STUDIES ON PIGMENTS AND LIPIDS IN *RHODOPSEUDOMONAS SPHEROIDES* Y REACTION CENTER

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1. Introduction

In photosynthetic bacteria light is absorbed in a set of 'light harvesting' pigment molecules (Bchls* and carotenoids): its energy is then transferred to special pigment complexes, called reaction centers, where the primary photochemical oxidoreduction takes place [1]. Reaction center preparations enriched in photochemical activity and devoid of light harvesting Bchl have been now isolated from various bacterial species; the best characterized to-date are from *R, spheroides*, especially those from a carotenoid-less mutant, R-26, obtained by extracting the membranes with LDAO [2,3]. As reported by several authors, these reaction center particles contain three polypeptide chains [4,5] and no lipids [2]: Bchl and Bpheo are present in a 2:1 ratio [6,7]. Several arguments favor a model with 4 Bchl and 2 Bpheo by reaction center unit (see [1]).

Reaction center preparations have been obtained from wild type *R. spheroides* strains, by extraction with either SLS [8,9], CTAB [10] or LDAO [11]. In the two later cases, we have found a number of similarities with the R-26 reaction center preparations, as regard the spectral properties and the polypeptide composition [11]; however, we noted that in the wild type preparation only, residual phospholipids and carotenoids were still present [11]. We did not determine the pigment composition; for SDS extracted reaction centers, Slooten concluded on the basis of absorption and fluorescence data that the Bchl/Bpheo ratio should be at least 2 [12].

In this communication, we will report the results of pigment analysis, which indicated a 2:1 ratio for Bchl/Bpheo, as in the mutant case. Succesful attempts to remove residual phospholipids from the preparation were done, but carotenoids were still present in the lipid depleted preparation. These results are discussed as regard the pigment organization.

2. Materials and methods

2.1. *Materials*

R. spheroides Y cells were grown in the synthetic 'L, 17 μM iron' medium as described previously [10]. Isolation and purification of reaction center particles were made using either CTAB of LDAO as reported [11]; they were stored as concentrated suspensions at 5°C respectively in 0.1 M Na-phosphate buffer, 0.2% Brij, pH 7.5 or in 0.1 M Na-phosphate buffer, 0.22_5% LDAO, pH 7.5.

CTAB and Na Doc were obtained from Sigma; LDAO (Onyx Chemical CO.) was freed from H_2O_2 traces as described by Straley et al. [7]. All solvents were from Merck, reagent grade.

Bchl was isolated at 4°C in dim light from *R. spheroides* Y by extraction of wet packed cells by acetone. The extract, diluted to a 7:3 acetone–water mixture, was chromatographed on a polyethylene column (GUR 412 Hoechst) in the same solvent [13]. Following elution, crystallisation of the pigment was induced by removing partly acetone under vacuum. Bchl was converted quantitatively into Bpheo by

* *Abbreviations*: Bchl: bacteriochlorophyll, Bpheo: bacteriopheophytin, CTAB: cetyltrimethylammonium bromide, LDAO: lauryldimethylammonium oxide, Na DOC: sodium deoxycholate, *R.: Rhodopseudomonas,* SLS: sodium lauryl sulfate.

adding HCl 5 N, 0.05 vol to an acetone solution, and crystallised from acetone–water. The absorption spectrum and extinction coefficients of Bpheo in ether, acetone–methanol (7/2, v/v) and acetone agreed with the values published by Kim [14] and Straley et al. [7].

2.2. *Pigment analysis*

Bpheo and carotenoids in neutral reaction centers.

These were determined by thin-layer chromatography on cellulose plates (Merck, 1mm thickness; Schleicher and Schull G 1805, 5 mm thickness); plates were activated 20 min at 60°C. Samples of neutral acetone extracts were applied under dim light on the plates which were developed at 5°C in the dark for 90 min in acetone/methanol/water (4/20/3, v/v). The Bchl band moved ahead; the Bpheo and carotenoid bands were scraped off, eluted with acetone, concentrated under vacuum and spotted on a thin-layer plate developed with petroleum ether/acetone (9/1, v/v). A complete separation was thus obtained. The Bpheo and carotenoids spots were recovered, eluted and their contents determined by spectrophotometry [Bpheo in acetone: ϵ_{mM} (745 nm) = 47.5; spheroidene $E^{1\%}_{1\,cm}$ (456 nm) = 2700 and spheroidenone $E^{1\%}_{1\,cm}$ (482 nm) = 2120 [15], in acetone/methanol (7/2)].

Determination of pyrrole (Bchl plus Bpheo) content.

This was measured as Bpheo after acidification of the reaction center sample with HCl 5 N (0.05 vol) followed by extraction with acetone and centrifugation. The pellet was again extracted until colourless. Aliquots of combined extracts were dried under vacuum and redissolved for spectral analysis. We checked that detergent at 0.2% did not affect the absorption spectrum of isolated Bpheo.

2.3. *Delipidation of reaction center*

The method of Helenius and Simons [16] was followed to remove residual phospholipids from the preparations. Reaction centers were isolated with LDAO [11] then dialyzed as a concentrated suspension (approx. 10 mg protein/ml) during 48 hr at 5°C against a 50 mM Tris–HCl, 50 mM NaCl, 10 mM Na DOC, pH 9.0 buffer. Solid Na DOC (11 mg per g protein) was then added to the sample which was stirred 2 hr in the dark at 21°C, then applied to a G-75 sephadex column equilibrated with the 50 mM Tris–HCl, 50 mM NaCl, 10 mM Na DOC, pH 9.0 buffer; elution was done with this buffer. 2 ml fractions were collected, checked by spectrophotometry for reaction center activity as described in [10], and their lipid phosphorus content determined by the method of Bartlett [17]. Protein and ubiquinone contents of the pooled reaction center fractions were determined as previously described [10].

3. Results

3.1. *Delipidation of R. spheroides Y reaction center*

Fig.1 illustrates the separation of phospholipids from LDAO-reaction centers, using the method of Helenius and Simons [16]. The phospholipids were solubilized by the incubation with concentrated Na DOC and separated on the Sephadex column from the reaction center particles which migrated in the void volume. This reaction center fraction contained only 10% of its initial lipid phosphorus (1.3 μg/mg protein, instead of 13 μg/mg protein before treatment); ubiquinone was not removed (16 μmol/g protein after treatment against 10 μmol/g protein before). The carotenoids were also unaffected, as their absorption bands were not modified. After delipidation, the bleaching by light of the 865 nm band and the whole absorption spectrum were unchanged. We should note that some initial attempts to delipidate reaction

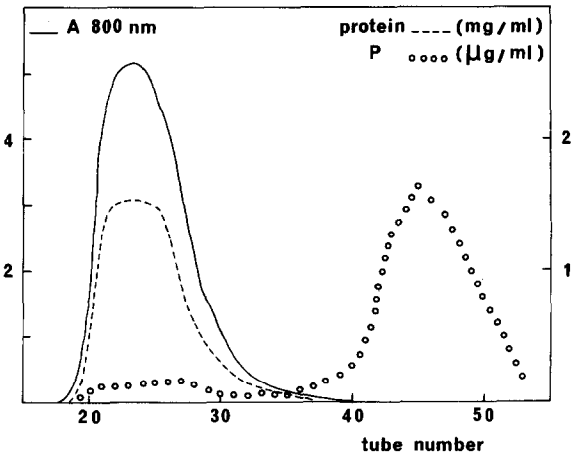

Fig.1. Separation of phospholipids from reaction center particles by chromatography on Sephadex G75 of a Na DOC treated sample.

centers using a similar protocole but LDAO instead of Na DOC resulted in pheophytinization and inactivation of the sample.

3.2. *Pigment composition of reaction centers*

The published data on the reaction centers pyrrole pigments were based in part on the identification of Bchl and Bpheo by thin-layer chromatography. However, this technique was not used quantitatively, as the yield of recovery was found to be low and irreproducible: this was attributed either to experimental losses during extraction and handling [6,18] or to abnormal behavoir linked to the presence of detergent [7]. Hence the determination of the Bchl/Bpheo molar ratio was based on spectral analysis of organic extracts. In the most accurate and definitive experiments of Straley et al. [7], this ratio is determined from the absorbances due to Bchl and Bpheo in a neutral extract. We found this method unsuitable in our case, for two reasons. First, the Bpheo absorbance in the 520 nm range was overlapping with the carotenoids one, which were difficult to abstract; secondly, Bchl could not be totally extracted from neutral reaction centers, whatever the solvent used. Hence, we used another method: Bpheo was determined in a neutral reaction center extract by thin layer chromatography (see Methods); total pyrrole (Bchl + Bpheo) was measured in an acidified reaction center extract by spectral analysis. From the two values the ratio Bpheo/Bchl was easily deduced.

In view of the signalled difficulties inherent to the chromatography, special care was taken to control any losses during the determination of native Bpheo. We used as control samples pure Bpheo treated with the same protocole as the reaction center preparation. Surprisingly, the most crucial factor was the chromatographic plates themselves. With Merck ones, the recovery of control Bpheo was never better than 70%; with Schleicher and Schull ones, it was at least 95% reproducible: then these only were used for the reaction center analysis.

The total pyrrole content was determined as described in the Methods by spectral analysis of an acidified reaction center extract. We observed that in our reaction center samples acidification had to be made before extraction in order to insure a total pigment recovery; this was checked in ether, acetone and acetone–methanol 7/2 (v/v).

The values of the Bchl/Bpheo ratio and the number of pyrrole mol in a sample of absorbance 1 at 800 nm are given in table 1; they are in good agreement with the determination done on the R-26 reaction center.

The carotenes were identified in LDAO-reaction centers by thin-layer chromatography (see Methods). Spheroidenone was present as traces; spheroidenone content is reported in table 1.

4. Discussion

In these experiments, we have determined for wild type *R. spheroides* reaction centers a pigment ratio of 2 Bchl per Bpheo, which agrees with the previous determinations on the carotenoid-less preparations [6,7]. Thus, in both cases, the pigment complexes are identical and the same conclusions are reached: as several pieces of evidence argue in favor of an even number of Bpheo and P865 per reaction center (see [1]), the minimal pigment composition should be 4 Bchl and 2 Bpheo per functional unit.

Our results indicated further that the presence of more than one set of 6 pigment molecules per particle is unlikely. As a number of reaction center preparations were analyzed, the total pyrrole content, expressed on a protein basis, was found to vary along with the specific activity in the range 50 to 70 nmol/g, the last value corresponding to the 'best' preparations. Theoretically, this content is equal to 6 nM^{-1}, for a

Table 1
Pigment analysis

Detergent used for isolation	Total pyrrole/$A_{800\ nm}$	Bchl/Bpheo molar ratio	Bchl/spheroidene molar ratio
CTAB (2 prep.)	18.0 ± 0.1 μM	1.9 ± 0.1	5.9 ± 0.4
LDAO (3 prep.)	18.3 ± 0.3 μM	2.0 ± 0.1	5.5 ± 0.6

model with 6 n (n = 1,2. . .) pyrrole molecules in a particle whose protein mass is M daltons. 70 nmol/g corresponded therefore to 6 pyrrole molecules for a protein mass of 85 000 daltons, or 12 molecules for 170 000 daltons, etc. The protein mass per reaction center particle was evaluated to 70 000 daltons in the R-26 case [2] as well as in the wild type one [11]: thus our result seemed to favour the model with 6 pyrrole molecules. We would therefore conclude that the reaction center unit contains 4 Bchl and 2 Bpheo, and that each particle in our preparation bears only one such unit.

Carotenoids are present in wild type reaction center preparations obtained from *R. spheroides* with various detergents [8,9], as well from other wild type strains [19]. However, they did not seem in our case to be an integral part of the pigment complex, as we found less than one spheroidene molecule per 4 Bchl, that is per reaction center unit. Yet they seemed tightly bound, perhaps to the protein as they were not extracted along with the phospholipids during the solubilisation of the later with Na DOC. We may not that they were still present when a reaction center was isolated without detergent by action of a protein denaturant, lithium di-iodosalicylate (unpublished experiments). Phospholipids on the reverse were easily displaced by Na DOC without perturbing the Bchl–Bpheo complex, which is probably deeply buried in the protein. The quite lower detergent concentration used in preparing wild type reaction centers [11], as compared to the case of the carotenoid-less mutant [2,3], explains probably why phospholipids are only partially replaced by detergent during the wild type reaction center isolation.

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