THE PHOTOSYNTHETIC REACTION CENTER

Front cover photo: Top view of the *Rhodobacter sphaeroides* reaction center protein showing the surface exposed to the cell periplasmic aqueous phase. The three protein subunits, L, M, and H, are shown in red, green, and blue, respectively.

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7 Bacterial Reaction Centers with Modified Tetrapyrrole Chromophores

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I. Introduction

Several different methods are available for preparing reaction centers with nonnative chromophores: (1) (re)assembly from the polypeptides, pigments, and other components such as quinones, iron, and possibly others; (2) exchange of chromophores into native or partly denatured reaction centers; (3) modification of chromophores in native or partly denatured reaction centers by chemical or enzymatic means; (4) modification of binding sites to incorporate different chromophores, for example, by mutagenesis, and (5) mutagenic modifications of the pigments biosynthetic pathways. With the exception of the last one, all these approaches have been used on different

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(bacterio)chlorophyll proteins, but only Methods 2 and 4 have been successful with reaction centers.

Complete reconstitution of the complexes (Method 1) from the isolated constituent polypeptides and suitably modified pigments is likely to have the greatest potential, but to date has been impossible with reaction centers. This technique has been applied successfully to the core light-harvesting complexes (LHCI) of purple bacteria (Miller *et al.*, 1987; Ghosh *et al.*, 1988; Parkes-Loach *et al.*, 1988,1990; Chang *et al.*, 1990a,b; Heller and Loach, 1990) and to the peripheral light-harvesting chromoprotein LHCII-β of higher plants (Plumley and Schmidt, 1987). The method has been extended by Paulsen *et al.* (1990,1991) to the full-length and modified LHCII-β precursor polypeptides expressed in and isolated from *Escherichia coli* and by Schmidt *et al.* (1991) to other green plant peripheral light-harvesting complexes.

Site-directed mutagenesis of the binding pockets (Method 4) has been used extensively to exchange bacteriochlorophylls (Bchls) with their metal-free derivatives, for example, bacteriopheophytins (Bphes) and vice versa in reaction centers (RCs) from *Rhodobacter* (*Rb.*) *capsulatus* and *Rb. sphaeroides* (Table I). With only a single exception, whenever the histidine(s) ligating the central magnesium atom(s) of the Bchl(s) were replaced by hydrophobic amino acids, stably assembled RCs were obtained in which the Bchls at the respective site(s) were replaced by Bphe to yield stably assembled and isolatable RCs (Bylina and Youvan, 1988; Coleman and Youvan, 1990; Robles *et al.*, 1990; Schenck *et al.*, 1990; Woodbury *et al.*, 1990). In reversing this reaction, replacement of leucine or isoleucine next to the central N-4 cavity of Bphe with histidine resulted in binding of Bchl at the respective site. Some results are available with amino acid residues of intermediate ligation strength. Serine or threonine selected binding of Bchl at B_A and B_B (Bylina *et al.*, 1990); glutamine (mutants H-L173Q and H-M200Q[2]) at the sites of the special pair resulted in binding of Bchl also (Bylina and Youvan, 1988). Omission of Bphe H_L was found in a mutant of *Rb. capsulatus* in which part of the D helix of subunit L was replaced by the symmetrically equivalent stretch of helix D_M. The resulting RC mutant, called D_{LL}, lacked Bphe H_A, presumably because a tryptophan residue was now occupying (part of) the space usually occupied by Bphe (Robles *et al.*, 1990). The reader interested in this approach is referred to an excellent review (Coleman and Youvan, 1990) and to a series of papers in the book edited by Michel-Beyerle (1990). A natural example of this type of pigment exchange exists in *Chloroflexus aurantiacus:* RCs are of the purple bacteria type (type II) but a significant difference is the exchange of His M180, which is normally the ligand of Bchl B_B, by

[2]Residue numbering according to "best alignment" with *Rps. viridis* RCs and the single letter amino acid code are used throughout this chapter when naming mutants.

TABLE I **BChl/BPhe Modifications Induced by Site-Directed Mutagenesis of Potential Ligands to the Central Magnesium**

Site	Residue number[a]	Amino acid[b]	Species[c]	Pigment bound	Remarks	Reference
PA	L173	H	Type II	BChla or b	Native situation	
		L	*Rb. caps.*	BPhea	Heterodimer, PS^-	Bylina and Youvan (1988)
		Q[d]	*Rb. caps.*	BChla	Heterodimer, PS^+	Bylina and Youvan (1988)
		L	*Rb. sphe.*	BPhea	Heterodimer	Huber *et al.* (1990); Schenck *et al.* (1990)
PB	M200	H	Type II	BChla or b	Native situation	
		L	*Rb. caps*	BPhea	Heterodimer, PS^-	Bylina and Youvan (1988)
		F	*Rb. caps.*	BPhea	Heterodimer, PS^-	Bylina and Youvan (1988)
		Q	*Rb. caps.*	BPhea	Heterodimer, PS^+	Bylina and Youvan (1988)
		L	*Rb. sphe.*	BPhea	Heterodimer	Huber *et al.* (1990); Schenck *et al.* (1990)
BA	L153	H	Type II	BChla or b	Native situation	
		S[e]	*Rb. caps.*	BChla		Bylina *et al.* (1990)
		L	*Rb. caps.*	?	PS^+	Bylina *et al.* (1990)
BB	M180	H	Type II	BChla or b	Native situation	
		L	*Cf. aur.*	BPhea	Native situation	Ovchinnikov *et al.* (1988) Shiozawa *et al.* (1989)
		S	*Rb. caps.*	BChla		Bylina *et al.* (1990)
		T	*Rb. caps.*	BChla		Bylina *et al.* (1990)
		L	*Rb. caps.*	?	PS^+	Bylina *et al.* (1990)
		R	*Rb. caps.*	?	PS^-	Bylina *et al.* (1990)
HA	M212	L	Type II	BPhea or b	Native situation	
		W	*Rb. caps.*	None	D_{LL} + mutant	Robles *et al.* (1990)
		L	*Rb. caps.*	BPhea	Revertant of D_{LL} Mutant	Breton *et al.* (1990) Robles *et al.* (1990)
HB	L187	L	Type II	BPhea or b	Native situation	
		F	*Rb. caps*	BPhea	D_{MM} mutant	Robles *et al.* (1990)

[a] Residue numbering according to "best alignment" with *Rp. viridis* RC.

[b] One-letter coding of amino acids.

[c] Type II refers to purple bacterial PSII and related RC. *Rb. caps.*, *Rhodobacter capsulatus*. *Rb. sphe.*, *Rhodobacter sphaeroides*. *Cf. aur.*, *Chloroflexus aurantiacus*.

[d] Double mutant H-L173-Q/H-M200-Q binds two BChl a at $P_{A,B}$ and is PS^+.

[e] Double mutant H-L153-S/H-M180-S binds two BChl a at $B_{A,B}$.

Ile M178 (Ovchinnikov *et al.*, 1988; Shiozawa *et al.*, 1989). In these RCs, for one of the four Bchlas is exchanged for Bphea at the same time, and it is likely to be the one located at B_B. A series of histidine mutations in the core antennas of photosystem II (PSII) resulted in distinct reductions of photosynthetic efficiency, but the pigment contents of these mutant complexes are not yet established (Kuhn and Vermaas, 1991; Shen *et al.*, 1991). Due to heterotrophic growth problems, mutagenesis has proven difficult with *Rps. viridis*, but progress in two laboratories shows promise that mutated RC with Bchlb/Bpheb-exchanges will soon be available from this organism (Bylina, 1992; Laußermair and Oesterhelt, 1992).

Genetic modification of Bchl biosynthesis (Method 5) has, to our knowledge, not yet been applied to obtain modified Bchl proteins. Little was known about the last steps of this process until the publishing of several reports (Burke *et al.*, 1991; Leeper, 1991; Richards and Fidai, 1991; Suzuki and Bauer, 1991).

One example of the apparent selective modification of one pigment in RCs (Method 3) is presented in the literature. Treatment of isolated RCs from *Rb. sphaeroides* R26 with borohydride (BH_4^-) resulted in the loss of about 40% of the absorption at 800 nm normally ascribed to the two Bchls at sites $B_{A,B}$ (Ditson *et al.*, 1984). Therefore, the researchers concluded that one of these pigments (most likely B_B) was attacked selectively by the reagent and subsequently lost from the RC (Ditson *et al.*, 1984; Maroti *et al.*, 1985; Beese *et al.*, 1987; Chadwick *et al.*, 1987; Frank, 1990; Frank and Violette, 1989). However, this interpretation has been challenged by the finding of (1) an unchanged pigment composition in BH_4^--treated RCs (Struck *et al.*, 1992a), (2) a tetrapyrrolic pigment at the B_B site in the poorly resolved X-ray structure (J. Allen, personal communication, 1991), and (3) at least partial proteolysis of the M subunit (Beese *et al.*, 1987). The BH_4^- reduction also leads to distinct changes in chlorophyll proteins of higher plants, but these changes have not yet been investigated at a molecular level (Scheer *et al.*, 1989). An enzymatic modification of several chlorophyll proteins with the degradative enzyme chlorophyllase has been reported by Schoch and Brown (1986). Modifications on other complexes involve the demetalation of the Fenna-Matthews-Olson (FMO) antenna protein of *Clorobium* (Ghosh *et al.*, 1968) and the selective loss of the 800-nm absorption band in the B800-850 antenna complex of *Rb. sphaeroides* (Clayton and Clayton, 1981). In the latter case, the decrease again has been shown to be due not to loss of any pigment but to a detergent effect (Chadwick *et al.*, 1987).

The first attempt to exchange pigments into native or partially denatured reaction centers (Method 4) is likely to be that published by Loach *et al.* (1975), who worked on the so-called alcaline urea-triton X100 (AUT) particles of *Rhodospirillum* (*Rs.*) *rubrum*. From line narrowing of the light-induced

signal after treatment with deuterated Bchla, an exchange of the primary donor Bchl $P_{A,B}$ was concluded. This interpretation was challenged (Norris, see discussion in Loach *et al.*, 1975) and the authors concluded subsequently that the line narrowing was artefactual and no exchange had taken place (P. Loach, personal communication, 1980). The method was modified, and an exchange of the monomeric Bchla at sites $B_{A,B}$ or of Bphea at sites $H_{A,B}$ with chemically modified pigments became possible in RCs of *Rb. sphaeroides* R26 and some other purple bacteria (Scheer *et al.*, 1988; Beese, 1989; Struck *et al.*, 1990a,b,1992; Struck, 1991). This chapter details the procedures involved and summarizes the results obtained with this method.

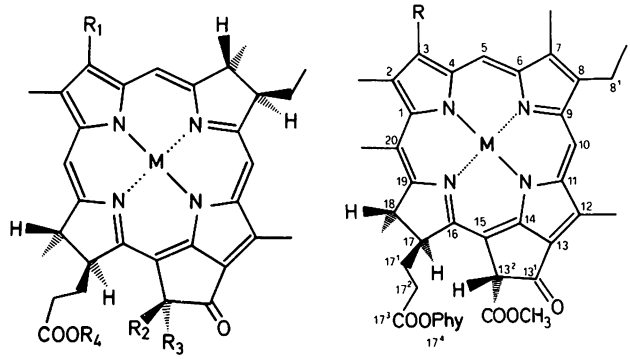
II. Methods

Successful exchange experiments rely on three combined methods: (1) synthesis and structure analysis of the pigments to be introduced, (2) optimum conditions for the exchange itself, and (3) a reliable, sensitive, and selective pigment analysis for optimization and verification. All three conditions must be met, but in every special case the emphasis may be on a different parameter(s) which shall, therefore, be discussed separately.

A. Pigment synthesis

A brief description of partial pigment synthesis, especially of selective modifications at positions C-3 and C-13^2, and some of the physical and spectroscopic properties of these pigments are described in Struck *et al.* (1992b). A selection of the modified pigments is presented in Figure 1.

The starting material for all syntheses was Bchla extracted from *Rb. sphaeroides* or Chla extracted from *Spirulina geitleri* (Scheer, 1988; Struck, 1991; Svec, 1991). Purification was done on DEAE-cellulose (Omata and Murata, 1983). Following the rather lengthy procedure of washing and equilibrating the material and increasing the methanol concentration in small increments is critical to preventing poor adsorption of the pigments to the material and, hence, poor separations. Light must be dim throughout, and the extraction and chromatography should be done without interruptions. The purified pigments are dried with a stream of Ar or N_2, then in a vacuum exsiccator, and stored under Ar in the deep freezer. The purity of the material should be checked by visible/near-infrared (Vis/NIR) absorption spectroscopy *and* chromatography, because some common alteration products have absorption spectra very similar to those of the respective parent pigments (see, for example, Scheer, 1988).



Pigment	Substituent			
	R_1	R_2	R_3	R_4
Bacteriochlorophylls (M = Mg)				
BChla	$COCH_3$	$COOCH_3$	H	Phytyl
BChla′	$COCH_3$	H	$COOCH_3$	Phytyl
(3-Vinyl) BChla	C_2H_3	$COOCH_3$	H	Phytyl
(3-Vinyl)-13²-hydroxy BChla	C_2H_3	$COOCH_3$	OH	Phytyl
(3-α-Hydroxyethyl) BChla	$CHOHCH_3$	$COOCH_3$	H	Phytyl
(3-α-Hydroxyethyl)-13²-hydroxy BChla	$CHOHCH_3$	$COOCH_3$	OH	Phytyl
13²-Hydroxy BChla	$COCH_3$	$COOCH_3$	OH	Phytyl
13²-Demethoxycarbonyl BChla	$COCH_3$	H	H	Phytyl
(17⁴-Methyl) BChla	$COCH_3$	$COOCH_3$	H	CH_3
13²-Hydroxy-(17⁴-methyl) BChla	$COCH_3$	$COOCH_3$	OH	CH_3
Bacteriopheoiphytins (M = H_2)				
BPhea	$COCH_3$	$COOCH_3$	H	Phytl
BPhea′	$COCH_3$	H	$COOCH_3$	Phytyl
(3-Vinyl) BPhea	C_2H_3	$COOCH_3$	H	Phytyl
(3-Vinyl)-13²-hydroxy BPhea	C_2H_3	$COOCH_3$	OH	Phytyl
(3-α-Hydroxyethyl) BPhea	$CHOHCH_3$	$COOCH_3$	H	Phytyl
(3-α-Hydroxyethyl)-13²-hydroxy BPhea	$CHOHCH_3$	$COOCH_3$	OH	Phytyl
13²-Hydroxy BPhea	$COCH_3$	$COOCH_3$	OH	Phytyl
13²-Demethoxycarbonyl BPhea	$COCH_3$	H	H	Phytyl
Zn-Bacteriopheophytins (M =Zn)				
Zn-BPhea	$COCH_3$	$COOCH_3$	H	Phytyl
Zn-13²-hydroxy BPhea	$COCH_3$	$COOCH_3$	OH	Phytyl
Zn-(3-Vinyl) BPhea	C_2H_3	$COOCH_3$	H	Phytyl
Zn-(3 Vinyl)-13²-hydroxy BPhea	C_2H_3	$COOCH_3$	OH	Phytyl
Chlorophylls (M = H_2)				
Chla	C_2H_3	$COOCH_3$	H	Phytyl
(3-Acetyl) Chla	$COCH_3$	$COOCH_3$	H	Phytyl
Pheophytins (M = H_2)				
Phea	C_2H_3	$COOCH_3$	H	Phytyl
(3-Acetyl) Phea	$COCH_3$	$COOCH_3$	H	Phytyl

FIGURE 1 BChla (left), Chla (right), and modified pigments. Stereochemistry at C-13² is generally undefined, except for BChla, BChla′, BPhea, and BPhea′.

1. [3-α-Hydroxyethyl] Bchla

Bchla was reduced to [3-α-hydroxyethyl] Bchla with $NaBH_4$ using a procedure modified after Ditson *et al.* (1984): 3-5 mg Bchla was dissolved in 100 ml ethanol and stirred under nitrogen at 4° C. After 10 min, 10 mg $NaBH_4$ was added and the reaction mixture stirred continuously. The reaction is followed by Vis/NIR absorption spectroscopy (blue shift of the Q_Y band from 770 nm to ≈728 nm). The reaction generally is completed after 30-60 min. The mixture is then separated between diethylether and water. The ether phase, which contains the pigments, is washed repeatedly with water and then dried over NaCl. The products are purified on a DEAE-cellulose column (see previous section). Nuclear magnetic resonance (NMR) indicates the presence of four stereoisomers by a characteristic splitting of, for example, the methine singlets in the range of 8-9.5 ppm and methyl singlets in the range of 3-4 ppm.

2. [3-Vinyl] Bchla

The pigment is synthesized from [3-α-hydroxyethyl] Bchla by elimination of H_2O in refluxing toluene. Purified [3-α-hydroxyethyl] Bchla (1 mg) is dried in vacuum over $CaCl_2$ for 12 hr and then dissolved in dry toluene (50 ml) stored over molecular sieves (3 Å). The mixture is refluxed under argon for 1-2 hr. The reaction usually is followed by absorption spectroscopy (red shift of the Q_Y band from 739 nm to 750 nm). After the reaction is completed, the solvent is removed by heating at 35° C in vacuum. The final product, which is already relatively pure, is purified on a DEAE-cellulose column. The 13^2 epimers are not separated. The high yield of the reaction is confirmed by high performance liquid chromatography (HPLC) analysis using a diode array absorption detector (see subsequent text). The silica gel system used (Watanabe *et al.*, 1985; Kobayashi *et al.*, 1988), allows the separation of the 13^2 diastereomers. No colored by-products are observable by HPLC analysis of the final product. Substituents at position 13^2 have a remarkable influence on the reactivity at C-3^1 and vice versa (Struck *et al.*, 1992b). In the context of pigment synthesis, it is important that, for example, the elimination of H_2O proceeds well with [3-α-hydroxyethyl] Bchla but not at all with [3-α-hydroxyethyl]-13^2-hydroxy Bchla or related modifications, which hinder enol(ate) formation at position C-13^2.

3. [13^2-Hydroxy] Bchls

Oxidations at C-13^2 are, in the historical literature, generally referred to as "allomerization;" the several products are often unwanted contaminants of (bacterio)chlorophylls (Schaber *et al.*, 1984; Hynninen, 1991; Svec, 1991). For hydroxylation at C-13^2, Bchla, [3-vinyl] Bchla or [3-α-hydroxyethyl] Bchla (5 mg) is dissolved in methanol (250 ml) and kept in the dark for 6 days at 4° C in the presence of air. Several products are separated on a DEAE-

cellulose column. One of the by-products is probably the 13^2-methoxy derivative (Markl, 1991). A subsequent repurification on RP-18 columns (Adsorbex, Merck) using methanol/water (≈95:5) as eluent, is sometimes necessary. The 13^2 epimers of [13^2-hydroxy] Bchla are not separated by this method, but this is possible by preparative thin-layer chromatography (TLC) on silica gel under Ar at 4° C (Markl, 1991). Because of the reactivity and large surface of the gel, the separated bands must be scraped off while the plates are still wet and eluted without delay.

4. [13^2-Demethoxycarbonyl] Bchla

This pigment often is referred to also as pyro-Bchla. Demethoxycarbonylation at C-13^2 was done as described elsewhere (Pennington *et al.*, 1964) in refluxing pyridine under Ar. The reaction was completed after 12 hr, and the reaction product was purified, if necessary, on DEAE-cellulose.

5. (Bacterio)pheophorbides

The (bacterio)pheophorbides can be obtained by demetalation of the respective (bacterio)chlorophylls. The reaction generally is carried out in diethylether under N_2 with 15% HCl at 4° C for 15 min. The final products are purified by preparative TLC on silica gel. Rosenbach-Belkin (1988) has described an alternative method in which the (bacterio)chlorophylls are dissolved in acetic acid, which is evaporated in vacuum after 15 min (Rosenbach-Belkin, 1988). In contrast to the (bacterio)chlorophylls, the (bacterio)pheophytins can be separated with less danger of degradation or alterations, by preparative liquid chromatography (LC) or TLC on silica (Scheer, 1988).

6. 13^2-Hydroxy-[17^4-Methyl] Bchla and other transesterifications

[13^2-Hydroxy] Bchla is treated for 5 min under Ar in methanolic NaOH (2%) and the mixture worked up. The method can be used for transesterification with other alcohols as well. Under these conditions, Bchla yields three products: [17^4-methyl] Bchla (often referred to as methyl-bacteriochlorophyllide a), 13^1a-oxa-[13^2-oxo]-[17^4-methyl] Bchla containing an enlarged oxygen-containing ring V, and the ring-opened bacteriochlorin e_7-trimethylester-Mg. The ratio of these products depends on the reaction conditions. The first product is obtained in good yields if the reaction time is kept to 2 min; the final two prevail at longer times (see Section II,A,8). The pigment containing the enlarged ring V also is obtained during transesterification of 13^2-hydroxy Bchla (Struck, 1991).

7. Chlorins

Chla is isolated from *S. geitleri* (Sosa Texcoco) by methanol extraction and chromatography on DEAE-cellulose (Omata and Murata, 1981). [3-Acetyl] Chla is prepared from Bchla according to Smith and Calvin (1966).

8. Pigments without isocyclic rings

Bchla is treated with methanolic NaOH according to Struck (1991) to yield bacteriochlorin e_7-trimethylester-Mg. A by-product of the reaction is 13^1-oxa-[13^2-oxo]-[17^4-methyl] Bchla. This product can be obtained in much better yield from [13^2-hydroxy] Bchla by the same treatment.

9. Pigments with central metals other than Mg

A variety of metals other than Mg can be inserted readily into Phes (=chlorins) (Hynninen, 1991). Few reports exist on the metalation of Bphes (=bacteriochlorins) (xxx). It has proved more difficult in our hands, and was also dependent on the substitution pattern. 13^2-hydroxy-Bphea reacted more smoothly than Bphea with Cu^{2+} and Zn^{2+}, but all complexes could be prepared in good yield (L. Fiedor, G. Hartwich, A. Scherz, and H. Scheer, unpublished results). This distinction may be related to the competition of the peripheral β-ketoester system with the central hole for the metal (Scheer and Katz, 1978). A more general method takes advantage of the transmetalation of complexes with large metals, which do not fit into the central hole. Starting with the Cd-complexes of Bphea and 13^2-hydroxy-Bphea, the respective Ni^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Pd, and Cu^{2+}-complexes were obtained (G. Hartwich, L. Fiedor, H. Scheer, and A. Scherz, unpublished results).

B. Pigment exchange

In this section, we will discuss in detail the exchange of pigments in RCs of *Rb. sphaeroides* and *Rps. viridis*. The influence of the central Mg to select the binding pocket is remarkable (see subsequent text), so we will distinguish between exchange experiments of modified Bchls with the native Bchl in the $B_{A,B}$ binding sites and exchange experiments of modified Bphes and Phes with the native Bphe in the $H_{A,B}$ binding sites.

1. Exchange of bacteriochlorophylls

Exchange experiments of Bchla, which target the $B_{A,B}$ binding sites (see subsequent text), were performed mainly with the carotenoidless mutant R26.1 of *Rb. sphaeroides*, as published elsewhere (Struck *et al.*, 1990a,b; 1992a; Struck, 1991). (For exchange experiments with RCs of other purple bacteria, see Section III,D.)

The modified pigments are dissolved in methanol; this solution is added to the RC in Tris-lauryldimethylamine-*N*-oxide (LDAO) buffer (20 m*M*, 0.08% LDAO, 10% MeOH). The concentrations are chosen to make the final reaction mixture 10 ± 1% in methanol and provide a 20-fold excess (in terms of Q_Y absorption units) of the pigment added. The exchange is carried out by incubating the RCs at 42.2° C for 90 min in this solvent mixture. This and all subsequently given temperatures may need to be optimized depending on the details of the RC preparation. The wild-type RC of *Rb. sphaeroides* also

exchanges, but the temperature must be increased to about 46° C and only an exchange of one Bchla per RC, most likely B_A, occurs (see subsequent text). Attempts to exchange both monomeric Bchlas by incubating at even higher temperatures resulted only in irreversible denaturation of the entire protein complex. For RCs of *Rps. viridis,* no exchange of $B_{A,B}$ occurs under comparable conditions (see subsequent text).

2. Exchange of bacteriopheophytins

Bphes and Phes are exchanged using a slightly modified procedure that adjusts for their poorer solubility in methanol. We use acetone or dissolve the pigments first in a minimum amount diethylether; then the concentrated solution is diluted with methanol. This mixture then is added to the RCs, followed by incubation at elevated temperature and finally purification, as in Bchl exchange.

3. Exchange mechanism

The "secret" to the exchange experiments is to heat the protein in Tris-LDAO buffer close to its "melting point" in the presence of an excess of "free" modified pigments. Under these conditions, the proteins (probably with bound detergent) start "breathing," thus providing external material with a more ready access to the protein interior, and equilibration seems to occur between external pigments and the original ones bound to sites $B_{A,B}$ and $H_{A,B}$. The preferred detergent is LDAO, but the RCs also can be solubilized in Triton X-100 buffer. The state of the free pigments under these conditions is not monomeric. (Bacterio)chlorophylls can aggregate in detergent solutions (Katz *et al.,* 1991; Scherz *et al.,* 1991). Pronounced red shifts are observed in the reaction mixture (M. Meyer and H. Scheer, unpublished observations). However, the type and extent of aggregation depends on many factors and has not yet been determined in detail for any of the pigments in the exchange mixture. Principally, other treatments with denaturing agents such as urea, guanidinium chloride, or detergents could be expected to provide alternative routes to exchanges. However, our experiments in this direction proved unsuccessful.

From the exchange results with wild-type strains of *Rb. sphaeroides* and *Rs. rubrum,* the environment of B_B appears to be stabilized by the presence of the carotenoids (see subsequent text). These pigments simply may block access to the B_B site. Alternatively, the carotenoid may have a "stiffening effect" on its protein environment, which prevents the necessary "breathing" of the binding site(s) region. This idea can be extended to RCs of *Rps. viridis* (see subsequent text), in which bound cytochrome *c* is likely to prevent "breathing". Also, in the native RC of this organism, exchange proved difficult, possibly because the cytochrome *c* on one side and the H subunit on

the other "clamp" the L and M subunits together. Exchange of Bphe occurs more readily in H-less RCs since the $H_{A,B}$ binding sites are more remote from the cytochrome *c* than the (nonexchangeable) Bchlb at the $B_{A,B}$ sites (Müller, 1993).

C. Analysis

Essential tools for verifying exchange experiments are selective, sensitive, reliable, and quantitative analytical systems. They involve chemical analysis including pigment extraction and quantitation, protein analysis, and spectroscopic techniques that can differentiate among the different binding sites for a certain pigment. Unambiguous evidence is necessary to establish whether, to what extent, and where an exchange was successful. Sensitivity is important for screening studies because of the need to analyze small amounts of modified RCs. Reliability means that no side reactions or irreproducible losses occur during the analytical procedures and no ambiguities occur in the spectroscopic assignments.

1. Pigment extraction and chromatography

Qualitative and quantitative analysis with the required selectivity, sensitivity, and reliability is possible by methanolic extraction of RCs absorbed on DEAE-cellulose, and subsequent HPLC analysis of the extract with multiwavelength absorption detection without delay (Struck, 1991). The extraction of pigments from RCs for HPLC analysis is done on small DEAE-cellulose columns (5 × 20 mm). RCs ($\leq$2 OD_{800}·ml) in Tris-HCl buffer (10 m*M*, pH 7.6) containing 0.08% LDAO are adsorbed, then washed extensively with distilled water. After removing most of the water by flushing the column with Ar, the pigments are extracted with methanol. Bphes elute after the Bchls and may be lost if the extraction is not done exhaustively. A check with pure unmodified RC is recommended. The methanolic solution is dried immediately under a stream of N_2 and the pigments are redissolved in toluene for HPLC analysis. The entire procedure is performed under safety light and completed within 30 min.

The subsequent HPLC analysis generally gives best results with silica as the adsorbent (Watanabe *et al.*, 1985; Kobayashi *et al.*, 1988). Reverse-phase HPLC is required, however, if pigments differing only slightly in their esterifying alcohols must be separated, for example, $Bchla_P$ and $Bchla_{GG}$ (see Scheer, 1988). The detector for the system is a diode array photometer HP 8451A or 8452A. It is controlled by self-designed programs (available on request) that allow simultaneous detection at selected wavelengths or recording, in-stream, of complete spectra at rapid intervals (>2 sec). With this detection method, pigments can be discriminated in a fast and reliable manner. Under these extraction and analytical conditions, even the highly sensitive

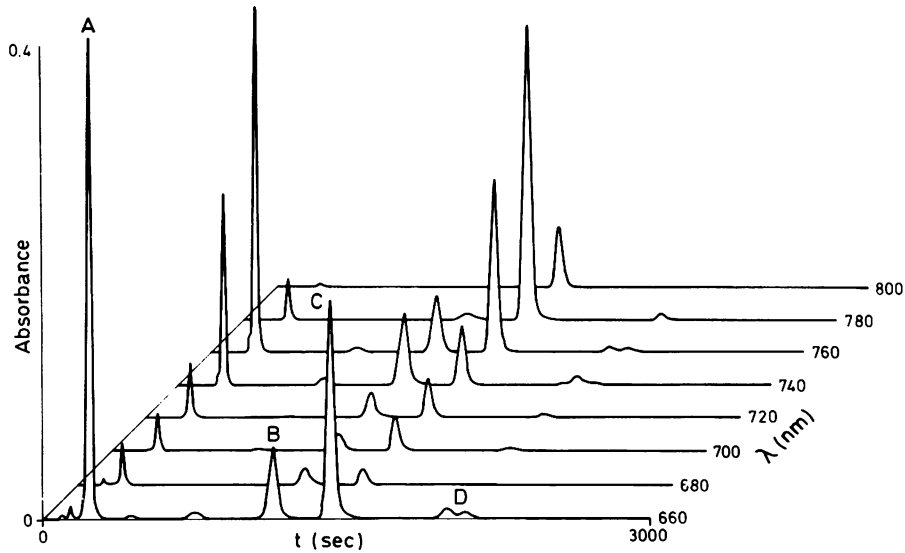


FIGURE 2 HPLC analysis of an extract from reaction centers of *Rb. sphaeroides* R26, in which BChla at sites $B_{A,B}$ has been replaced by (3-vinyl)-13.^{2}-hydroxy BChla. Column: SiO_2, 5 μm; mobile phase: toluene/methanol/2-propanol (A = 100:0.2:0.2, B = 100:1.6:0.2); hyperbolic gradient (Waters No. 7, 22 min) from 100% A to 40% A/60% B; flow, 1.5 ml/min. Peak assignments: A = BPhea; B = (3-vinyl) BChla *plus* BChla'; C = BChla; D = (3-vinyl)-13.^{2}-hydroxy BChla *plus* 13.^{2}-hydroxy BChla. The presence of two components in peaks B and D is, in the 660-nm trace, only indicated by a broadening and a shoulder, respectively. The peaks are resolved clearly in the traces >700 nm, at which the 3-vinyl and 3-acetyl substituted pigments have different absorption maxima (see Fig. 3).

Bchlb extracted from *Rps. viridis* can be analyzed, with only minor amounts of side products ($\lambda_{max} \approx 680$ nm; see Steiner *et al.*, 1983) formed. An example of HPLC analysis with multiwavelength detection is given in Fig. 2.

Early attempts to quantify the exchange were made by using ^{14}C-labeled RCs or Bchla. The error with this method was much larger, probably because of unspecific labeling after feeding with [^{14}C] succinate or insufficient purification of the final products (Beese, 1989).

2. Protein analysis

Protein analysis can be done by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Since the absorption at 870 nm has remained unaffected in all exchange experiments at this point (see subsequent text), the ratio of the 280/870-nm absorptions is also a good method if no other components, for example, the detergent, absorb at 280 nm. The ratio of the 280/800-nm absorptions (see, e.g., Feher and Okamura, 1978) is not suitable for modified pigments at sites B_A or B_B, because they often have different extinc-

tion coefficients. The absorption at 800 nm can also be affected by modified pigments at sites $H_{A,B}$. The necessity for protein analyses must be stressed, because Bchls tend to form a variety of aggregates in aqueous detergent solutions. Depending on detergent type, concentration, and so forth, these complexes exhibit extraordinarily wide variations in their spectroscopic (absorption, circular dichroism) properties that can mimic those of almost any Bchl protein (see Katz *et al.*, 1991; Scherz *et al.*, 1991, for reviews).

3. Spectroscopic methods

Since the exchange involves incubation with an excess of extraneous pigments, it is important to establish that no unspecifically bound pigments remain in the RCs after purification. Absorption spectroscopy can be used only in cases in which the unspecifically bound pigment absorbs at distinct and otherwise unobstructed wavelengths. Fluorescence is a much more sensitive method, particularly at low temperatures, because the adsorbed pigments have a high quantum yield of fluorescence compared to the RC (Beese, 1989). Obviously, this method fails with nonfluorescent pigments such as many transition metal complexes.

A variety of methods has given independent proof that the two Bchlas of the primary donor are neither exchanged nor modified during the exchange of the other four pigments (see Section IV). Absorption, electron-nuclear double-resonance (ENDOR) (H. Käs, J. Rauther, W. Zweygart, A. Struck, H. Scheer, and W. Lubitz, unpublished observations); absorption-detected magnetic resonance (ADMR) (W. Greis, A. Struck, H. Scheer, and A. Angerhofer, unpublished observations); linear dichroism (J. Breton, A. Struck, and H. Scheer, unpublished observations), and vibrational spectroscopy (W. Mäntele, A. Struck, and H. Scheer, unpublished observations) give no indication of changes. Using uniformly deuterated Bchla, a self-exchange experiment resulted in no electron paramagnetic resonance (EPR) line narrowing (Beese, 1989). The EPR method must be met with caution, however, because Loach *et al.* (1975) observed an artefactual line narrowing in AUT particles incubated with (and measured in the presence of) an excess of [^{2}H] Bchla.

Static spectroscopy also can be used sometimes to distinguish between equivalent sites. With this method, it is not possible to discriminate sites B_A and B_B by absorption spectroscopy, because Bchls bound to these positions absorb very similarly. Due to triplet interaction, a distinction seems possible, however, after reconstitution with carotenoids and triplet spectroscopy of the latter (G. Hartwich, V. Aust, A. Angerhofer, H. Scheer, unpublished results). The Bphes at H_A and H_B can be distinguished by their Q_X absorptions around 535 nm; the former absorbs at longer wavelengths (Clayton and Yamamoto, 1976; Vermeglio *et al.*, 1978; Breton, 1985; Kirmaier *et al.*, 1985; Tiede *et al.*, 1985). The two bands overlap at room temperature but can be deconvoluted; at low temperatures they are well resolved.

4. Functional analysis

The exchange of pigments is no guarantee that the RCs are functional, that is, that they exhibit light-driven electron transfer. If the exchange is not complete, analytical methods are required that allow quantification of the amount of pigment exchanged in the different binding sites. These requirements can be met only by spectroscopic methods that work on the intact RC.

Light-induced difference spectroscopy can be used in both cases as a valuable tool. In the simplest version of this method, the sample is irradiated with a strong actinic light source. Under these conditions, $P^{+\cdot}Q_B^{-\cdot}$ accumulates as the longest living species in the futile electron transport cycle. This state can be monitored readily by the absorption decrease of the Q_Y band of the primary donor ($\lambda_{max} \sim 870$ and 960 nm for RCs containing Bchla and b, respectively) or by the concomitant increase of the band of $Bchl_2^{+\cdot}$ in the 1250-1350 nm region. If the kinetics of the late electron transfer reactions are unaffected and the irradiation is saturating, the change is related quantitatively to the concentration of RCs with functional pigments.

Much more information can be gained from time-resolved spectroscopy. With the necessary resolution (<1 ps) and the wavelength range extending into the near infrared, this technique requires highly sensitive and correspondingly sophisticated equipment that is currently only accessible in few specialized laboratories. From the amplitude of the light-induced bleaching at 870 nm (*Rb. sphaeroides*), the yield of the primary charge separation can be obtained. In the case of changed spectral properties of the pigments introduced, an incomplete exchange can be detected and quantified; sometimes the site can be determined (see subsequent text).

III. Selectivity of the exchange

A. Influence of central metals

In all pigments studied, the influence of the central Mg has been very distinct. Irrespective of the substituents at the periphery, whenever an exchange has been possible, the presence of Mg in the Bchls directed them into the $B_{A,B}$ binding sites; its absence in the [B]Phes directed them into the $H_{A,B}$ binding sites. This effect can be compared with the results of mutagenic studies in *Rb. capsulatus* and *Rb. spheroides* that established the selecting influence of amino acid residues close to the central N-4 cavity of the tetrapyrrole macrocycle. When this residue is histidine, Bchla is bound to that site; when the residue is hydrophobic, for example, isoleucine, the metal-free Bphea is present in the isolated RCs (Table I). The only site at which no such exchange has been possible to date is B_A. In native RCs, this amino acid residue close to the center of the macrocycle is the most probable directing influence to bind

Bchl at the sites $P_{A,B}$ and $B_{A,B}$ and Bphe at $H_{A,B}$. This behavior also explains the presence of three Bchls and three Bphes in RCs from *Cfl. aurantiacus,* in which histidine M180 is replaced by isoleucine. The Mg-histidine interaction then appears to be a dominant factor in the pigment-protein interaction, which also is obvious for most Bchls from the X-ray structures (see Deisenhofer *et al.,* 1984; Allen *et al.,* 1986,1987; Chang *et al.,* 1986,1991b; Michel, 1992; Michel and Deisenhofer, 1988; Deisenhofer and Michel, 1989; El-Kabbani *et al.,* 1991).[3] The interaction is related to the coordinatively unsaturated magnesium, which was recognized long ago from studies of Bchl aggregation *in vitro* (Katz *et al.,* 1978). The only pigments with a different central metal that have been exchanged into RCs were the Zn complexes, which seem to prefer the $B_{A,B}$ sites (G. Hartwich and H. Scheer, unpublished observations).

A comparison with other Bchl proteins is useful. The only other crystal structure to date is solved for the FMO protein of *Chloropseudomonas ethylicum,* which is involved in energy transfer from the chlorosome to the RC (Olson, 1978). Here, five of the seven Bchls are ligated to histidines, one to a backbone peptide carbonyl, and one to water (Fenna *et al.,* 1977). In bacterial antenna polypeptides, a highly conserved histidine residue exists within the central hydrophobic stretch of all α and β chains (Zuber and Brunisholz, 1991). The latter is believed to form a membrane-spanning helix, and the histidine serves as the binding site for Bchl. Another Bchl probably is bound to a second histidine residue in the β polypeptides of the bacterial LHCII. The situation is less clear in other complexes in which often more Bchls are present than there are histidine residues. Examples are LHCIIs of oxygenic photosynthetic organisms (Zuber and Brunisholz, 1991; Thornber *et al.,* 1991) and, in particular, the chlorosomes of green bacteria (Smith *et al.,* 1983; Brune *et al.,* 1988; Griebenow and Holzwarth, 1989; Griebenow *et al.,* 1990; Olson and Pederson, 1990; Niedermeyer and Feick, 1991; Redlinger *et al.,* 1991; Wullink *et al.,* 1991; Niedermeyer *et al.,* 1992). Two possibilities are discussed currently: (1) other polar amino acid residues, such as lysine, glutamine, and asparagine, serve as ligands to the central Mg and by this means bind the pigments or (2) some of the pigments are bound as oligomers rather than as monomers, with a variety of possible mechanisms for the pigment-pigment interactions (Katz *et al.,* 1991; Scherz *et al.,* 1991). First detailed data on the high-resolution electron-diffraction structure of plant LHCII have recently added glutamate to binding amino acids (Kühlbrandt, 1992).

Some results regarding the selective influence of amino acid residues of intermediate ligating power are given in Table I and in Section I. No good data are available at present on the binding of pigments with central metals other than magnesium. We have been able to exchange several Zn bacterio-

[3]The notion of an unusually large Mg-histidine distance in *Rb. sphaeroides* RCs (Allen *et al.,* 1987; Chang *et al.,* 1991) has recently been corrected in favor of a situation similar to *Rps. viridis* by two laboratories (Chang *et al.,* 1991b; El-Kabbani *et al.,* 1991; Michel, 1992).

pheophorbides into RCs of *Rb. sphaeroides,* but these await a detailed characterization (G. Hartwich and H. Scheer, unpublished observations). To our knowledge, no other metal complexes have been studied with this protein. Paulsen *et al.* (1990,1991) have performed reconstitution experiments with LHCII-related polypeptides from plants that indicate that pigments with central metals other than Mg can replace at least part of the 14 - 15 Chl a/b complement. H. Paulsen *et al.* (personal communication, 1991) have been able to incorporate several chlorophylls containing metals other than Mg into the LHCII precursor of green plants. No detailed data are available on these preparations to date.

The high selectivity of the $B_{A,B}$ pockets for Mg complexes, that is Bchl, and of the $H_{A,B}$ pockets for free bases, that is, Bphe, may be pertinent to the assembly of RCs. At least two mechanisms are possible: (1) binding of Bchl into all sites and a subsequent selective demetalation or (2) selective binding from pools of both pigments. The available evidence seems to indicate the second possibility. This option was indicated by the finding of Walter *et al.* (1979) that different esterifying alcohols are present in Bphea and Bchla of *Rs. rubrum* RCs, in conjunction with the finding that these RCs did not discriminate strongly between pigments bearing phytol or geranylgeraniol (Scheer *et al.,* 1987; Beese, 1989; Struck, 1991). The mutagenesis experiments (Table I) suggest the same mechanism if the exchange of the amino acid next to the central N-4 cavity at any one of the positions is not assumed to bestow these sites indiscriminately with dechelatase activities. Note, however, that the Mg complexes of cyclic tetrapyrroles are labile. Dechelatase activities have been reported (Ziegler *et al.,* 1988; Owens and Falkowsky, 1982), but the enzymes have not been purified, and no information on their active sites is available. The exchange experiments can be explained in this way also.

Since Bchl and Bphe have very similar shapes and the central N-4 cavities of both pigments are fairly polar, this selective binding poses the interesting question of how the sites select for (B)Phes versus BChls. One possibility is the strong driving force of Mg to avoid a coordination number of 4 (Katz *et al.,* 1978). In the absence of suitable ligands (e.g., leucine at the correct location), this avoidance would require for Bchl but not Bphe the introduction of, for example, water as fifth ligand into the binding site, which may be sterically or energetically unfavorable. Additional exchange experiments, for example, with pigments bearing other metals, are therefore desirable.

B. Peripheral substituents in bacteriochlorophylls

The influence of substituents of the modified Bchls on their exchange capacity can give new insight into the interaction between amino acids of the protein with the pigments. However, without a more detailed knowledge of

the exchange process, exchangeability is a purely operational criterion involving the solubility and aggregation state of the pigment in micellar solution, competition between different binding sites, stability of the pigment, and kinetic and/or thermodynamic controls that can operate at each stage. Under these conditions, only positive exchanges can yield information on pigment-protein interactions, whereas negative results (for example, those with Chla derivatives) may originate for a variety of reasons that cannot be distinguished.

A previous overview of all modifications tested is given by Struck *et al.* (1990a). Most work has focused on substituents at C-3, C-13^2, and C-17^4. Pigments modified at these positions, alone or in combination, exchange into both the B_A and B_B pockets. Isotopically labeled Bchla (^{14}C, ^2H) has been introduced by this procedure as well.

1. C-3 substituents

At C-3, the substituents acetyl (native Bchla), α-hydroxyethyl, and vinyl have been tested, alone or in combination with 13^2-OH. The first substituent is an H-bond acceptor only, the second a potential acceptor or donor, the last none of these. Pigments with a 3-vinyl substituent are also important in distinguishing between the binding properties of Chla, which contains this group, and Bchla, which contains an acetyl group instead. Exchange of 50% corresponding to 100% of $B_{A,B}$ is difficult (but possible) with [3-vinyl] Bchla. On the other hand, a partial exchange is achieved readily, which can be interpreted as a differential exchange at the two sites. This result agrees with kinetic studies, which indicate a more ready exchange in the B_B site. This is also supported by the complete exchange with [3-vinyl]-13^2-hydroxy Bchla into the $B_{A,B}$ sites. The formation of stable and functional reaction centers with [3-α-hydroxyethyl] Bchla at sites B_A and B_B is of particular interest with regard to the reaction of RCs with borohydride (Struck *et al.*, 1992).

2. C-13^2 substituent

[13^2-Hydroxy] Bchla and several other pigments bearing a hydroxy substituent at C-13^2 are exchangeable; this substituent even appears to increase exchangeability, perhaps because of selective hydrophilic interaction within the binding sites. Inspection of the $B_{A,B}$ environment in the crystal structures of *Rps. viridis* (Deisenhofer *et al.*, 1984; Deisenhofer and Michel, 1989) and *Rb. sphaeroides* RCs (Chang *et al.*, 1991; El-Kabbani *et al.*, 1991) indicates not only that there is enough space at the expected sites to accommodate a hydroxy substituent instead, but also that H bonding is possible between the peptide carbonyl of glycine M201 and 13^2-OH Bchla at the B_B site and be-

tween serine L178 and 13^2-OH Bchla at the B_A site. Hydroxylation at C-13^2 also occurs (with epimerization) in RCs when they are incubated under the exchange conditions without extraneous pigments. Since isolated RCs containing [13^2-hydroxy] Bchla are functionally and kinetically indistinguishable from native ones (see subsequent text), it is surprising that this pigment, which *in vitro* is formed easily from the parent Bchla, has not yet been found in photosynthetic complexes.[4] If identified in photosynthetic organisms, it was believed to be a degradation product (Haidl *et al.*, 1985; Schmidt, 1985; see also Brown *et al.*, 1991, for leading references).

3. Other modifications of the isocyclic ring

None of the pigments with modifications other than 13^2-OH exchange into RCs. It is possible neither with [13^2-demethoxycarbonyl] Bchla, which lacks the $COOCH_3$ substituent nor with 13^1-oxa-[13^2-oxo]-Bchlide a-methylester, in which the ring is enlarged. An exchange is impossible, too, with bacteriochlorin e_7-trimethylester, in which the isocyclic ring is opened.

4. Esterifying alcohol (C-17^4 substituent)

Only few pigments bearing alcohols other than phytol have been tested to date, but the experiments indicate a low specificity of the binding sites. $Bchla_P$ (bearing a phytyl substituent at this position) can be replaced by $Bchla_{GG}$, the geranylgeraniol-ester present in *Rs. rubrum* (among others). The exchange of $Bchla_P$ into RCs of *Rs. rubrum* appeared to be more difficult, but these RCs are also less stable. Some short-chain aliphatic alcohols have been tested and found to be exchangeable. In spite of the relatively good definition of most parts of the phytyl chains in the crystal structures of RCs (Deisenhofer *et al.*, 1984; Allen *et al.*, 1987; Michel and Deisenhofer, 1988; Deisenhofer and Michel, 1989; Chang *et al.*, 1991; El-Kabbani *et al.*, 1991) as well as of its considerable size and its presence in most chlorophylls, it still seems difficult to assign a functional significance to them in the assembled RCs.

5. Macrocylic conjugation

The bacteriochlorin-conjugation system appears to be essential for an exchange into the $B_{A,B}$ sites, since neither Chla nor [3-acetyl] Chla is exchangeable. The latter differs from Bchla only by the removal of the two extra hydrogens at C-7 and C-8. Inspection of the crystal structure (Chang *et al.*, 1991)

[4]Another hydroxylated pigment, [8^1-hydroxy] Chla, has been identified in RC preparations from *Chlorobium limicola* and suggested to be an acceptor in electron transfer (van de Meent *et al.*, 1991).

does not indicate an obvious reason for the failure to incorporate chlorins instead of bacteriochlorins. If this result reflects a true thermodynamic effect and is not due to a problem of the exchange procedure, this selectivity would be surprising at first glance. A possible explanation is that the flexibility of the macrocycle is important for binding. Bacteriochlorophylls are known to be more flexible than chlorophylls (Barkigia *et al.*, 1989; Forman *et al.*, 1989; Fajer *et al.*, 1990). Unfortunately, current X-ray resolution is not good enough to obtain reliable information on the details of such distortions.

C. Peripheral substituents in bacteriopheophytins

Much less is known currently about the exchange of Bphea. Whenever an exchange is possible, the pigment is directed toward the Bphea-binding sites, that is, $H_{A,B}$ (see previous text). In contrast to the exchange of Bchl derivatives, Bphe derivatives also exchange in RCs of *Rps. viridis*. There appears to be no difference, whether the carotenoid is present (*Rb. sphaeroides* 2.4.1 wild-type) or absent (mutant R26.1).

Both pigments modified at C-3, that is, [3^1-OH] Bphea and [3-vinyl] Bphea, do exchange. Position 13^2 seems to exert a remarkable influence on the site specifity. 13^2-OH and 13^2-demethoxycarbonyl Bphes only exchange with one Bphe, which is likely to be the one in H_B because the exchange at this position is distinctly more rapid (compared with H_A) with pigments not modified at C-13^2. The crystal structures (Allen *et al.*, 1987; Michel and Deisenhofer, 1988; Chang *et al.*, 1991; El-Kabbani *et al.*, 1991) and vibrational spectra (Lutz and Mäntele, 1991) of *Rps. viridis* and *Rb. sphaeroides* RCs indicate a selective interaction of Glu L104 with the 13^1-carbonyl group of the bound pigment. This interaction is responsible for the red shift of the Q_X band of Bphe at H_A, but *not* for the binding and function of this pigment (see Coleman and Youvan, 1990). This interaction may, however, be responsible for the fact that pigments bearing a 13^2-OH substituent do not exchange. This substituent inhibits the ready enolization of the β-ketoester system at ring V. The lack of exchange thus perhaps relates to the role keto-enol tautomerism plays in (bacterial) RCs. Enolization of Bphea at H_A has been suggested from Raman resonance data (Bocian *et al.*, 1987). ENDOR spectra of $Bphe^-$ indicate strong H-bonding between the 13^1-keto group and the neighboring glutamate (see Lubitz, 1991), a state that is probably not easily distinguishable from (partial) enolization (Hanson *et al.*, 1988). In view of the occurrence of the enolizable β-ketoester system in most (B)chls and in all those present in RCs, an investigation of pigments in which enolization is modified or prevented seem useful.

In contrast to the situation with the $B_{A,B}$ sites, both $H_{A,B}$ sites accept chlorin derivatives, for example, pheophytin a and [3-acetyl] pheophytin a (Scheer *et al.*, 1992).

TABLE II **Species Specificity of Pigment Exchange in Bacterial Reaction Centers**

Species	Pigments[a]		
	$P_{A,B}$	$B_{A,B}$	$H_{A,B}$
Rhodobacter sphaeroides R26	—	2	1 - 2[b]
Rhodobacter sphaeroides 2.4.1	—	1[c]	1 - 2[b,d]
Rhodospirillum rubrum G9	—	2	1 - 2[b,d]
Rhodospirillum rubrum S1	—	1[c]	1 - 2[b,d]
Rhodopseudomonas viridis[e]	—	—	1 - 2[b,e]

[a] The nearest integer number of exchanged pigments is given.
[b] Pigments bearing a 13^2-hydroxy substituent exchange only at site H_B.
[c] Probably B_A, see text.
[d] Not investigated in detail; by inference only from *Rb. sphaeroides*.
[e] Reaction centers lacking the H-subunit give best results.

D. Species specificity

Extensive exchanges have been performed mainly with the carotenoidless strain R26 of *Rb. sphaeroides*[5] and with *Rps. viridis*. Other species tested are listed in Table II with their exchange results. Since the exchange conditions, which have been optimized for *Rb. sphaeroides* R26, are rather harsh on many of the more labile RCs, for example, those from *Rb. capsulatus*, the methods probably must be modified for tests of the latter species.

From the few species tested, three conclusions appear to be relevant for the exchange. First, in the two wild-type strains of Bchla-containing species, exchange of only one Bchla occurred, compared with two for the carotenoidless mutants. We assume that the carotenoid located close to B_B (Arnoux *et al.*, 1989; Michel and Deisenhofer, 1988; Allen *et al.*, 1989) shields or stiffens this site and thereby inhibits exchange at the neighboring B_B site. Second, in *Rps. viridis* RCs (from which the H subunit is dissociated), none of the four Bchlb is exchangeable with Bchla. Here, the "inhibitor" is suggested to be the tightly bound cytochrome *c*, which "clamps" the L and M subunits together and inhibits the "breathing" necessary for the exchange (see Section II,B,3). Note, however, that the native Bchlb of *Rps. viridis* carries an 8-ethylidene group. Attempts to exchange pigments carrying this group (for example [3-α-hydroxyethyl] Bchlb) failed because of the lability of this sub-

[5] The parent strain R26 is known to be unstable. The laboratory culture used was similar to the strain R26.1 described by Davidson and Cogdell (1981) by its more rapid growth and its lack of fluorescence in culture.

stituent. No Bchla-containing RC with a similarly attached cytochrome has been studied yet. Finally, exchange at the Bphe sites $H_{A,B}$ is possible in *Rps. viridis* RCs, but yields are poor with the complete RC containing the H subunit. The efficiency of exchange becomes similar to that in *Rb. sphaeroides* with *Rp. viridis* RCs lacking this subunit.

IV. Static spectroscopy of reaction centers with modified pigments

A. Structural integrity of modified reaction centers

The absence of crystal structure data on RCs with modified chromophores causes us to rely currently on spectroscopic methods. Several complications in the analysis are possible, alone or in combination: (1) changed quaternary structure, (2) changed tertiary structure, and (3) incorrect orientation of the exchanged pigments. Exchange with the authentic pigments (self-exchange) serves as a control as does, to some extent, exchange with 13^2-hydroxy Bchla. In both cases, the subunit composition is unchanged. From the similar absorption, CD,[6] light-induced bleaching, linear dichroism (J. Breton, A. Struck, and H. Scheer, unpublished observations), ENDOR (H. Käs, J. Rauther, W. Zweygart, A. Struck, H. Scheer, and W. Lubitz, unpublished observations), vibrational (W. Mäntele, A. Struck, and H. Scheer, unpublished observations), and triplet spectra (W. Greis, A. Struck, H. Scheer, and A. Angerhofer, unpublished observations), it is likely that no major change in the tertiary structure and no false orientation has occurred. Two points are noteworthy regarding the self-exchange experiments: the resulting purified reaction centers contained a decreased amount of Bchla and instead contained varying amounts of Bchla′, its 13^2-epimer, and [13^2-hydroxy] Bchla. From the selectivity data, these modified pigments are most likely located at sites $B_{A,B}$. Obviously, none of them seems to change the structure of the RC significantly, which is evidenced for the latter compound by the possibility of a full exchange without strongly affecting any of the spectroscopic (or kinetic) data.[6] No similar experiments have been possible for Bchla′, which epimerizes under the exchange conditions.

The protein subunit composition is unaltered in all exchange experiments. A false insertion into the binding sites is unlikely, in light of the pronounced asymmetry of the Bchls (see Wright and Boxer, 1981; Boxer *et al.*, 1982; Moog *et al.*, 1984; for studies on chlorophyll-containing heme-apoproteins

[6]The small shift of the Q_X band of RCs containing [13^2-hydroxy] Bchla is discussed in Section IV,B.

called "chloroglobins"). Other structural changes are, of course, possible and must be clarified.

B. Environmental effects on pigments at sites $B_{A,B}$

Most chemical modifications of Bchls are accompanied by distinct variations in their absorption spectra in solution (see Scheer, 1988) that are retained after incorporation into RCs. Reactions of the conjugated 3-acetyl substituent in Bchla lead to pronounced hypochromic shifts and reduction of the extinction coefficients in monomeric solution (Fig. 3). The shifts are similar to the ones in the Chla series. More unexpectedly, even the substituent changes at C-13^2 produce characteristic, albeit much smaller, effects that are most distinct in the Q_X band (Struck, 1991). Two examples are given in Fig. 4. The small red shift on demethoxycarbonylation of C-13^2 ("pyrolysis") and the small blue shift on hydroxylation have been observed in a variety of Bchl and Bphe derivatives with other peripheral substituents also (Struck, 1991). The circular dichroism of all free pigments in monomeric solution is small. In the Phe series, it has been related to a combined effect of some twist of the macrocycle, induced by the asymmetric C-17, and the perturbations by the other asymmetric C atoms (Wolf and Scheer, 1972).

In native reaction centers, all Bchl absorptions are shifted to the red compared with monomeric solutions of the pigment, for example, in diethylether. In RCs of *Rb. sphaeroides,* this environment-induced red shift (EIRS) amounts

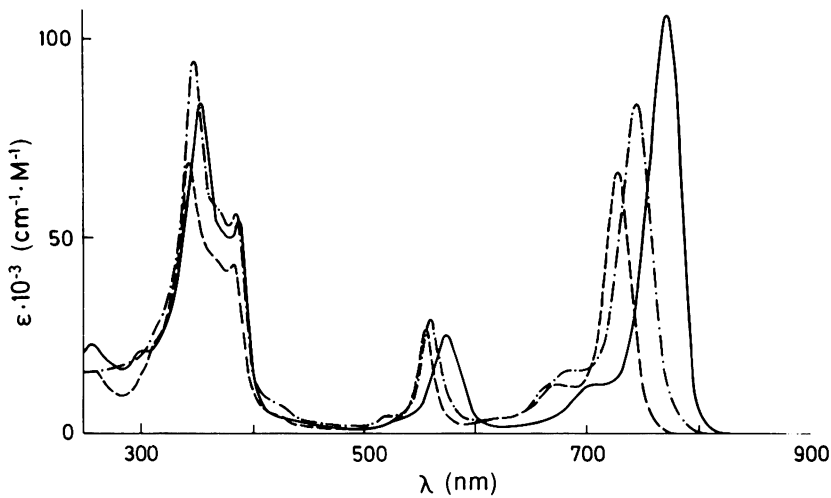


FIGURE 3 Absorption spectra in diethylether of BChls modified at C-3. BChla (—), [3-vinyl] BChla (- · - · -), [3-α-hydroxyethyl] BChla (----).

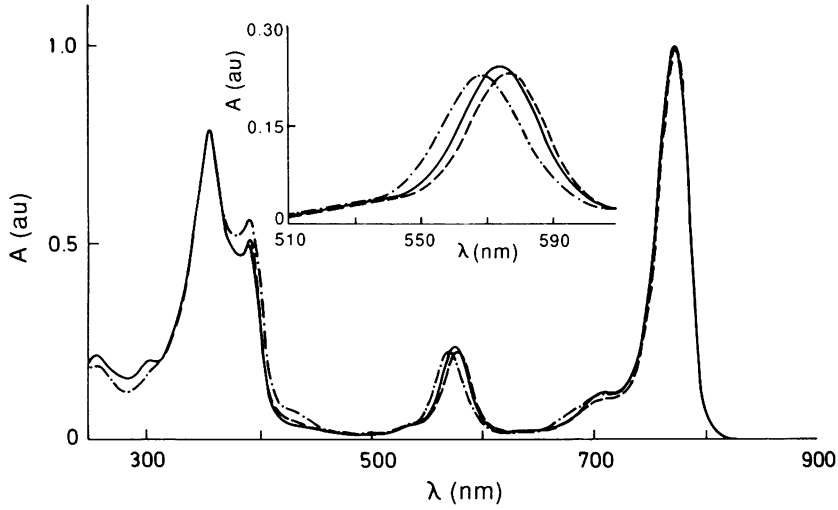


FIGURE 4 Absorption spectra in diethylether of BChls modified at C-13². BChla (—), 13²-hydroxy BChla (-·—·-), 13²-demethoxycarbonyl BChla (----). *Inset:* The Q_X region expanded. Similar small but distinct variations in this region are observed for otherwise modified series of BChl derivatives differing only in their 13² substitution.

to 501 cm^{-1} (Q_Y) and 729 cm^{-1} (Q_X)[7] for Bchla at sites $B_{A,B}$. The EIRS of the Q_Y band(s) in the monomeric pigments is surprisingly large compared with the much smaller solvent-induced shifts ($\leq$220 cm^{-1}) observed by Connolly *et al.* (1982) in a variety of solvents, suggesting a nonisotropic environmental effect of the surrounding polypeptides or the neighboring pigments. The positions of the Q_X band are known to be more strongly solvent dependent and have been related to a change in the Mg coordination number (Evans and Katz, 1975; but see Belanger and Rebeiz, 1984). Also, a strong induced circular dichroism of the monomeric Bchl(s) is seen that is more than an order of magnitude larger than that for Bchl in monomeric solution. In the Q_Y region, the RCs show a strong negative extreme centered to the red and a positive one centered to the blue of the absorption maximum.

In RCs containing modified pigments at $B_{A,B}$, the observed EIRS is very similar to that of Bchla in native RCs (Table III). For the modifications at C-3 tested (with and without a concomitant change at C-13²), the induced red shift of the Q_Y band varies between 469 and 554 cm^{-1}. Similar EIRS are observed for pigments with modifications at C-13² or C-17⁴ also, which give only minor absorption changes in isotropic solution. The EIRS of the Q_X bands shows much stronger variations (Fig. 5; Table III), reflecting the larger sensi-

[7]The EIRS of the primary donor is even larger; here it is mainly caused by pigment-pigment interactions in the special pair.

TABLE III **Environment-Induced Red Shifts of Monomeric Bacteriochlorophylls at Sites $B_{A,B}$**

Pigment	Energy[a] ΔQ_Y (cm^{-1})	ΔQ_X (cm^{-1})
BChla (3-acetyl)	−501	−729
[3-α-hydroxyethyl] BChla	−526	−381
[3-vinyl] BChla	−469	−733
13^2-hydroxy BChla (3-acetyl)	−501	−742
[3-α-hydroxyethyl]-13^2-hydroxy BChla	−543	−514
[3-vinyl]-13^2-hydroxy BChla	−554	−596

[a] ΔQ_Y and ΔQ_X are defined as the energy of the pigment in the reaction center environment minus the energy in diethylether solution and refer to the Q_Y and Q_X bands, respectively.

tivity of this band. However, even here the substituent-induced shifts do not appear to be washed out by the RC environment. As an example, the small blue shift on hydroxylation at C-13^2 is clearly discernible in the RC bearing this pigment at $B_{A,B}$ (Struck and Scheer, 1990).

Obviously, none of the chromophore substituents hitherto modified seems to be involved strongly in the EIRS. Possible origins for this and the strong CD

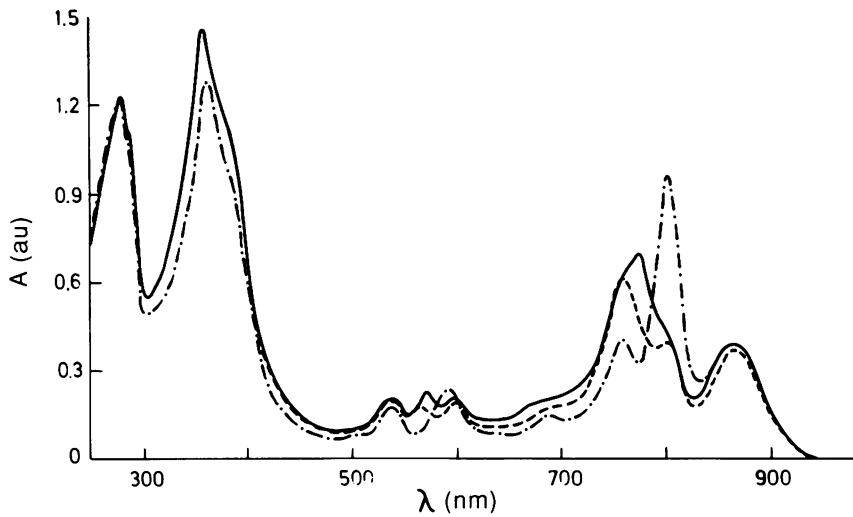


FIGURE 5 Absorption spectra of reaction centers of *Rb. sphaeroides* R26 in which the monomeric BChla at sites $B_{A,B}$ has been replaced by BChls modified at C-3. BChla (- · - · -), [3-vinyl] BChla (—), [3-α-hydroxyethyl] BChla (----).

activity (the positive and some of the negative bands are shifted with the absorptions) are discussed in the next section.

C. Interactions of pigments at sites $B_{A,B}$ with the primary donor (P)

The EIRS of chlorophylls has been subject to numerous theoretical treatments (see Hanson, 1991; Katz *et al.*, 1991; Scherz *et al.*, 1991; for reviews). For the primary donor, the dominant contribution of pigment-pigment interactions within the special pair is generally accepted. How much pigment-pigment interactions also contribute to the EIRS of the monomeric Bchls and Bphes is much less clear. In view of the close distances among all pigments in the RC, this problem has received considerable attention (Scherer and Fischer, 1991). Modified pigments provide an experimental approach to this question.

1. Singlet state

None of the modifications of pigments in the $B_{A,B}$ sites that were tested produces a significant change in the long-wavelength band of the primary donor (Fig. 5). This band remains unchanged in its position, shape, and intensity. This result can be extended even to the CD spectra (Fig. 6). Since the latter are very sensitive to pigment interactions, we conclude that little such interaction takes place among the orbitals participating in the absorption, at least as far as substitutions at the modified positions are concerned.

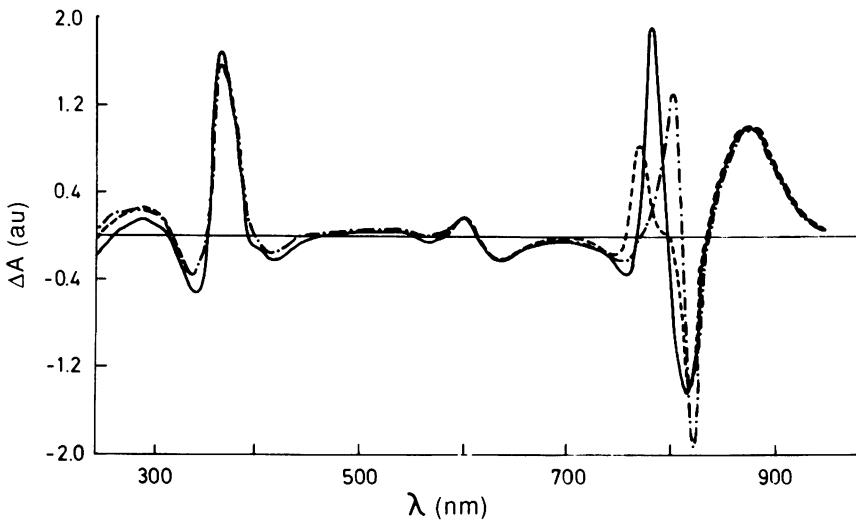


FIGURE 6 Circular dichroism spectra of reaction centers of *Rb. sphaeroides* R26 in which the monomeric BChla at sites $B_{A,B}$ has been replaced by BChls modified at C-3. BChla (-·-·-), [3-vinyl] BChla (—), [3-α-hydroxyethyl] BChla (----).

The Q_Y band of the primary donor commonly is interpreted as the lower excitonic band (P_-) of this dimer or special pair of Bchls. Its upper excitonic band (P_+) generally is buried under the Q_Y absorption of the monomeric Bchls, where it can be identified only with difficulty. Since the latter absorption is blue-shifted in RCs bearing pigments modified at C-3 at sites $B_{A,B}$, the upper excitonic band of the primary donor should become disentangled. Such a change is observed (Fig. 6); the negative lobe of the CD spectrum splits into a constant and a variable component. The former, located at about 812 nm, is assigned to the upper exciton band, in agreement with previous indirect assignments by Vermeglio *et al.* (1978). If our assignment is correct, the large negative and positive CD bands that shift with the absorptions must be derived from the monomeric (modified) pigments. This optical activity is more than one order of magnitude higher than in Bchls in monomeric solutions and indicates very specific interactions with the environment. In view of the negligible interactions with the primary donor, two explanations are currently considered to be most likely: (1) a pronounced twist of the partly flexible macrocyclic systems or (2) (excitonic) interactions with the neighboring Bphes or amino acid side chains (see subsequent text).

2. Doublet and triplet states

The cation radical doublet state $P^{\cdot+}$ of the primary donor is obtained after light-induced electron transfer and can be investigated selectively by electron spin resonance. The most sensitive method, ENDOR spectroscopy in solution (Lubitz, 1991), gives no indication of changes induced by pigment modifications in the $B_{A,B}$ site (H. Käs, J. Rautter, W. Zweygart, A. Struck, H. Scheer, and W. Lubitz, unpublished observations). Apparently, in the doublet state of the primary donor there is little interaction with the neighboring monomeric Bchls.

The triplet state has been investigated by optical-magnetic double-resonance methods. In microwave-induced absorption difference spectra (MIA, often also referred to as singlet-triplet difference; Lous and Hoff, 1986), the band located at 802 nm shifted with the Q_Y absorptions of [3-vinyl]-13^2-hydroxy Bchla to 776 nm and with that of [3-α-hydroxyethyl]-13^2-hydroxy Bchla. This result may be relevant for the triplet energy transfer from the primary donor to the carotenoid located close to B_B. This transfer is likely to involve the monomeric Bchla at B_B (Frank and Violette, 1989). It is then tempting to assign the shifting MIA signal to a contribution from the latter to the primary donor triplet.

In a more roundabout sequence, a RC preparation with [3-Vinyl]-13^2-hydroxy-Bchla at the B_B-site only, has recently been obtained (G. Hartwich and H. Scheer, unpublished results). First, both B-sites have been exchanged with [3-Vinyl]-13^2-hydroxy-Bchla in RC from *Rb. sphaeroides* R26, followed by a reconstitution with spheroiden(on)e, and finally by a second exchange with

Bchla into the B_A-site only. The triplet spectra of both the 15,15'Z-carotenoid and the primary donor contain contributions from the [3-Vinyl]-13²-hydroxy-Bchla. Since the latter is located between the former two, this is good evidence for a triplet energy transfer from P to the carotenoid *via* the intervening pigment at B_B (V. Aust, G. Hartwich, H. Scheer, and A. Angerhofer, unpublished results).

D. Interactions of pigments at sites $B_{A,B}$ and $H_{A,B}$

The influence of pigments at sites $B_{A,B}$ on the neighboring ones at sites $H_{A,B}$ is difficult to assess from current exchange experiments at the former sites. The Q_Y bands of most of the modified Bchls are blue-shifted and overlap strongly with those of Bphea. In the Q_X region, the bands are better resolved. Small changes are observed, for example, a minor red shift of the maximum of the broadened Q_X band of Bphea around 545 nm on exchange of [3-vinyl] 13²-hydroxy Bchla into $B_{A,B}$. The 3-acetyl substituent of Bphea at $H_{A,B}$ is only 4.7 and 5 Å, respectively, from the Mg of the pigment at $B_{A,B}$ (Allen *et al.*, 1987; Chang *et al.*, 1991; El-Kabbani *et al.*, 1991). These changes are on the order of those observed on exchange of 13²-hydroxy Bphea into $H_{A,B}$, which may occur under exchange conditions.

Currently, fewer data are available on the influence of Bphe modifications on Bchl absorptions. One example is the replacement of H_A with [3-vinyl] Bphea, which leads to a decreased absorption around 810 nm and a broadened Q_X band. These spectral shifts could indicate an (excitonic) interaction with the pigments at sites B_A or P, but also can originate from a change in the protein structure. Tyr L210 is a potential candidate that interacts with both sites (Gray *et al.*, 1990; Parson *et al.*, 1990). Further experiments are necessary to clarify these interactions, which are relevant to the extraordinarily fast electron transfer (see Chapter 4, Volume 2).

V. Time-resolved spectroscopy

The catalytic function of RCs is the conversion of excitation into electrochemical energy. The kinetic details of this process are reviewed in Chapter 4, Volume 2. Modifications of pigments may help clarify the assignment of kinetic processes to the different molecular events that are discussed currently. To date, two modifications at the $B_{A,B}$ sites have been characterized kinetically (Finkele *et al.*, 1992; Lauterwasser *et al.*, 1992). RCs containing 13²-hydroxy Bchla at sites $B_{A,B}$ behave identically to native ones within the limits of error, indicating (1) that this modification is "neutral" to the processes involved and (2) that structural changes are unlikely in these preparations (see previous text). The other complex investigated contains [3-vinyl]-13²-hydroxy Bchla at sites $B_{A,B}$. At all wavelengths studied, the kinetics could

be fit by four components, that is, 0.9, 3.5, 30, and 200 ps. This number is one greater than in native RCs, which lack the 30-ps component. The most straightforward explanation of the kinetics is that this RC preparation is heterogeneous. One fraction of the preparation consists of (kinetically) native RCs (0.9, 3.5, and 200 ps) the other of modified RCs in which the 3.5-ps component is replaced by a 30-ps one. This conclusion is supported by the absorption spectroscopic observation that, in this preparation, the 800-nm band is relatively intense. The relative amplitudes of the 30-ps component are highest on excitation at 756 nm, which is the absorption maximum of the modified pigments at sites $B_{A,B}$. Since the 13^2-OH substituent alone has no influence on the kinetics, the tenfold decrease of the intermediate component is probably the result of a replacement of the 3-acetyl by a 3-vinyl substituent. The redox potentials of the chromophores are not known, but in first order the substituent exchange is expected to render the redox potential more negative (Watanabe and Kobayashi, 1991). This slowing would be consistent with a more difficult electron transfer to the 3-vinyl pigment at B_A. A 0.9-ps component has been related to the electron transfer from P to B_A (Holzapfel *et al.*, 1989; Finkele *et al.*, 1990). Some evidence exists for a kinetic component with $\tau \approx 4$ ps in the modified RCs but this concept needs further work (Finkele, 1992). First, results with RC in which Bphea at sites $B_{A,B}$ has been replaced with Phea indicate that to the preparation is functional. The 3 ps electron transfer component is increased to about 4.5 ps, and the 0.9 ps one remains, and the yield of charge separation is decreased by only 20% (T. Arlt, S. Schmidt, M. Meyer, H. Scheer, and W. Zinth, unpublished results; A. Shkuropatov, V. Shuvalov, private communication).

VI. Prospective

Bchl modifications, improved pigment analysis, and exchange optimization have been equally important to the development of procedures for pigment exchanges into RCs. Although the method of choice has been tested only with a limited number of pigment modifications and species to date, it seems to be a valuable complement to site-directed mutagenesis because it allows a more direct manipulation of the pigments. A major effort will be necessary to provide basic data for the modified pigments, not only *in situ* but also in solution, to evaluate the effects of the native environment fully.

The method is restricted so far to the monomeric Bchls and Bphes. Currently it is unclear whether the principle can be extended to the primary donor as well. There is hope, however, that the recent advances in the folding of previously unwieldy proteins, will render this site accessible in the forseeable future as well. In view of the similarities between bacterial RC and those of PSII, the technique may also be applicable to the latter.

Acknowledgments

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