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Chlorophyll *a* and β-carotene content in the D_1/D_2/cytochrome *b*-559 reaction center complex from spinach

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Pigment stoichiometries in the D_1/D_2/cytochrome *b*-559 reaction center complex from spinach have been studied by spectrophotometry and normal-phase silica HPLC of the solvent extracts. Based on the well-accepted molar ratio Pheo *a*/P680 = 2, the results are summarized by Chl *a*/P680 = 6.01 ± 0.37 ($n = 24$) and β-carotene/P680 = 1.84 ± 0.11 ($n = 4$). These stoichiometries are significantly different from those of bacterial reaction center complexes.

Photosystem II reaction center; Chlorophyll; Pheophytin; β-Carotene; High-performance liquid chromatography analysis

1. INTRODUCTION

It is now widely accepted that the primary charge separation in photosystem (PS) II occurs at the D_1/D_2/cytochrome (Cyt) *b*-559 reaction center complex [1]. In discussing or even speculating on the as yet unclarified mechanism of PS II photochemistry, a correct knowledge on the pigment composition (and hopefully their spatial arrangement) within the complex is indispensable. Such knowledge would, in addition, help us resolve a more fundamental question whether and to what extent there exists a homology on the molecular level between the PS II and the purple bacterial reaction center, which should be regarded as two organs quite different from each other in view of the redox processes carried out therein. To date, however, a significant controversy still prevails as to the pigment stoichiometries in this complex.

In their first report in 1987, Nanba and Satoh [1] evaluated, by reversed-phase HPLC, the chlorophyll (Chl) *a*/pheophytin (Pheo) *a*/β-carotene (β-Car) molar ratio in the complex from spinach to be around 5:2:1. A later work by Barber et al. [2] on the complex from peas suggested the molar ratio to be 4:2:1. Recently, Gounaris et al. [3] obtained the molar ratio of 16:2:2 by reversed-phase HPLC on extracts of the D_1/D_2/Cyt *b*-559 complex from a cyanobacterium. Further, a more recent spectrophotometric investigation by Dekker and coworkers [4] indicates that the PS II reaction center complex from spinach comprises 10–12 Chl *a* and 2–3 Pheo *a* molecules.

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In the present work we measured the Chl *a*/Pheo *a*/β-Car molar ratio within the D_1/D_2/Cyt *b*-559 reaction center complex from spinach by both spectrophotometry and silica normal-phase HPLC on the extracts, by paying enough attention to keep the pigments from undergoing molecular alterations in the course of extraction/analysis. The result is very close to an integer ratio of 6:2:2. This is hence substantially different from the well-established BChl/BPheo/Car molar ratio of 4:2:1 in bacterial reaction center complexes [5,6]. The possible roles of additional two Chl *a* and one β-Car molecules in the PS II reaction center are briefly discussed.

2. MATERIALS AND METHODS

2.1. *Preparation of the reaction center complex*

The D_1/D_2/Cyt *b*-559 reaction center complex suspended in a 50 mM Tris-HCl (pH 7.2) and 0.2% digitonin aqueous solution was prepared from spinach as described by Nanba and Satoh [1], with modifications which include isoelectric focusing in the presence of digitonin [7].

2.2. *Pigment extraction*

20 ml of acetone was added to 10 μl of the reaction center complex solution (~13 μg Chl *a*/ml), and pigments were extracted by sonication at 4°C for 1 or 2 min. The solution was then filtered through a Teflon filter (German Science Japan, Ekicrodisc-AcroLC25CR, prewashed with acetone) at 4°C. The filtrate was immediately dried in a rotary evaporator under ~10^{-2} Torr. These operations were conducted under dim light.

2.3. *Spectrophotometric quantitation of Chl* a *and Pheo* a

The green solid material obtained by the above procedure was redissolved in acetone or benzene, and Chl *a* and Pheo *a* were quantitated from the absorption spectrum of the solution based on the molar extinction coefficient of each pigment [8].

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2.4. *HPLC quantitation of Chl* a, *Pheo* a *and β-Car*

The green solid material mentioned above was separately redissolved in ~20 μl of chloroform, and a 2 μl aliquot of the solution was injected to a silica HPLC column cooled to approx. 4°C. The Chl *a*/Pheo *a* molar ratio was calculated from their HPLC peak area ratio with an absorbance detector (425 nm), which had been calibrated repeatedly by injecting a standard solution containing known amounts of authentic Chl *a* (>99.9% in purity) and Pheo *a* (99.9%). The extinction coefficient of 135000 $M^{-1} \cdot cm^{-1}$ (453 nm) in hexane [9] was used for β-Car quantitation. Further details of the HPLC analytical conditions are described elsewhere [10].

3. RESULTS

3.1. *Chl* a/*Pheo* a *molar ratio determined by spectrophotometry*

The molar ratio of Chl *a*/Pheo *a* was determined in acetone and benzene, after redissolving the acetone extract (sonication time, 1 min) of the D_1/D_2/Cyt *b*-559 complex in these solvents. As a typical example, the absorption spectrum in acetone is shown in fig.1. This spectrum gives a molar ratio Chl *a*/Pheo *a* = 3.01. The values summarized in table 1 (Run 1–6) in the form of Chl *a*/2Pheo *a* (i.e. one photoactive Pheo *a*) are 5.95 ± 0.29 (n = 6) and 5.86 ± 0.36 (n = 6) in acetone and benzene, respectively.

3.2. *Chl* a/*Pheo* a/*β-Car molar ratio determined by HPLC*

The molar ratio of Chl *a*/Pheo *a*/β-Car was determined by HPLC analysis. A typical HPLC trace is given in fig.2. Only 3 pigments (β-Car, Pheo *a* and Chl *a* eluted in this order) are detected by the present high-resolution HPLC, and neither Chl *a'* (C-10 epimer of Chl *a*) associated with PS I [10] nor Chl *b* is present in this reaction center complex. The results are summarized in table 1. The Chl *a*/2Pheo *a* molar ratios found by spectrophotometry and by HPLC are in good agreement with each other and, when combined together, the mean Chl *a*/2Pheo *a* value is 6.01 (n = 24). The β-Car/2Pheo *a* molar ratio is close to 2, namely 1.84 ± 0.11 (n = 4).

Table 1

Pigment stoichiometries in D_1/D_2/cytochrome *b*-559 reaction center complex as determined by spectrophotometry and HPLC analysis of the extracts

Run	Chl *a*/2Pheo *a*			β-Car/2Pheo *a*
	Spectrophotometry		HPLC	HPLC
	In acetone	In benzene		
1	6.02	6.41	–	–
2	5.57	5.47	6.58	–
3	6.49	5.45	5.80	–
4	5.90	5.96	6.20	–
5	5.72	6.17	6.70	–
6	6.00	5.67	6.14	–
7	–	–	5.76	–
8	–	–	6.14	–
9	–	–	5.40	–
10	–	–	6.14	1.72
11	–	–	6.56	1.88
12	–	–	5.76	1.76
13	–	–	6.22	2.00
Mean ± SD	5.95 ± 0.29	5.86 ± 0.36	6.12 ± 0.37	1.84 ± 0.11
			6.01 ± 0.37	

Highly purified Chl *a*, containing 0.03% Chl *a'* as the sole impurity, was subjected to the same extraction/HPLC procedure as in the analysis of the reaction center complex, to see the extent of pigment alteration. As a result, less than one out of 500 Chl *a* molecules was converted into Pheo *a*, probably during the extraction stage; this ensures that the HPLC results well reflect the in vivo pigment composition. Moreover, the sonication time (1 min for Runs 2–9 and 2 min for Runs 10–13 in table 1) showed practically no influence on the final results. We thus conclude that a D_1/D_2/Cyt *b*-559 complex comprises six Chl *a* and two β-Car molecules per two Pheo *a* molecules.

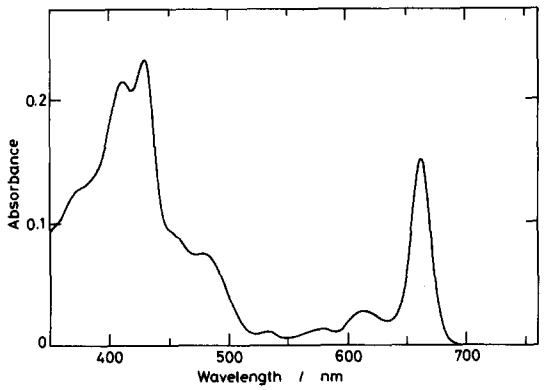

Fig.1. Absorption spectrum in acetone for an acetone extract of the D_1/D_2/Cyt *b*-559 reaction center complex.

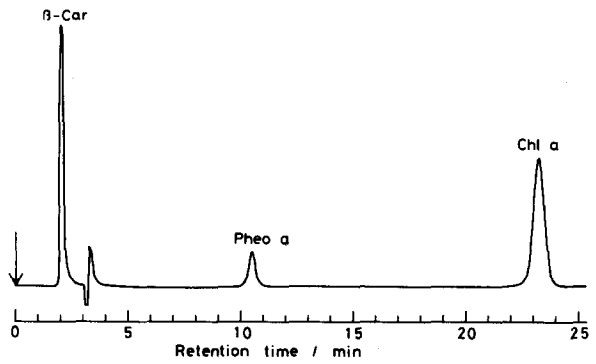

Fig.2. HPLC trace for an acetone extract of the D_1/D_2/Cyt *b*-559 reaction center complex. Detection wavelength: 425 nm.

4. DISCUSSION

X-ray crystallography of purple bacterial reaction centers has unambiguously shown that 4 BChl, 2 Pheo and 1 carotenoid constitute the reaction center [5,6]. In contrast, the present study has demonstrated that the D_1/D_2/Cyt *b*-559 reaction center complex of PS II contains 6 Chl *a*, 2 Pheo *a* and 2 β-Car; namely, there are additional 2 Chl and 1 Car molecules as compared to the bacterial reaction center.

In a bacterial reaction center, all of the BChl molecules are coordinated by histidine residues on the protein subunits [5]. The D_1 and D_2 subunits of PS II reaction center contain 18 histidine residues; 10 on D_1 and 8 on D_2 proteins [11]. A remarkable feature in the arrangement of these residues in their primary sequences [11], as well as in the putative three-dimensional structures [12], is that most of them are present as symmetric pairs on both proteins, i.e. D_1-92 and D_2-88, D_1-118 and D_2-118, D_1-190 and D_2-190, D_1-198 and D_2-198, D_1-215 and D_2-215, D_1-272 and D_2-269, and D_1-337 (or 332) and D_2-337. Based on the sequence homology between L and M subunits of purple bacterial reaction center and D_1 and D_2 subunits of PS II, it is anticipated that 4 histidine residues on D_1 and D_2 proteins (D_1-215, D_2-215, D_1-272 and D_2-269) are responsible for the binding of the non-heme iron [11,12]. Similarly, the binding sites for the special pair of Chl *a* (P680) are assumed to be the two histidine residues at positions 198 of both D_1 and D_2 proteins [11,12].

A pair of histidine residues at positions 118 of both D_1 and D_2 proteins is present in the middle part of the putative membrane spanning helices (helices β) in the hydrophobic environment. These histidines might be involved in binding two additional Chls *a* in PS II; one of which may be the intermediary for electron transfer from P680 to Pheo *a*, as in the case of 'accessory' BChl in purple bacterial reaction center. The planes of the Chl ring system, however, are expected to be perpendicular to the membrane, in this case, and not nearly parallel as found for the accessory BChls in purple bacterial systems. Histidine residues at positions 190 of both D_1 and D_2 proteins are alternative candidates for the additional Chl binding. They are rather close to the putative P680 binding sites. Parallel orientation to the membrane of chromophores is expected in this case. One of these Chls may correspond to the EPR species oriented parallel to the membrane [13], which is susceptible to photo-oxidation and is engaged in the putative protective electron transport cycle around P680 [14].

Other two histidine pairs on D_1 and D_2 proteins (D_1-92 and D_2-88, and D_1-337 (or 332) and D_2-337) are present in the hydrophilic environment on the lumenal side. They might be candidates for being ligands to manganese atoms of the oxygen-evolving system. However, another possibility is that one of the pairs binds two Chl *a* molecules, which are engaged in the excitation energy transfer from an antenna system to the oxygen-evolving Mn complex in line with a recent proposal [15].

In a bacterial reaction center complex, one molecule of Car is located near the 'accessory' BChl in the M subunit [16]. However, the surprisingly nice symmetry between the L and M subunits incites us to consider that there should have been in the L subunit another Car which had been lost during sample preparation. The existence of two β-Car molecules in the D_1/D_2/Cyt *b*-559 complex, as verified by the present work, is in line with this view. The role of these β-Car molecules is to be clarified in future investigations.

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REFERENCES

[1] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Barber, J., Chapman, D.J. and Telfer, A. (1987) FEBS Lett. 220, 67–73.
[3] Gounaris, K., Chapman, D.J. and Barber, J. (1989) Biochim. Biophys. Acta 973, 296–301.
[4] Dekker, J.P., Bowlby, N.R. and Yocum, C.F. (1989) FEBS Lett. 254, 150–154.
[5] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature (Lond.) 318, 618–624.
[6] Allen, J.P., Feher, G., Yeates, T.O., Rees, D.C., Deisenhofer, J., Michel, H. and Huber, R. (1986) Proc. Natl. Acad. Sci. USA 83, 8589–8593.
[7] Satoh, K. and Nakane, H. (1989) The VIIIth International Congress on Photosynthesis, Abstr.542, Stockholm.
[8] Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M. and Saitoh, S. (1984) Anal. Chem. 56, 251–256.
[9] Davis, B.H. (1965) in: Chemistry and Biochemistry of Plant Pigments (Goodwin, B.H. ed.) pp.489–532, Academic Press, London.
[10] Kobayashi, M., Watanabe, T., Nakazato, M., Ikegami, I., Hiyama, T., Matsunaga, T. and Murata, N. (1988) Biochim. Biophys. Acta 936, 81–89.
[11] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[12] Trebst, A. (1986) Z. Naturforsch. 41C, 240–245.
[13] Rutherford, A.W. (1985) Biochim. Biophys. Acta 807, 189–201.
[14] Thompson, L.K. and Brudvig, G.W. (1988) Biochemistry 27, 6653–6658.
[15] Watanabe, T., Kobayashi, M. and Sagara, T. (1989) The VIIIth International Congress on Photosynthesis, Abstr. 815, Stockholm.
[16] Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1988) in: The Photosynthetic Bacterial Reaction Center (Breton, J. and Vermeglio, A. eds) pp.5–11, Plenum, New York.