Low-Temperature Optical and Resonance Raman Spectra of a Carotenoid Cation Radical in Photosystem II

John S. Vrettos,[†] David H. Stewart,[†,‡] Julio C. de Paula,[§] and Gary W. Brudvig*[,†]

Department of Chemistry Yale University, P.O. Box 208107, New Haven, Connecticut 06520-8107, Department of Chemistry, Haverford College, Haverford, Pennsylvania 19041-1392

Received: May 5, 1999; In Final Form: June 21, 1999

Low-temperature (77 K) illumination of manganese-depleted *Synechocystis* PCC 6803 photosystem II core complexes caused the reversible photooxidation of a carotenoid, forming a carotenoid cation radical with an absorbance maximum at 984 nm. Resonance FT-Raman spectra obtained with 1064 nm excitation gave a spectrum characteristic of carotenoid cation radicals in solution. This is the first example of a resonance Raman spectrum of a carotenoid cation radical in a protein. The carotenoid photooxidation requires prior chemical oxidation of cytochrome b_{559} which implicates the carotenoid in the secondary electron-transfer pathway of photosystem II that may play a role in photoprotection. The possible nature of the pathway and the structure of the carotenoid cation radical are discussed.

Photosystem II (PSII) is a multi-subunit transmembrane protein complex which catalyzes the oxidation of water to dioxygen and concomitant reduction of plastoquinone to plastoquinol. Electron transfer from a chlorophyll species, P_{680}, proceeds via a pheophytin molecule to the quinone pool. $P_{680}^{+\bullet}$ is rereduced by a tetranuclear manganese cluster, the active site of water oxidation.[1] Under conditions in which the oxidation of water is impaired or the reduction of the quinone pool becomes rate-limiting, oxidative damage (photoinhibition) of PSII may occur as $P_{680}^{+\bullet}$ accumulates.[2] A secondary electron-transfer pathway consisting of alternate donors to $P_{680}^{+\bullet}$ may function to prevent photoinhibition in these circumstances.[3] This pathway is known to include cytochrome b_{559} (cyt b_{559}) and an accessory chlorophyll (chl_z). The sequential flow of electrons from cyt b_{559} to chl_z to P_{680} has been previously demonstrated.[4−6]

Accumulation of $chl_z^{+\bullet}$ can be achieved by illuminating PSII at temperatures low enough to prevent the oxidation of the manganese cluster, or in samples in which the manganese has been removed. It has also been shown that a reaction center (RC) carotenoid (car) can be photooxidized under similar conditions.[7−9] To be observed, both species require prior chemical oxidation of cyt b_{559}.[5,10,11] This requirement suggests that photooxidation of the carotenoid occurs as part of the secondary cyt b_{559}/chl_z electron-transfer pathway.

Redox activity is an unexpected role for the carotenoids in the PSII RC when contrasted against the bacterial RC (BRC). The carotenoid within the BRC protects the protein from photoinhibition by quenching triplet states of bacteriochlorophyll or singlet oxygen.[12] However, in PSII D1/D2/cyt b_{559} RC complexes, which contain two β-carotene molecules per RC,[13] only a very low yield of ^{3}car forms, suggesting that its method of photoprotection is not by quenching ^{3}chl.[14] The different functions of the reaction center carotenoids—electron donors in PSII versus triplet quenchers in the BRC—may be attributable to their different structures and local environment. Evidence strongly suggests that the BRC-bound carotenoid has a 15-cis configuration.[12,15−17] On the other hand, the two β-carotenes in the PSII RC adopt an all-trans configuration[18,19] and may be excitonically coupled.[20] In addition, $P_{680}^{+\bullet}$ is a much stronger oxidant than the primary electron donor (P_{870}) in the BRC, and it may be that only $P_{680}^{+\bullet}$ is capable of oxidizing a carotenoid while $P_{870}^{+\bullet}$ cannot.

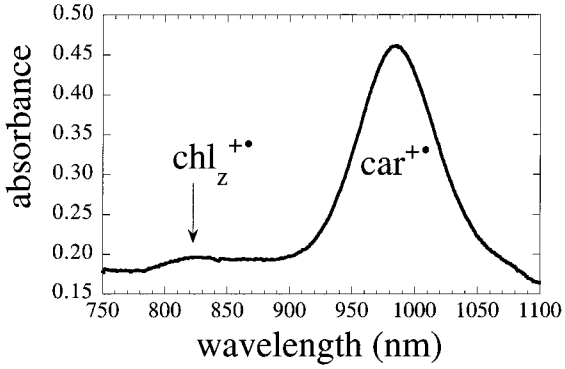

Figure 1. Illuminated-minus-dark difference near-IR spectrum of PSII core complexes at 77 K. The spectrum is the difference between the average of two scans each of the same dark and illuminated sample.

Other groups have shown evidence for the photooxidation of a PSII RC carotenoid by observation of either irreversible bleaching of the neutral carotenoid near ambient temperature[9−11] or the formation of the cation radical itself at cryogenic temperatures.[8,21,22] Here we present the low-temperature near-IR and resonance FT-Raman spectra of a $car^{+\bullet}$ in PSII core complexes after continuous illumination at 77 K, which forms a stable charge-separated state in which a carotenoid is photooxidized.[23] The advantage of using optical and Raman spectroscopy is the selectivity of the methods. For example, electron paramagnetic resonance (EPR) spectra of carotenoid and chlorophyll radicals are virtually identical; indeed, mixtures of both radicals may explain variations in the EPR line width of chl_z.[24] This is the first example of a resonance Raman spectrum of a carotenoid cation radical in a protein. The probable

* Author to whom correspondence should be addressed. Telephone: (203)-432-5202. Fax: (203)-432-6144. E-mail: gary.brudvig@yale.edu.
† Yale University.
‡ Current address: Xanthon, Inc., 104 Alexander Dr., Research Triangle Park, NC 27709.
§ Haverford College.

10.1021/jp991464q CCC: $18.00
Published on Web 07/16/1999

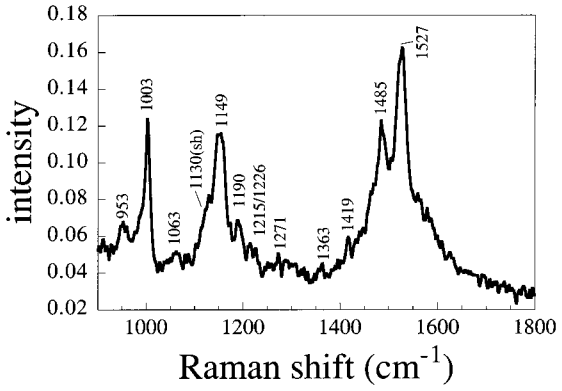

Figure 2. Resonance FT-Raman illuminated-minus-dark difference spectrum of PSII core complexes at 77 K. Instrumental settings: resolution, 4 cm^{-1}; laser incident power, 300 mW; 2000 scans.

participation of the car in the photoprotection pathway of PSII and the structure of the carotenoid are discussed.

Materials and Methods

Photosystem II core complexes from *Synechocystis* PCC 6803 were prepared according to literature methods.[25] O_2 evolution was measured with a Clark electrode; typical rates were 2100−3200 μmol O_2 (mg Chl)$^{-1}$ h^{-1}. The core complexes were depleted of manganese by treatment with 5 mM NH_2OH and subsequently washed with 5 mM sodium ethylenediaminetetraacetate to remove Mn(II).[26] Chlorophyll concentrations were determined by methanol extraction using an extinction coefficient of 79.24 mL (mg Chl)$^{-1}$ cm^{-1} at 665 nm.[27] The final PSII concentration was 86 μM for the absorbance measurements and 38 μM for the Raman measurements based on 38 chl/PSII.[25] To oxidize cyt b_{559}, both samples were treated with ferricyanide (100 and 230 μM, respectively) and were dark-adapted at 0 °C for at least 30 min. The sample used for the absorbance measurements was also treated with 1 mM hexachloroiridate before dark adaptation, although a similar yield of $car^{+\bullet}$ was obtained in samples treated only with ferricyanide.

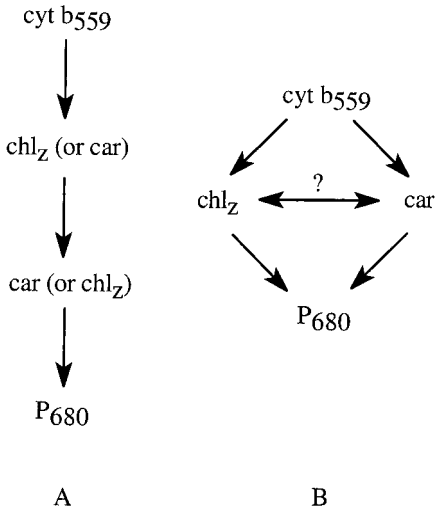

Figure 3. Two possible roles for β-carotene in the photoprotection pathway. In scheme A, the car is a part of a linear path from cyt b_{559} to P_{680}. In B, the car and chl_z are parallel paths for electron transfer.

Low-temperature optical spectra were collected using a home-built Plexiglas flat cell with a path length of 0.75 mm, whereas an EPR tube was used for the Raman measurements. Both vessels were immersed in liquid N_2 in a quartz finger dewar. Spectra were collected before and after illumination in the dewar with white light from a 200 W quartz-halogen bulb for 10 min at 77 K. Near-IR spectra were recorded on a Perkin-Elmer Lambda-20 UV−vis spectrophotometer. Raman spectra were collected using a Nicolet Raman 950 spectrophotometer with excitation at 1064 nm from a Nd:YVO_4 laser.

TABLE 1: Comparison of Raman Spectra of Neutral and Cation Radical Forms of β-Carotene and Model Carotenoids

β-carotene normal mode analysis[a]		experimental Raman frequencies (cm^{-1})					
						β-carotene in PSII	
assignment	calculated frequency (cm^{-1})	β-carotene neutral[b]	8′-apo-8′-al cation (Δ)[c]	7′-7′-dicyano cation (Δ)[c]	canthaxanthin cation (Δ)[c]	neutral[d]	cation (Δ)[e]
7=8, 9=10, 7H	1591	1586		1546 (−27)			
11=12, 15H, 12H	1573	1562	1545 (−40)	1511 (−29)	1542 (−33)		1527
13=14, 11=12, 12H	1524 (ν1)	1516	1481 (−46)	1478 (−22)	1507 (−13)	1529	1485 (−44)
20ad, 20r	1447	1442		1440 (−5)		1445	1419 (−26)
20sd	1393	1390					
13−20, 14H, 20sd	1347	1352	1369 (+13)	1365 (+10)	1365 (+9)		1363
12H, 11=12, 15=15′	1314	1310					
15H, 14H, CCC15b	1308	1281					
11H, 8−9, 12−13	1261	1270	1281 (+10)	1271	1274		1271
8H, 7H, 5−18	1248	1255				1251	
12−13, 14H, 15=15′	1212	1210	1231 (+16)	1231 (+27)			1215/1226
10H, 11H, 8−9	1192	1190	1199 (+14)		1193	1187	1190 (+3)
5−4, 18r, 6−7	1172	1173					
14−15, 15H, 15=15′	1151 (ν2)	1157	1157 (−2)	1158 (+3)	1162 (?)	1161	1149 (−12)
10−11	1137	1128				1133	1130(sh)
19r, 8−9, 19ad	1012	1018					1063
20r, 12−13, 20ad	1004	1008				1006	1003 (−3)
T(11=12), 12Hw, 11Hw	967	963				964	
T(7=8), 7Hw, 8Hw	952						953

[a] The normal mode analysis is for all-trans β-carotene, ref 31. It is provided for comparison to the observed frequencies for β-carotene (solid and bound to PSII) as well as the model carotenoids. Abbreviations for the modes are as in the following examples: 15=15′, stretch of C_{15}=$C_{15'}$ bond; 12−13, stretch of C_{12}−C_{13} bond; 14H, in-plane bending of C_{14}−H bond; 20sd and 20ad, symmetric and asymmetric deformations of methyl group 20; CCC15b, bending of the $C_{14}C_{15}C_{15'}$ bond angle; 18r, rocking of methyl group 18; T(11=12), torsion about the C_{11}=C_{12} bond; 7Hw, out-of-plane wagging of the C_7−H bond. [b] From ref 36. [c] From ref 32. 8′-apo-8′-al is 8′-apo-8′−β-caroten-8′-al; 7′,7′-dicyano is 7′,7′-dicyano-7′-apo-β-carotene. Shifts (Δ) are relative to the neutral species (not listed). [d] From ref 18. [e] This work. Shifts (Δ) are relative to neutral β-carotene in PSII. The absence of some neutral PSII β-carotene peaks does not allow shifts to be assigned for all of the PSII β-carotene cation peaks, but these may be qualitatively compared to solid neutral β-carotene.

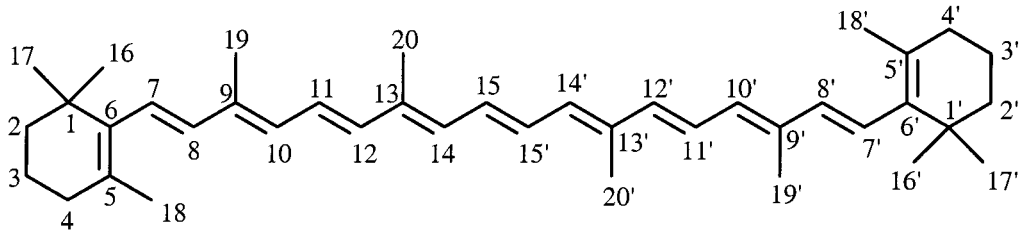

Figure 4. Chemical structure of β-carotene.

Results

Near-IR Spectroscopy. The near-IR difference spectrum of *Synechocystis* PSII core complexes illuminated at 77 K is shown in Figure 1. The spectrum shows two features: a weak absorbance centered at 820 nm attributable to $chl_z^{+\bullet}$[28] and a strong absorbance at 984 nm known to arise from a carotenoid cation radical.[8,9,11] Using extinction coefficients of 94000 M^{-1} cm^{-1} at 950 nm for $car^{+\bullet}$ and 7000 M^{-1} cm^{-1} for $chl_z^{+\bullet}$,[9] the yield of each cation radical is about 30%. The $car^{+\bullet}$ observed is assigned to the cation radical of β-carotene, because *Synechocystis* PCC 6803 cells have been found to contain predominantly β-carotene[29] and it is thought that PSII D1/D2/cyt b_{559} RC complexes contain only β-carotene.[13]

Resonance FT-Raman Spectroscopy. A resonance Raman spectrum of the carotenoid cation radical was collected using an FT-Raman spectrometer exciting at 1064 nm. The difference spectrum obtained at 77 K is shown in Figure 2. No features were observed in the dark spectrum, indicating that all of the observed peaks arise due to resonance enhancement of the carotenoid cation. Large downshifts in the high-frequency C═C modes (the ν1 region) and small upshifts in the C−C modes (the ν2 region) relative to neutral β-carotene[18] are observed (Table 1), and are characteristic of carotenoid cation radicals electrochemically oxidized in solution.[30−32] The photooxidation of the carotenoid was reversible by annealing the sample to room temperature in the dark. Subsequent reoxidation by 77 K illumination gave a spectrum with equivalent intensities (not shown).

Discussion

The photooxidation of a PSII RC β-carotene only when cyt b559 is preoxidized implicates the carotenoid as a cofactor in the secondary cyt b_{559}/chl_z electron-transfer pathway. In previous experiments, we have shown that there is a linear pathway for electron transfer from cyt b_{559} to P_{680} through chl_z.[5,6] The carotenoid may be involved in this pathway in one of two ways, as depicted in Figure 3. The linear pathway in Figure 3A involves sequential electron transfer from cyt b_{559} to P_{680}, while Figure 3B depicts parallel electron donation from either chl_z or β-carotene to P_{680}. On the basis of recent electron crystallographic structure of PSII,[33] which shows a close homology between the D1/D2 and L/M subunits of PSII and the BRC, respectively, the linear pathway seems more likely. The axial ligand to chl_z has been identified as H118 in the D1 polypeptide of PSII[34] which is homologous to L-C92 in the BRC. The distance of closest approach from the sulfur atom of L-C92 to the nearest carbon atoms in the phytol group of the special pair bacteriochlorophylls is approximately 30 Å. This places chl_z almost 30 Å from P_{680} on the basis of structural homology to the L subunit of the BRC. This distance is too great for efficient electron transfer in a single step. For example, relying on the analysis of Dutton and co-workers of the rates of electron transfer as a function of distance for proteins,[35] a 30 Å distance between P_{680} and chl_z would correspond to a rate of about 0.1 s^{-1}, which is about 2000 times slower than the rate of recombination of $P_{680}^{+\bullet}/Q_A^{-\bullet}$. This is too slow for chl_z to be an effective electron donor to $P_{680}^{+\bullet}$ upon illumination at low temperature. Sequential electron transfer to a carotenoid positioned between chl_z and P_{680} would shorten this distance spanned by a single electron-transfer step and increase the electron-transfer efficiency. While these considerations support the linear pathway of Figure 3A, further experiments are required to differentiate pathways A and B of Figure 3.

The optical and Raman spectra of the carotenoid cation radical are consistent with those previously reported.[8,32] An analysis of the Raman spectrum and a comparison with Raman data for other carotenoids[18,31,32,36] are presented in Table 1, with the chemical structure of β-carotene shown in Figure 4. The model carotenoid data are presented to illustrate the character and magnitudes of the shifts expected between the neutral and oxidized species. The Raman spectrum shown in Figure 2 does not contain contributions from $chl_z^{+\bullet}$, which is evident by comparison to previous resonance Raman spectra of $chl_z^{+\bullet}$ obtained with 820 nm excitation.[28] The lack of the strong band at 1501 cm^{-1}, present in the resonance Raman spectrum of $chl_z^{+\bullet}$, and the presence of the strong $car^{+\bullet}$ ν2 mode at 1149 cm^{-1} exclusively in the spectrum obtained with 1064 nm excitation indicate selective resonance Raman scattering from $car^{+\bullet}$ at this wavelength. In the model carotenoids, oxidation results in a large downshift and decrease in intensity of the ν1 mode from ca. 1520 cm^{-1} to ca. 1480 cm^{-1}, as well as a downshift and increase in intensity of a mode from ca. 1580 cm^{-1} to ca. 1540 cm^{-1}.[32] We observe similar changes for the $car^{+\bullet}$ in PSII when compared to the spectrum of its neutral form.[18] These downshifts in C═C bond vibrations are consistent with the hole being delocalized over the entire polyene chain. The observation of an absorbance maximum at 984 nm for $car^{+\bullet}$ also indicates that the hole is delocalized over all eleven double bonds of β-carotene.[32]

In conclusion, we have shown that one of the RC-bound β-carotenes in PSII is involved in the secondary cyt b_{559}/chl_z pathway. The low-temperature optical and resonance FT-Raman spectra of photooxidized β-carotene allow the selective measurement of $car^{+\bullet}$ and will aid in the characterization of the redox-active role of β-carotene in PSII.

The authors thank Dr. Harry Frank for useful comments and discussion. Funding was provided by NIH GM32715 (GWB) and by the Kresge Foundation (JdP).

References and Notes

(1) Debus, R. J. *Biochim. Biophys. Acta* **1992**, *1102*, 269−352.
(2) *Photoinhibition of Photosynthesis*; BIOS Scientific Publishers: Oxford, UK, 1994.
(3) Stewart, D. H.; Brudvig, G. W. *Biochim. Biophys. Acta* **1998**, *1367*, 63−87.
(4) de Paula, J. C.; Innes, J. B.; Brudvig, G. W. *Biochemistry* **1985**, *24*, 8114−8120.
(5) Thompson, L. K.; Brudvig, G. W. *Biochemistry* **1988**, *27*, 6653−6658.

(6) Thompson, L. K.; Miller, A.-F.; de Paula, J. C.; Brudvig, G. W. *Isr. J. Chem.* **1988**, *28*, 121−128.
(7) Dawe, E. A.; Land, E. J. *J. Chem. Soc., Faraday Trans.* **1975**, *71*, 2162−2169.
(8) Schenck, C. C.; Diner, B.; Mathis, P.; Satoh, K. *Biochim. Biophys. Acta* **1982**, *680*, 216−227.
(9) Telfer, A.; De Las Rivas, J.; Barber, J. *Biochim. Biophys. Acta* **1991**, *1060*, 106−114.
(10) Velthuys, B. R. *FEBS Lett.* **1981**, *126*, 272−276.
(11) Yamashita, K.; Konishi, K.; Itoh, M.; Shibata, K. *Biochim. Biophys. Acta* **1969**, *172*, 511−524.
(12) Frank, H. A.; Codgell, R. *Photochem. Photobiol.* **1996**, *63*, 257−264.
(13) Gounaris, K.; Chapman, D. J.; Booth, P.; Crystall, B.; Giorgi, L. B.; Klug, D. R.; Porter, G. *FEBS Lett.* **1990**, *265*, 88−92.
(14) Durrant, J. R.; Giorgi, L. B.; Barber, J.; Klug, D. R.; Porter, G. *Biochim. Biophys. Acta* **1990**, *1017*, 167−175.
(15) Koyama, Y. *J. Photochem. Photobiol., B: Biol.* **1991**, *9*, 265−280.
(16) Lutz, M.; Agalidis, I.; Hervo, G.; Cogdell, R. J.; Reiss-Husson, F. *Biochim. Biophys. Acta* **1978**, *503*, 287−303.
(17) de Groot, H. J. M.; Gebhard, R.; van der Hoef, I.; Hoff, A. J.; Lugtenburg, J.; Violette, C. A.; Frank, H. A. *Biochemistry* **1992**, *31*, 12446−12450.
(18) Ghanotakis, D. F.; de Paula, J. C.; Demetriou, D. M.; Bowlby, N. R.; Petersen, J.; Babcock, G. T.; Yocum, C. F. *Biochim. Biophys. Acta* **1989**, *974*, 44−53.
(19) Yruela, I.; Tomás, R.; Sanjuán, M. L.; Torrado, E.; Aured, M.; Picorel, R. *Photochem. Photobio.* **1998**, *68*, 729−737.
(20) De Las Rivas, J.; Telfer, A.; Barber, J. *Biochim. Biophys. Acta* **1993**, *1142*, 155−164.
(21) Noguchi, T.; Mitsuka, T.; Inoue, Y. *FEBS Lett.* **1994**, *356*, 179−182.
(22) Hillmann, B.; Schlodder, E. *Biochim. Biophys. Acta* **1995**, *1231*, 76−88.
(23) Vrettos, J. S.; Stewart, D. H.; Cua, A.; de Paula, J. C.; Bocian, D. F.; Brudvig, G. W. *Biophys. J.* **1999**, *76*, A248.
(24) Miller, A.-F.; Brudvig, G. W. *Biochim. Biophys. Acta* **1991**, *1056*, 1−18.
(25) Tang, X.-S.; Diner, B. A. *Biochemistry* **1994**, *33*, 4549−4603.
(26) Tamura, N.; Cheniae, G. *Biochim. Biophys. Acta* **1987**, *890*, 179−194.
(27) Lichtenthaler, H. K. *Methods Enzymol.* **1987**, *148*, 350−382.
(28) Cua, A.; Stewart, D. H.; Brudvig, G. W.; Bocian, D. F. *J. Am. Chem. Soc.* **1998**, *120*, 4532−4533.
(29) Schubert, H.; Fulda, S.; Hagemann, M. *J. Plant Physiol.* **1993**, *142*, 291−295.
(30) Merlin, J. C. *Pure Appl. Chem.* **1985**, *57*, 785−792.
(31) Saito, S.; Tasumi, M. *J. Raman Spectrosc.* **1983**, *14*, 310−321.
(32) Jeevarajan, A. S.; Kispert, L. D.; Chumanov, G.; Zhou, C.; Cotton, T. M. *Chem. Phys. Lett.* **1996**, *259*, 515−522.
(33) Rhee, K. H.; Morriss, E. P.; Barber, J.; Kühlbrandt, W. *Nature* **1998**, *396*, 283−286.
(34) Stewart, D. H.; Cua, A.; Chrisholm, D. A.; Diner, B. A.; Bocian, D. F.; Brudvig, G. W. *Biochemistry* **1998**, *37*, 10040−10046.
(35) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796−802.
(36) Weesie, R. J.; Merlin, J. C.; Lugtenburg, J.; Britton, G.; Jansen, F. J. H. M.; Cornard, J. P. *Biospectroscopy* **1999**, *5*, 19−33.