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The Photosynthetic Bacterial Reaction Center

Structure and Dynamics

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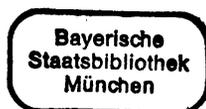
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REACTION CENTERS OF PURPLE BACTERIA
WITH MODIFIED CHROMOPHORES

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INTRODUCTION

Reaction centers (RC*) of purple bacteria are generally composed of three subunits designated H(igh), M(edium) and L(ow) according to their apparent molecular weights on SDS PAGE. Four molecules of bacteriochlorophyll (Bchl) are bound to it, together with two bacteriopheophytins (Bphe), two quinones (Q) and one non-heme iron. The crystal structure of RC from the BChl b-containing purple photosynthetic bacterium, *Rp. viridis* (Deisenhofer et al., 1984) and from the Bchl a-containing *Rb. sphaeroides* (Chang et al., 1986; Allen et al., 1987) shows a C2 - symmetry axis which divides the reaction center into two very similar sets of pigments interacting mainly with the L and M-subunits, respectively. The reaction center is asymmetric, however, in functional terms. The primary charge separation takes place most probably from the special pair situated on the symmetry axis, via Bphe_L and Q_A situated on the L-(or 'active') branch of the complex, to Q_B on the M or 'inactive' branch (Deisenhofer et al., 1984; Vermeiglio and Paillotin, 1982; Zinth et al., 1985).

*) Abbreviations: RC = reaction center; Bchl = bacteriochlorophyll; Bphe = bacteriopheophytin, the location of these pigments on the L- or M-branch of the RC is indicated by the respective subscript; P870 = primary donor; Chl = chlorophyll; Q = quinone; subscript indicates the primary (A) or secondary acceptor (B) located on the L- and M-branch, respectively; cd = circular dichroism, ESR = electron spin resonance; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis, LDAO = dimethyldodecylamineoxide, TX-100 = Triton X-100; Rb. = Rhodobacter; Rp. = Rhodopseudomonas, Rs. = Rhodospirillum, Cf. = Chloroflexus, Cr. = Chromatium.

This provides a function for only three of the six chlorophyllous pigments. Much less is known on the function of the remaining two monomeric BChl *a* molecules absorbing around 800nm (Bchl_L, Bchl_M), and of the second pheophytin, Bphem, which shows a well resolved absorption from the 'active' Bphe_L in the Q_x spectral region. Bchl_L is situated between the primary donor, P870, and the "primary" acceptor, Bphe_L. It is still unclear, however, if it acts as "pre-primary" acceptor in this process (Shuvalov and Duysens, 1986), or if some other mechanisms like super-exchange, rapid hopping between a close and a distant site or yet another process is operative (Holten, 1986; Ogrodnik et al., 1982; Breton, 1986; Zinth et al., 1985; Knapp et al., 1985; Wasielewski, 1986; Parson, 1982, Michel-Beyerle et al., 1987; Fischer and Scherer, 1987). The participation of the pigments on the M-branch, Bchl_M and Bphem, is unclear. The latter can also accept an electron (Robert et al., 1985; Michel-Beyerle et al., 1987), but it is questionable if this also takes place under physiological conditions.

In view of the tight packing of the pigments and the highly optimized charge separation, modified RC are of considerable interest to understand the mechanism of charge separation and the function of all pigments present in RC. The possibility to remove one of the six tetrapyrrolic pigments in bacterial reaction centers (Ditson et al., 1984) provided for the first time an experimental tool to modify the tetrapyrroles in this complex. By treatment with sodium borohydride, about 50% of the bacteriochlorophyll (Bchl) absorbing at 800nm can be removed. From kinetic (Maroti et al., 1985; Holten, 1986; Shuvalov and Duysens, 1986; Breton 1986) and spectroscopic evidence (Robert et al., 1986; Scherer and Fischer, 1987), it has been suggested that the 'inactive' Bchl_M located between the primary donor P870 and the bacteriopheophytin Bphem on the M-branch, is reduced at the 3-acetyl group and can then be dissociated from the remaining complex (Maroti et al., 1985). A basic requirement for evaluating the results is a thorough characterization of the modified preparations with respect to chemical composition, homogeneity, spectroscopy and kinetics. This is of particular importance because the homogeneity of the sample has been questioned more recently by Shuvalov et al. (1986), who discuss a product mixture in which the Bphem is partly reduced as well.

Principally, an exchange of pigments with modified ones would be most useful. Much of the progress in the retinylidene-protein research relies on this technique. However, comparably few such experiments have been reported on (bacterio)chlorophyll proteins, and it is moreover very difficult to verify the exchange. Loach et al. (1975) provided ESR-spectroscopic evidence for an exchange of Bchl in AUT-particles from *Rs. rubrum*. The line narrowing observed upon incubation of ¹H-RC with ²H-Bchl was reversed, however, after subsequent washing of the samples (P.Loach, private communication, Beese, 1984). The reincorporation of Chl *a*' into ether-washed PSI particles led to a light induced difference spectrum similar to that of P700, but the bleaching was at shorter wavelengths, and more important it was irreversible (Hiyama et al., 1987). Verification problems are even more severe with antenna complexes (Clayton and Clayton, 1982; Parkes-Loach et al., 1987; Plumley and

Schmidt, 1987, Chadwick et al., 1987) for difficulties in establishing reliable functional criteria in an isolated antenna. These verification problems are mainly related to three factors: pigment adsorption on RC (vide supra) detergent effects on the spectra of pigment-protein complexes (Chadwick et al., 1987), and formation of micellar (bacterio)chlorophyll-detergent complexes which have spectroscopic and chemical properties very similar to the ones of protein complexes with the respective pigments (Gottstein and Scheer, 1983; Scherz and Parson, 1984, 1986, Scheer et al., 1985; Scherz and Rosenbach-Belkin, 1987).

Here, we want to summarize data characterizing the "NaBH₄-removable" Bchl a, its interactions with near-by pigments, and the homogeneity of treated RC from Rb. sphaeroides, as well as the extension of the modification method to other pigment complexes. In addition, results are presented on an improvement of the method of Loach et al. (1975) by which the 'extra' Bchl molecules Bchl_L and Bchl_M are exchanged with extraneous pigment.

MATERIALS AND METHODS

Preparations

RC from Rb. sphaeroides were prepared by a modification of the method of Feher and Okamura (1978). Bchl_M was removed according to the original method of Ditson et al. (1985) by addition of solid borohydride in the presence of LDAO as detergent. To stop the reaction in kinetic experiments, aliquots were diluted 1:1 with a glucose solution (for spectroscopic studies), or treated with acetone to precipitate the protein (for polypeptide analysis).

Exchange experiments

RC were treated with a 10-20 fold excess of free pigment according to Loach et al (1975). The temperature was raised to 40°C. Purification of RC after modification and detergent

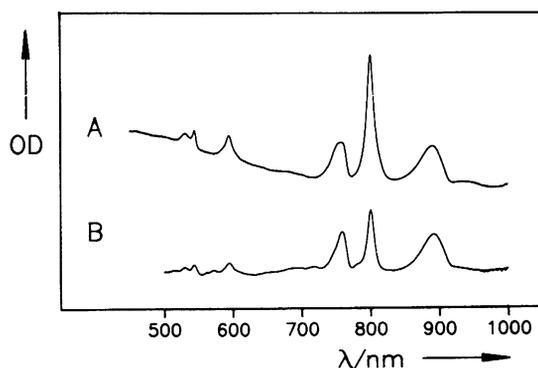


Fig. 1. Absorption spectra at 4 K of RC from Rb. sphaeroides R26 before (A), and after treatment with borohydride and subsequent purification on DEAE cellulose (B).

exchange (if necessary) was done by chromatography on DEAE cellulose (DE 52, Whatman). Chemical oxidation of reaction centers was performed by titration with potassium ferricyanide.

Spectroscopy

Extinction coefficients were determined with respect to the 870nm transition ($\epsilon=1.28 \times 10^5 \text{ cm}^{-2} \text{ M}^{-1}$, Clayton and Wang, 1971). Since the intensity of this band remained unchanged during treatment with borohydride or exchange, the same value was used for modified RC. For the determination of molar ellipticities, the reaction was followed in the CD cuvette, and the decrease at the 870nm band then used to calibrate all other bands in purified, modified RC. Low temperature absorption and fluorescence spectra were recorded in the apparatus described by Angerhofer et al. (1986), fluorescence-ODMR and microwave-induced absorption-difference (MIA or ADMR) spectra were measured with the ODMR set-up described by Angerhofer et al. (1985).

RESULTS

Treatment of RC with borohydride from Rp. sphaeroides

Upon treatment with borohydride, the 800nm absorption decreases by about 55% as compared to its starting value. Distinct changes occur also in the Q_x spectral region between 500 and 630nm (Fig.1). Typical preparations have an 800/870nm absorption ratio of 1.1 to 1.3. These spectral changes are similar to the ones described by Ditson et al. (1984). The reaction is less complete in the presence of TX-100 instead of LDAO. The low-temperature spectrum shown in Fig.1 reveals some details (Beese et al., 1987) which were not discernible in the room temperature spectra. Two minor bands become apparent at 780 and 572nm, and the shape of the Bphe band at 757nm is different. Instead of consisting of two poorly resolved bands of equal intensity, it now appears to be a main band shifted to longer wavelengths with a short wavelength shoulder.

Since the reaction is accompanied by an increase of the pH to 10-10.5, the effect of high pH alone was tested in an independent experiment. Up to pH 10.5, the spectrum remains

Table 1. Reaction of different bacteriochlorophyll-proteins with potassium borohydride

RC	<u>Rb. sphaeroides R26</u>	decrease 804nm
RC	<u>Rb. sphaeroides 2.4.1</u>	no effect
RC	<u>Rp. viridis</u>	some decrease 820nm
B880	<u>Rs. rubrum</u>	no effect
B800-850	<u>Rb. sphaeroides</u>	decrease 800nm
B800-850	<u>Rp. acidophila (type I)</u>	decrease 800nm
B800-850	<u>Rp. acidophila (type II)</u>	decrease 800nm
B800-840	<u>Cr. vinosum</u>	decrease 800nm
B800-820	<u>Cr. vinosum</u>	decrease 800nm

unchanged. At higher pH, the absorption around 860 nm decreases first, followed by the 800 nm band. Obviously, the high pH obtained under the experimental conditions has no effect on the spectrum. It should be noted, however, that the reaction is accelerated by it.

Treatment of other chlorophyll - proteins and micellar chlorophyll-detergent complexes with borohydride

The reactions of other bacteriochlorophyll - proteins with borohydride are summarized in Table 1. Except for the RC from the wild-type strain *Rb. sphaeroides* 2.4.1 and from *Rp. viridis*, which gave little to no reaction, all other complexes showed a selective and very pronounced reaction of those chromophores absorbing around 800nm, whereas the ones absorbing at longer wavelengths did not react. This indicates some common property of these chromophores, which sets them apart from the ones absorbing at longer wavelengths. Part of this reactivity may be related to a better accessibility to the hydrophilic borohydride. That the aggregation state of the pigments may be another factor, is indicated by model studies with micellar complexes in the detergent, TX-100. The reaction of monomeric chlorophyll a or b is faster by two orders of magnitude as compared to the respective aggregates. More recently, some experiments were carried out with chlorophyll-protein complexes from green plants showing that they react as well with borohydride in a differential manner (Scheer, Anderson and Porra, unpublished). Another noteworthy and common feature is the regioselectivity of the reaction. Bchl a and the plant pigment, Chl b, bear two carbonyl groups. In solution, e.g. in methanol, the 13¹ C=O group is always much less reactive than 3 C=O or 7¹ C=O, respectively. This is shown in Fig. 2 for the reaction of Chl b. The same regioselectivity has been observed in all experiments, both with the pigment-protein and pigment-detergent complexes.

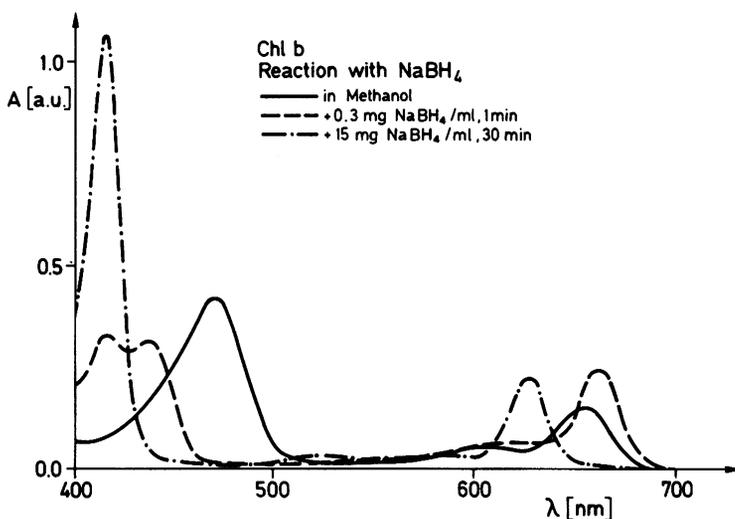


Fig. 2. Reaction of Chl b in methanolic solution with borohydride. See inset for experimental details.

Sample homogeneity: Spectroscopy

The excitation spectra of the P870 fluorescence at low temperatures are similar to the absorption spectra. The emission spectra of modified RC (Beese et al., 1987) have two bands at 917nm (reaction center), and around 776nm (contamination with free (= non-aggregated) pigments). The latter are strongly fluorescent and thus picked up with high sensitivity in the RC, which is only weakly fluorescent itself. The only significant change of the sample after modification with sodium borohydride, is an increase of this free pigment emission by approximately 150%. There is in particular no new band above 800nm. This indicates a homogeneous emission of protein-bound pigments. Homogeneity of the preparation and unchanged hyperfine coupling of the primary donor triplet, is also supported by optically-detected magnetic resonance. The spectra of unmodified and modified RC are identical within the experimental error (Beese et al., 1987) both for absorption and fluorescence detection.

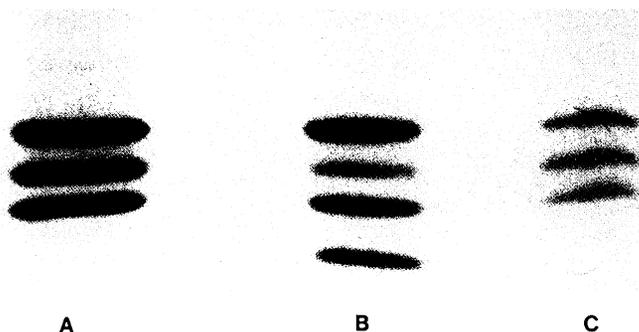


Fig. 3: Polypeptide composition of RC as analyzed by SDS-PAGE. Trace A: Untreated RC. Trace B: RC treated with borohydride. Absorbance ratio 800/870 = 1.3. Trace C: RC incubated for 14 hrs at pH 11.

Sample homogeneity: Polypeptide composition

The RC contains three polypeptides (H,M,L) with apparent molecular weights of 28, 24 and 21 kDa, respectively. An additional peptide (15 kDa) appears upon treatment with borohydride, and the intensity of the M-band is diminished at the same time (Fig. 3), indicating most likely a cleavage of M. Control samples incubated at pH 11 for similar length of time, but without the addition of borohydride, did not show the additional polypeptide. This indicates that only the NaBH_4 -reduction but not the accompanying pH change leads to cleavage of the M-polypeptide. Cleavage reactions of this reagents have been reported before (Crestfield et al., 1963).

Two conclusions can be drawn from these results: 1) The sample is biochemically heterogeneous in spite of its spectroscopic homogeneity. 2) Cleavage of the M-subunit has only minor effects on stability and functionality of RC. Reversible bleaching has been retained before in membranes after extensive proteolysis (Bachofen and Wiemken, 1984; Steiner et al., 1986; Theiler et al., 1984), but the reaction centers are generally not resistant to the same treatment in solution. This reflects a more specific proteolysis by borohydride.

Coupling of B800_M with P870

The results discussed so far indicate that removal of Bchl_M produces only localized changes and no disturbances of the remaining five tetrapyrrols. Such interactions are revealed, however, by methods which are more sensitive to interactions. As an example, the cd spectra of untreated and

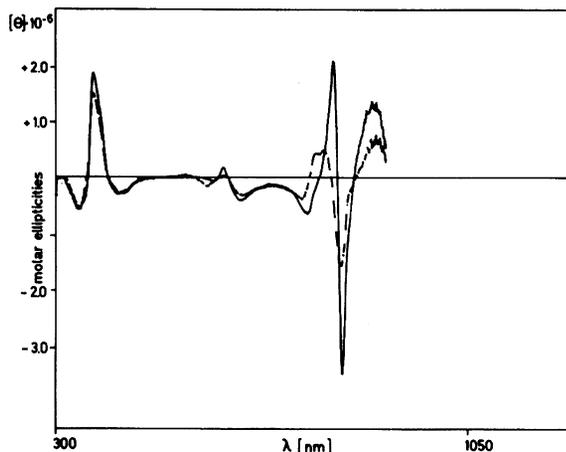


Fig. 4. CD spectra of untreated (—) and borohydride treated RC (---). The treated sample was purified over DEAE cellulose.

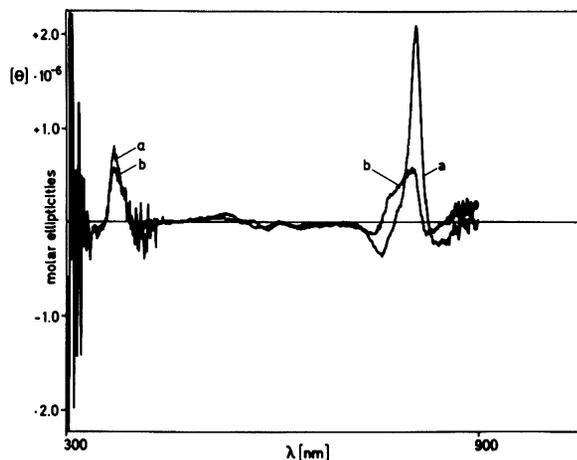


Fig. 5. Circular dichroism spectra of RC oxidized with $\text{Fe}(\text{CN})_6^{3-}$: a: Untreated RC. b: Sample after reaction with borohydride and purification.

modified RC are compared in Figs. 4,5. In relaxed (=reduced) RC the changes are no longer confined to the spectral region around 800nm. There is in particular a decrease in optical activity of the 870nm band, indicating some structural change and/or the coupling of B800_M with P870 in unmodified RC. In oxidized RC (Fig.5), the strong band around 800 nm is reduced to 33% in intensity, and the fine structure of the Bphe bands indicates a sign inversion for the one absorbing at longer wavelengths. Distinct changes have also been found in the microwave-induced absorption difference spectra of RC upon modification with borohydride. They could be rationalized by a structural change (Scherer and Fischer, 1987). The Raman resonance spectra show small but distinct shifts as well reflecting interactions of Bchl_M with both neighboring B870 and Bphe_M (Beese et al., 1987).

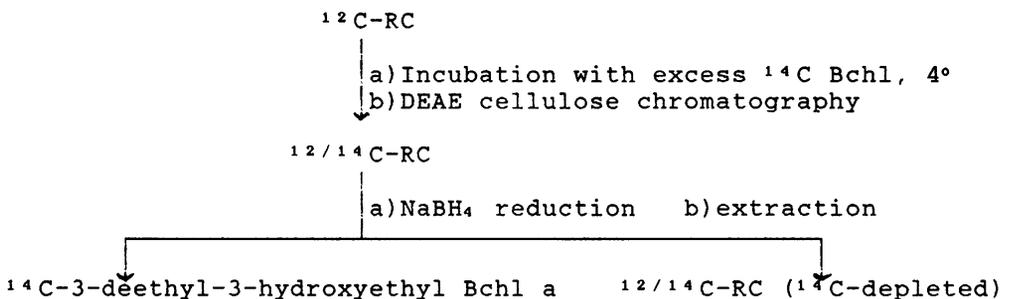
These examples show that removal of Bchl_M has very pronounced effects on its neighboring pigments, P870 and Bphe_M. They involve both minor geometric changes, probably due to a rearrangement in response to the hole created, and the elimination of couplings.

Exchange of 'extra bacteriochlorophylls' in RC

Application of the procedure of Loach et al. (1975) to RC from Rs. rubrum and Rb. sphaeroides did not show any exchange of pigments detectable by labeling with ¹⁴C (both ways) or with Bchl bearing a different alcohol. All samples were purified after the incubation by chromatography on DEAE cellulose, to remove any adsorbed pigments. This confirms findings of Loach (private communication), who observed a loss of ESR line-narrowing in ²H-treated RC if they were re-purified after the incubation. Whereas this reversible line-narrowing poses an interesting problem by itself, because it means spin-exchange between P870 and loosely adsorbed Bchl, the results defy the original goal.

When the method was pursued further, it could be modified, however, to produce what looks like a true exchange of the 'extra' Bchl. The main modification is an increase of the incubation temperature to about 40°, which is just at the point where reaction centers begin to unfold. The verification tests involved several different techniques. 1. Labelling of Bchl a with different esterifying alcohol and subsequent analysis by HPLC. 2. Incubation of ¹⁴C-RC with cold Bchl a, and vice versa.

Scheme 1. Experimental procedure to establish exchange of 'extra' Bchl in RC.



Exchanges of $\leq 50\%$ of the total Bchl present in RC were observed. In all cases, the purity of the treated RC with respect to free or adsorbed pigment was checked by absorption (intensity ratio of NIR bands) and/or fluorescence spectroscopy (observation of emission bands below 850nm).

Assignment of the exchanged pigment to Bchl_M and Bchl_L was done by two procedures. 1. Exchange of P870 was excluded by the fact that the ESR signal of RC treated with ²H-Bchl a was unchanged. 2. A positive correlation with the 'extra' Bchl a made use of the aforementioned NaBH₄ selectivity to Bchl_M (Scheme 1). The finding of ¹⁴C-3-deacetyl-3-hydroxyethyl Bchl a confirms that exchange occurs at least at Bchl_M, and the exchange yield then indicates that Bchl_L is accessible as well.

These results indicate that the way first pursued by Loach et al. (1975) could be useful after all. Experiments to explore this reaction further by using structurally modified pigments are in progress.

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