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Modified reaction centers from *Rhodobacter sphaeroides* R26

Exchange of monomeric bacteriochlorophyll with 13^2-hydroxy-bacteriochlorophyll

A. Struck and H. Scheer

Botanisches Institut der Universität, Menzinger Str. 67, D-8000 München 19, FRG

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Incubation of photosynthetic reaction centers from *Rhodobacter sphaeroides* R26 with exogenous 13^2-OH-bacteriochlorophyll a_P or a_{GG} according to Scheer et al. (1987) results in the exchange of endogenous bacteriochlorophyll a_P. The exchange amounts to $\leq 50\%$ according to HPLC analysis, corresponding to a complete replacement of the 'monomeric' bacteriochlorophylls, B_M and B_L, by exogenous pigment. The absorption spectra show small, but distinct changes in the Q_X-region of the bacteriochlorophylls, and bleaching of the modified reaction centers is retained. The corresponding binding sites must be accessible from the exterior, and allow for the introduction of a polar residue at C-13^2. This is supported by the observation of side reactions of the endogenous 'monomeric' bacteriochlorophylls within the reaction center pigments, e.g. epimerization and hydroxylation at C-13^2.

Photosynthesis; Reaction center; Pigment modification; Pigment exchange; Chlorophyll; Bacteriochlorophyll *a*; Bacteriochlorophyll a'; 13^2-Hydroxy-bacteriochlorophyll a; Chromatography; Esterifying alcohol; Epimerization; Oxidation; (*Rhodobacter sphaeroides*)

1. INTRODUCTION

The structure elucidation of bacterial raction centers [1–5] has raised the possibility and triggered many efforts to understand the light reactions of photosynthesis on a molecular basis. Among the biochemical techniques, site-directed mutagenesis [6,7], extraction of quinones and replacement with modified ones [8,9] and exchange of the non-heme iron atom [10] have yielded important information. By contrast, modification of the bacteriochlorophylls (BChl) and bacteriopheophytins (BPhe) which are involved in the primary reactions of charge separation [11–13], has remained a challenge. Treatment of reaction centers from *Rhodobacter (Rb.) sphaeroides* R26 with sodium borohydride leads to a selective decrease of the monomeric BChl absorption [14,15], but is only incompletely understood and, for example, complicated by the proteolytic cleavage of the M-subunit [16]. Replacement of BChl-binding histidine residues in *Rb. capsulatus* by non-liganding ones, have produced reaction centers in which the respective BChl a (**1a**) is replaced by its Mg-free analogue, e.g. BPhe a (**2a**) [17,18]. Both products have been valuable in studying electron transfer reactions, but no other pigments have as yet been introduced, and in both cases there are simultaneous changes in the protein *and* the pigments moieties.

A more general approach to modify the tetrapyrrolic pigments has been initiated by Loach et al. [19] as early as 1975, with an attempt to exchange exogenous pigments into reaction centers of *Rhodospirillum (Rs.) rubrum*. While this method proved doubtful subsequently (Loach, personal communication), a modification of it was more recently developed by which monomeric BChl in reaction centers from *Rb. sphaeroides* R26 can be exchanged [20,21]. This method is currently explored to test the importance of the various functional groups in the tetrapyrrole pigment for binding and function. Here, we wish to report that 13^2-OH-BChl a (**3**) can be exchanged and binds more tightly than the natural pigment. There is also evidence that oxygenation and epimerization at C-13^2 is possible, in situ.

2. MATERIALS AND METHODS

Rb. sphaeroides R26 reaction centers were prepared by repeated solubilization of chromatophores and subsequent chromatography on DEAE-cellulose as described previously [16]. Final purification was achieved by density-gradient centrifugation (0.2–0.8 M saccharose in 10 mM Tris-HCl buffer, pH 7.6, containing 0.08% LDAO, 20 h, 240 000 × *g*). Reaction centers were enriched in the 0.6 M region. They were withdrawn, dialyzed against Tris-HCl buffer (10 mM, pH 7.6, containing 0.08% LDAO) and stored at −20°C.

Behl a_p (**1a**) and Bchl a_{gg} (**1b**) were isolated from *Rb. spheroides* and *Rs. rubrum* G9, respectively, and purified on DEAE cellulose [22]. 13^2-OH-Bchl a_p (**3a**) and 13^2-OH-Bchl a_{gg} (**b**) were obtained as

Correspondence address: A. Struck, Botanisches Institut der Universität, Menzinger Str. 67, D-8000 München 19, FRG

Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; 13^2-OH-BChl, 13^2-hydroxy-bacteriochlorophyll; *Rb.*, *Rhodobacter; Rp., Rhodopseudomonas; Rs, Rhodospirillum*

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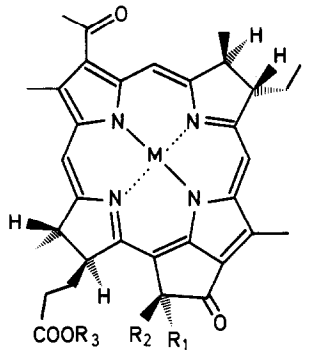

scheme 1

epimeric mixtures from the respective Bchl during standing in methanol for several days at 4°C. These products were also purified on DEAE cellulose.

Exchange experiments were performed by the method of Scheer et al. [20,21] using a 20-fold excess of exogenous pigments over the BChl a contained in the reaction centers. Excess pigments were removed from the incubation mixture by repeated chromatography on DEAE-cellulose.

The HPLC analysis was done on two different systems. The reversed phase system is described in [23]. The normal phase system is adapted from [24] (Alltech Cartridge SS150×4.6 mm, Rosil 5 μm start conditions 100% solvent A, final conditions 40% A and 60% B, 30 min nonlinear gradient no. 7 on Waters model 660 gradient programmer; solvent A is toluene/methanol/2-propanol (100:0.3:0.2) and solvent B is toluene/methanol/2-propanol (100:1.6:0.2). The column was thermostated at 4°C. The detector for both systems was a Diode Array Photometer HP 8451A. It is controlled by a home-made program, which allows to detect simultaneously at selected wavelengths at rapid intervals. In the present case, we detected at 8 wavelengths (360, 680, 700, 720, 740, 760, 780 and 800 nm) every 4 s. With this detection method, pigments can be discriminated in a rather fast and reliable manner. Details of this system will be described separately. The absorbtion spectra were recorded on a Perkin Elmer Lamda 2 spectrophotometer.

The extraction of pigments from reactions centers for HPLC analysis was done on small DEAE cellulose columns (5 × 20 mm). Reaction centers in Tris-HCl buffer (10 mM, pH 7.6) containing 0.08% LDAO were adsorbed, then washed extensively with destilled water. Afterwards, most of the water was removed by flushing the column with argon, and the pigments extracted subsequently with methanol. The methanol solution was dried with a stream of nitrogen, and the pigments resolubilized in toluene for HPLC analysis. The entire extraction procedure was completed within 30 min.

3. RESULTS AND DISCUSSION

Reaction centers treated with 13^2-OH-BChl a_p (**3a**) had adsorption spectra which were very similar to the original ones, and to controls treated in the same way, but without addition of extraneous pigment (fig.1). There is a distinct change, however, in the Q_x region of Bchl a to shorter wavelengths, which is more pronounced in the difference spectrum shown in the inset. This typical short-wavelength shift of the Q_x region is correlated with the increase of the 13^2-OH-BChl a and the decrease of BChl a analyzed by HPLC (data not shown). Reversible photobleaching of the exchange samples is retained.

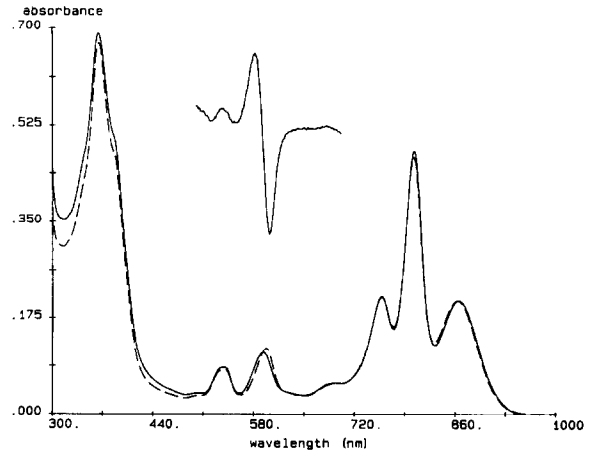

Fig.1. Absorption spectra of reaction centers from *Rb. spheroides* R26 before (dashed) and after exchange experiment with expogenous 13^2-OH-BChl a (solid line). The inset shows the difference spectrum (dashed line); ordinate expanded 20-fold.

In the HPLC chromatogram (fig. 2), there is a large decrease of BChl a (peak C, t_r = 26 min) and a concomitant increase of a new band belonging to 13^2-OH-BChl a (peak D, t_r = 33 min). The latter is very low in freshly prepared reaction centers. Some of this pigment is also formed in the control which was subjected to the same exchange/purification sequence, except for the omission of exogenous 13^2-OH-BChl a, but its amount is much less than in the exchange sample proper (see below).

The possibility of unspecific adsorption of the 13^2-hydroxylated pigment could be excluded. There is always a correlated decrease of the BChl a peak in the HPLC chromatogram, and the ratio of the total bacteriochlorophylls (BChl a plus 13^2-OH-BChl a) to BPhe a remains constant at 2:1 throughout. This and the small changes in the absorption spectra also exclude an exchange of extraneous or newly formed 13^2-OH-BChl a against BPhe a. In all experiments, the exchange rate is in the range of 30–50% of the total BChl a, corresponding to an exchange of a maximum of two of the four BChl a molecules present in reaction centers.

Handling of small amounts of BChl a, extraction from pigment proteins and HPLC is non-trivial and prone to artefact formation. One typical artefact is oxygenation at C-13^2 yielding 13^2-OH-BChl a (**3**). Our analytical procedure produces a very low amount of this artefact, and freshly prepared RC gives in all cases the same or a better trace than the top one shown in fig.2. However, since some of this pigment is formed in the controls (center trace), hydroxylation at C-13^2 obviously can take place under the exchange conditions, and the pigment formed remains bound. The origin of the much larger amounts in reaction centers treated with an excess of 13^2-OH-BChl a, then had to be ascertained. This was done by repeating the exchange experiments with a doubly modified BChl a, e.g. 13^2-OH-

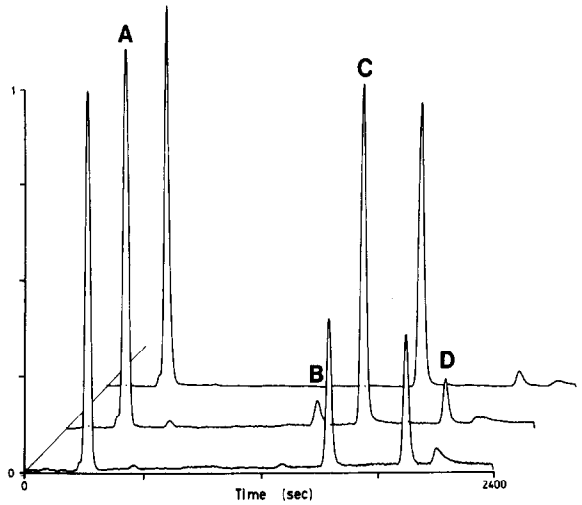

Fig.2. HPLC chromatogram of pigments extracted from *Rb. sphaeroides* reaction centers (on silica, see section 2 for details). Before (upper) and after exchange with 13^2-OH-BChl a_p (**3a**) (botton trace). Control reaction centers treated in the same way, without exogenous pigments are shown in the middle trace. The chromatograms are adjusted to absorbance 1 at the band A. (Band A) BPhe a_p (**2a**); (B) BChl a′ (**1c**); (C) BChl a_p (**1a**) and 13^2-OH-BChl a_p (**3a**).

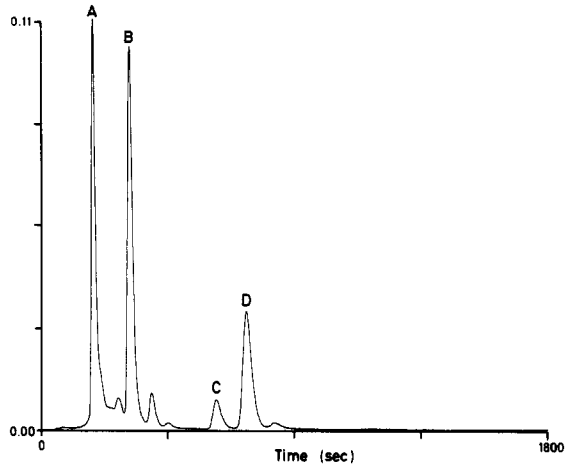

Fig.3. HPLC chromatogram from *Rb. spheroides* reaction centers (on reversed phase, see section 2 for details) after exchange with 13^2-OH-BChl a_{gg} (**3b**). (Band A) 13^2-OH-BChl a_{gg} (**3b**); (B) BChl a_p (**1a**); (C) 13^2-OH-BPhe a_p; and (D) BPhe a_p (**2a**). 13^2-OH-BPhe a_p (band C) seems to be an artefact of the reversed phase/methanol-water system, because this pigment is absent in HPLC on silica.

BChl a_{gg} (**3b**), which is hydroxylated at C-13^2 and esterified with geranyl-geraniol instead of phytol at the propionic acid side chain. Exchange of the parent pigment, BChl a_{gg} (**1b**), has been established before [20], as has more recently the exchange pf BChl a with other esters (unpublished).

Spectroscopically, the results are the same as shown above for the exchange with 13^2-OH-BChl a_p (**3a**). The HPLC analysis was done on a reversed phase system, which is superior for separation of BChl a-pigments esterified with different alcohols [25]. In reaction centers treated with excess 13^2-OH-BChl a_{gg} (**3b**), this pigment replaces up to 50% of the endogenous BChl a_p, and whereas little to no 13^2-OH-BChl a_p (**3a**) is found (fig.3). With this result, we can exclude that the 13^2-OH-BChl a is formed by hydroxylation within the RC, but rather prove that it is introduced from without.

Careful inspection of the HPLC-chromatograms gives evidence that yet another pigment can replace some of the BChl a in reaction centers, e.g. its 13^2-epimer BChl a′ (**1c**), which moves faster than BChl a (**1a**) on silica (fig.2, band B). This pigment is present in varying amounts in reaction centers purified extensively. In freshly prepared samples, it is usually barely detectable (fig.2, upper trace), but in aged samples larger amounts can be found, which are not removed by repeated chromatography (middle trace). This pigment is lost, however, during exchange with 13^2-OH-BChl a (bottom trace). This and results of competition experiments (unpublished) indicate an affinity which increases in the order BChl a′ (**1c**), BChl a (**1a,b**) and 13^2-OH-BChl a (**2**). It should be noted, that 13^2-OH-BChl a (**3**) is also present as a C-13^2-epimer mixture, but the effect of different stereochemistry has not yet been investigated.

Previous exchange experiments have demonstrated, that the 'monomeric' or 'voyeur' BChl a B_l (or B_a), and B_m (or B_b) are the ones exchanged [20,21]. The function of the former has recently been shown to be an intermediate electron carrier [13], the latter is possibly involved in (triplet) energy transfer [26]. The results presented show that both pigments can be replaced by 13^2-OH-BChl a (**3**) to yield stable, chemically modified reaction centers. Inspection of the environment of these pigments in the crystal structure of *Rp. viridis* reaction centers [1,2] indicate that there is enough space at the 13^2-hydrogen site of both 'monomeric' BChls to accomodate a hydroxy-substituent instead. There may even H-bonding be possible between the peptide-CO of gly-M201 with a 13^2-OH-BChl a at the L-site, and ser-L178 with a 13^2-OH-BChl a at the M-site. The primary structures of *Rp. viridis* and *Rb. spheroides* are very similar in this region, so the results seem to extendeable to the latter. The distinct differences in the phytol-chain geometries for the 'monomeric' BChls (and only those) among the two species, and their relatively open environments are further aspects which may contribute to the ready exchange, and also for the possibility to epimerize at C-13^2.

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