equation $A = A_o^{e-Kt}$, where A = absorbance value at time = t, and A_o = absorbance value at time = 0. The equation was solved for K, which was inserted into the equation $t_{½} = ln2/K$

Preliminary ultrafast transient absorption measurements on the neurosporene-reconstituted *Rs. rubrum* LH1 preparation indicated that some energy was transferred from the S_1 state, since the S_1 lifetime appeared to be shorter than for neurosporene in hexane (20 ps), but longer than that of LH2 from *R. sphaeroides* G1C, a neurosporene-only strain, in which essentially all S_1 energy is transferred to the BChl Q_Y bands (E. Papagiannakis, R. van Grondelle and R.A. Niederman, unpublished). In the reconstituted LH1, a neurosporene triplet was apparently generated at a higher yield than in the G1C LH2; however, definitive results await thorough target analysis and optimization of measurements conditions. It is also noteworthy that for neurosporene in hexane, no evidence was found for the S^* state. The inability to detect S^* in free neurosporene is not surprising, since spirilloxanthin is one of the few carotenoids in which this state appears in solution. Moreover, for spheroidene (n = 10), the S^* state was only generated when this carotenoid was associated with the *R. sphaeroides* LH2 complex, albeit with a 5% yield (10).

Discussion

These studies show that neurosporene was successfully incorporated into the carotenoidless *Rs. rubrum* LH1 protein, since most of the potential carotenoid binding sites were occupied in the reconstituted complex, and the carotenoid absorption spectrum showed a 12-nm redshift that is close to the 15-nm redshift in the LH1 complex of *R. sphaeroides* G1C. Moreover, the strength of the carotenoid association was confirmed by the photoprotection half-life of the LH1 BChl Q_y band which was comparable to that of the *Rs. rubrum* wild-type complex. It is now important to determine the conformation that the bound neurosporene has assumed in the reconstituted LH1 complex. This will be assessed in resonance Raman spectra, in which the extent of polyene chain twist is determined from the relative amplitudes of bands in the ν_4 region near 1000 cm^{-1} (11). It is noteworthy, however, that the photoprotective ability of the non-native neurosporene in the heterologous LH1 complex suggests that this polyene has assumed an appropriate orientation in the carotenoid binding sites, and that it is also in geometrical proximity to the BChls.

In addition to the apparent role played by the LH protein in increasing the probability of the localization of coexisting triplets on separate segments of the polyene chain during fission of the S^* state into triplet pairs, other roles have been proposed for protein-induced conformational distortion of the carotenoid backbone in their photophysical processes. These include a diminution of LH efficiency in carotenoids with twisted geometries (12), and enhancement of electronic coupling between carotenoid and BChl excited states during singlet-singlet energy transfer by protein-imposed polyene chain symmetry breaking (19). Furthermore, it has been postulated (1) that the protein environment has a capacity for fine tuning the energy of the S_2 state through regulation of the local polarizability of carotenoid binding, and in establishing the exact position of carotenoid transition dipole moments, which is also of key importance in singlet-singlet energy transfer to BChl. Since only a few studies have specifically addressed the molecular details of carotenoid binding in photosynthetic pigment-protein complexes, this important question will be the subject of future investigations in this laboratory.

Acknowledgments

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References

(1) Fraser, N.J., Hashimoto, H. and Cogdell, R.J. (2001) Carotenoids and bacterial photosynthesis: The story so far. Photosynth. Res. 70, 249-256.

(2) McDermott, G., Prince, S.M., Freer, A.A., Hawthornthwaite-Lawless, A.M., Papiz, M.Z., Cogdell, R. J. and Isaacs, N. W. (1995) Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria. Nature 374, 517-521.

(3) Koepke, J., Hu, X., Muenke, C., Schulten, K. and Michel, H. (1996) The crystal structure of the light-harvesting complex II (B800-850) from *Rhodospirillum molischianum*. Structure 4, 581-597.

(4) Papiz, M.Z., Prince, S.M., Howard, T., Cogdell, R.J. and Isaacs, N.W. (2003) The structure and thermal motion of the B800-850 LH2 complex from *Rps. acidophila* at 2.0 Å resolution and 100 K: New structural features and functionally relevant motions J. Mol. Biol. 326, 1523-1538.

(5) Fleming, G.R. and van Grondelle, R. (1997) Femtosecond spectroscopy of photosynthetic light-harvesting systems. Curr. Opin. Struct. Biol. 7, 738-748.

(6) Picorel, R., Bélanger, G. and Gingras, G. (1983) Antenna holochrome B880 of *Rhodospirillum rubrum* S_1. Pigment, phospholipid, and polypeptide composition. Biochemistry, 22, 2491-2497.

(7) Broglie, R.M., Hunter, C.N., Delepelaire, P, Niederman, R.A., Chua N.-H. and Clayton, R.K. (1980) Isolation and characterization of the pigment-protein complexes of *Rhodopseudomonas sphaeroides* by lithium dodecyl sulfate/polyacrylamide gel electrophoresis. Proc. Natl. Acad. Sci. U.S.A. 77, 87-91.

(8) Karrasch, S., Bullough, P.A. and Ghosh, R. (1995) The 8.5 Å projection map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits. EMBO J. 14, 631-638.

(9) Gradinaru, C.C., Kennis, J.T.M., Papagiannakis, E., van Stokkum, I.H.M., Cogdell, R.J., Fleming, G.R., Niederman, R.A. and van Grondelle, R. (2001) An unusual pathway of excitation energy deactivation in carotenoids: singlet-to-triplet conversion on an ultrafast time scale in a photosynthetic antenna. Proc. Natl. Acad. Sci. USA 98, 2364-2369.

(10) Papagiannakis E., Kennis J.T.M., van Stokkum I.H.M., Cogdell R.J., and van Grondelle R. (2002) An alternative carotenoid-to-bacteriochlorophyll energy transfer pathway in photosynthetic light harvesting. Proc. Natl. Acad. Sci. USA 99, 6017-6022.

(11) Papagiannakis E., Das, S.K., Gall, A., van Stokkum, I.H.M, Robert, B., van Grondelle R., Frank H.A. and Kennis J.T.M. (2003) Light harvesting by carotenoids incorporated into the B850 light-harvesting complex from *Rhodobacter sphaeroides* R-26.1: Excited-state relaxation, ultrafast triplet formation and energy transfer to bacteriochlorophyll. J. Phys. Chem. B 107, 5642-5649.

(12) Noguchi T., Hayashi, H. and Tasumi, M. (1990) Factors controlling the efficiency of energy transfer from carotenoids to bacteriochlorophyll in purple photosynthetic bacteria. Biochim. Biophys. Acta 1017, 280-290.

(13) Miller, J.F., Hinchigeri, S.B., Parkes-Loach, P.S., Callahan, P.M., Sprinkle, J.R., Riccobono, J.R., and Loach, P.A. (1987) Isolation and characterization of a subunit form of the light-harvesting complex of *Rhodospirillum rubrum*. Biochemistry 26, 5055-5062.

(14) Inamine, G. S. and Niederman, R. A. (1982) Development and growth of photosynthetic membranes of *Rhodospirillum rubrum*. J. Bacteriol. 150, 1145-1153.

(15) Fujii, R., Inaba, T.,Watanabe, Y., Koyama, Y. and Zhang, J.P. (2003) Two different pathways of internal conversion in carotenoids depending on the length of the conjugated chain. Chem. Phys. Lett. 369, 165-172.

(16) Olivera, L. M. and Niederman, R. A. (1993) Effect of phospholipase A_2 on the carotenoid and bacteriochlorophyll components of the light-harvesting complexes in *Rhodobacter sphaeroides* chromatophores. Biochemistry 32, 858-866.

(17) Desamero, R.Z.B., Chynwat, V., Van der Hoef, I., Jansen, F.J., Lugtenburg, J., Gosztola, D., Wasielewski, M.R., Cua, A., Bocian, D.F. and Frank H.A. (1998) Mechanism of energy transfer from carotenoids to bacteriochlorophyll: Light-harvesting by carotenoids having different extents of π-electron conjugation incorporated into the B850 antenna complex from the cartenoidless bacterium *Rhodobacter sphaeroides* R-26.1. J. Phys. Chem. B 102, 8151-8162.

(18) Davis, C.M., Bustamante, P.L. and Loach, P.A. (1995) Reconstitution of the bacterial core light-harvesting complexes of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* with isolated α- and β-polypeptides, bacteriochlorophyll α, and carotenoid. J. Biol. Chem. 270, 5793-5804.

(19) Ritz, T., Damjanovic, A., Schulten, K., Zhang, J. and Koyama, Y. (2000) Efficient light harvesting through carotenoids. Photosynth. Res. 66, 125-144.

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