M.-E. Michel-Beyerle (Editor)

Reaction Centers of Photosynthetic Bacteria

Feldafing-II-Meeting

With 165 Figures

Professor Dr. MARIA-ELISABETH MICHEL-BEYERLE
Institut für Physikalische und Theoretische Chemie
Technische Universität München
Lichtenbergstraße 4
W 8046 Garching, FRG

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Concluding Remarks

Beyond Native Reaction Centers

M. E. Michel-Beyerle[1] and H. Scheer[2]

[1] Institut für Physikalische und Theoretische Chemie, Technische Universität München,
  W 8046 Garching, FRG
[2] Botanisches Institut der Ludwig-Maximilians-Universität, Menzinger Straße 67,
  W 8000 München 19, FRG

The past five years after solving the crystal structure of the bacterial reaction center from *Rhodopseudomonas (Rp.) viridis* [1] - and the first Feldafing meeting [2] - have witnessed a remarkable concentration of interdisciplinary research on this pigment-protein complex. The importance of the reaction center is related to its key function in transforming photons into electrochemical energy and, in more general terms, to the fact, that it is the first membrane protein of which the three-dimensional structure is known. Beyond these central biological bearings, the reaction center has a stimulating effect on many developments in related fields. Its complex pigment-protein and pigment-pigment interactions required refinement of existing and development of novel spectroscopic techniques. Since the reaction center constitutes the best-defined supramolecular electron donor/acceptor system in chemistry, it has the potential of becoming a test case for electron transfer theories. This year's Feldafing II conference intended to provide a status-report on the present understanding of reaction center function, highlighting the new biochemical, spectroscopic and theoretical approaches. Still under the impression of the conference we will try to give our personal views and some leading references[*], preferentially to papers within this book, on the spectacular progress and limitations related to biochemical and genetic modifications of the reaction center collected in Part III.

Until recently, there were only few ways to introduce specific modifications into the reaction center structure which were suited for a parametric study of charge separation processes. Selective modifications were limited to the two quinones, Q_A and Q_B (Warncke & Dutton), the presence and nature of the divalent metal ion located between Q_A and Q_B [3], and the removal of the H-protein subunit [4]. The only possibility to interfere with one of the four bacteriochlorophylls, the one at the site B_B in reaction centers of *Rhodobacter (Rb.) sphaeroides*, consisted in the borohydride treatment [5] and the chemistry of this procedure is still under debate [6].

In the meantime, this situation has been fundamentally improved by the development of two new tools: Site-directed mutagenesis of the polypeptides and, most recently, thermally induced exchange of four of the six pigments, i.e. the two monomeric bacteriochlorophyll molecules (BChl) at the sites $B_{A,B}$ and the two bacteriopheophytins (BPh) at the sites $H_{A,B}$ by chemically modified analogues (see Appendix for nomenclature).

MUTAGENESIS

Site-directed mutagenesis of reaction centers has been pioneered with *Rb. capsulatus* [7], a bacterium which is genetically more stable than *Rb. sphaeroides*. However, reaction centers from this species have not yet been crystallized. As shown in various reports in this volume (Gray et al., Huber et al., Schenck et al., Stilz et al.), site-specific mutagenesis has also been successful in reaction centers from *Rb. sphaeroides* for which the three-dimensional structure is known (Reiss-Husson et al.) [8]. The techniques are presently established in three laboratories and rapidly advancing. It should be pointed out, however, that the structures of the "native" *Rb. sphaeroides* reaction center (from the wild type strain 2.4.1 and the carotenoid-less mutant R26) are by $\simeq 0.5$Å less resolved than the best defined structure of the *Rp.viridis* reaction center (2.2Å). Unfortunately, directed mutagenesis in the latter species still meets considerable problems and remains a challenge. Some interesting, spontaneously formed mutants are already now available (Sinning et al.). They show single- or multiple replacements of amino acid residues in the vicinity of (or at) the quinone (Q_B) binding site and for some of them a differential crystal structure has been performed.

In summary, comparing reaction centers from these three purple bacteria there is at present an inverse relationship between the capability to mutate selectively and the extent of structural information available, and a continuing effort is needed to balance the two opposing trends. In this context, it is still rewarding to explore reaction centers from other species which may be more amenable to both, crystallographic and genetic manipulations. The reaction center from the green bacterium *Chloroflexus (Cf.) aurantiacus* may be such a candidate. It shows some very interesting variations with respect to pigment composition and protein structure (Feick et al.), and has been crystallized recently.

The mutations focused on were designed or selected to influence specific electron transfer steps and thus addressed the protein environment of the cofactors participating in the electron transfer sequence. These are the primary donor, the BChl dimer (P) with its components P_A and P_B, the BChl monomer and the BPh at the sites B_A and H_A, respectively, and the two quinones, Q_A and Q_B.

(1) The first class of mutations are those in which amino acid residues close to the central four N-atoms of any of the six tetrapyrroles are exchanged. In "native" reaction centers the BChl molecules carry histidines (His) ligated to the central Mg-ion, while the BPhs have hydrophobic amino-acids like isoleucine (Ile) instead at the respective positions. Interchange of any one of these His-residues leads to a change of the pigment bound at the respective site, e.g. a His-Ile mutation results in a reaction center in which the respective BChl is replaced by BPh, and *vice versa* (Schenck et al., Woodbury et al.). Up to now there is only one report on an "intermediate" amino acid, the glutamate (Glu), which at least partly leads to BChl binding. Remarkably, in the reaction center of the green bacterium *Cf. aurantiacus* which has Ile instead of His at the relevant position of the B_B binding site, one BChl is replaced by BPh, most likely at the B_B-site (Feick et al.).

The pigment exchange experiments (see below) on reaction centers from *Rb. sphaeroides* and *Rp.viridis* fully corroborate this selection rule of the binding sites. Presently without any exception, pigments are selectively introduced at the sites $B_{A,B}$ and $H_{A,B}$ according to the presence or absence of a central Mg-ion, respectively. This selection principle has been observed irrespective of the structural modifications at the pigment periphery, provided an exchange was possible at all (Struck et al.).

(2) The second set of site-directed mutations involved aromatic amino-acids located in (or close to) the electron transfer path and preferentially those which are highly conserved in type-II bacterial reaction centers and photosystem-II of plants.

In photosystem-II, two such amino acids, tyrosine (Tyr-160 on D_2-subunit and Tyr-161 on D_1-subunit) seem to actively participate in the electron transfer sequence between the water-splitting site and the primary donor radical cation, $P680^+$ [9]. The homologous Tyr-M162 in reaction centers from *Rb. sphaeroides* is located between P and the putative binding site for the cytochrome-c_2 (acting as electron donor to P^+); its replacement results in a decreased rate of electron transfer (Gray et al., unpublished results).

Of special interest with respect to primary charge separation was the tyrosine Tyr-M208 in reaction centers of *Rb. capsulatus* (Parson et al.) and analogous Tyr-M210 of *Rb. sphaeroides* (Gray et al.). This amino acid residue is located between the P_A component of the primary donor and the BPh at H_A and is not conserved in *Cf. aurantiacus*. Another aromatic amino acid (conserved in all reaction centers sequenced up to now) is the tryptophane Trp-M250 in *Rb. capsulatus* (Coleman et al.) and the analogous Trp-M252 in *Rb. sphaeroides* (Stilz et al.) which is the only amino acid residue between the BPh at H_A and Q_A and in van der Waals-distance to both cofactors. The so-far studied replacements of these aromatic amino acids at

both positions lead to a pronounced decrease of the respective electron transfer rates; they also tend to reduce the binding affinity of the primary quinone, Q_A.

(3) A third set of mutations addressed the asymmetry of charge separation which seems to proceed exclusively along the active A-branch, the ratio of relative primary rates being $k_A/k_B > 25$ in *Rb. sphaeroides* [10]. Replacement of glutamate, Glu-L104, located close to the 13^1-CO-group of H_A in *Rb. capsulatus* was the first example [11] investigated; the latest attempts towards "symmetrization" of the two pigment branches involve the exchange of large structural motifs between the two protein subunits, L and M (Robles et al., Woodbury et al.). So far, it has not been possible to remove or diminish the asymmetry of charge separation. Not even after complete blocking of the A-branch, electron transfer along the B-branch became detectable (Robles et al.).

PIGMENT EXCHANGE

In a complementary approach to study structure-function relationships in the reaction center, the tetrapyrrole pigments are directly modified. Site-directed mutagenesis is at present restricted to BChl-BPh exchange (see above), and potentially to introducing biosynthetic precursors. A more general method suitable for exchanging the native BChl and BPh by chemically modified analogues, was developed very recently (Struck et al.).

Accessible sites are B_A and B_B, into which a variety of pigments bearing a central metal-ion have been introduced, and H_A and H_B, into which a corresponding set of free bases (bacteriopheophytins) have been exchanged. So far, the majority of studies has been performed with reaction centers from the carotenoidless *Rb. sphaeroides* R26, but the method is applicable to other purple bacteria. Again, BChla- or BPha-containing species are favourable, while in *Rp. viridis* containing b-type pigments, only the BPh sites are accessible, probably due to the stiffening by the bound cytochrome-c. By the proper choice of reaction center, pigment and reaction conditions, there is even the possibility of non-symmetric modifications. As an example, only the B_A site is accessible in reaction centers of the carotenoid-containing wild-type strain *Rb. sphaeroides* 2.4.1. The primary donor has as yet resisted any exchange, but it may be anticipated that the successful exchange of the other pigments triggers research which will eventually render the P-site accessible, too. Whenever a pigment exchange was possible, the reaction center showed reversible bleaching, although electron transfer was slowed down e.g. in the BChla-$B_{A,B}$-(3-vinyl)-BChla modification (see Appendix for the proposed nomenclature). Pigments like the aforementioned, which show distinctly blue-shifted absorption maxima, were also useful to study electronic interactions, and for the assignment of absorption bands in the crowded regions of the spectrum.

STRUCTURAL AND FUNCTIONAL CONTROL OF MODIFIED REACTION CENTERS

The final objective of modifications is certainly only achieved in control experiments which relate the modifications to changes in structure, interactions and electron transfer dynamics.

Control by crystal structure analysis. It is self-evident that the control of a modified reaction center by crystal structure analysis would be highly desirable, although hard to achieve. However, there are some arguments which make us think on this point in relative terms. For many problems related to electronic couplings in electron transfer and, in particular, for a full quantitative analysis, ultrahigh resolved structural data are needed. Calculations on the basis of the 2.2Å structure of *Rp. viridis* revealed much less charge-transfer character of the excited BChl-dimer $^1P^*$ than the ones based on the "intermediate" refinement [12](Scherer et al.). However, even on the basis of this ultimate refinement calculations of electronic overlaps turned out to be problematic. This is especially true when these depend on the proximity of two methyl groups, as it seems to be the case for the electronic overlap between BChl at B_A and BPh at H_A [12]. Moreover, it should be kept in mind that even the best-resolved structure of the ground state configuration at room temperature might differ from the one encountered after protein relaxation, or at the low temperatures to be used in many spectroscopic studies. And, last, but not least, non-periodic water molecules might play a large and different role in native and modified reaction centers.

Thus, such limitations together with the requirement of high resolution certainly lower the expectations linked to future differential crystal structures of modified reaction centers, in particular of those from *Rb. sphaeroides*, as long as the native structure is only resolved to $\simeq$3Å. Furthermore, the resolution of crystals from modified reaction centers might suffer from additional complications, such as an increase of structural or energetic inhomogeneity and stability problems, as previously encountered in e.g. borohydride treated reaction centers from *Rb. sphaeroides* (Allen et al., private communication).

Structural and functional control by spectroscopy . In order to study interactions and dynamics in the native and modified reaction centers the entire arsenal of spectroscopy has to be employed. The interpretation of dynamic data profits from more general developments in electron transfer theory [13] and new experimental results in the field of artificial donor/acceptor systems where specific features can be influenced in a parametric way. (Scherer et al., Wasielewski et al.).

The road to the identification of detailed changes in pigment-pigment and pigment-protein interactions opens through steady state optical (i) and magnetic resonance (ii) spectroscopy.

Temperature-dependent absorption (Sebban et al., Zhou et al.) and fluorescence spectroscopy, is complemented and supplemented by structure-based quantum calculations (Vrieze & Hoff) and more complex techniques, as LD- (Breton et al.), CD- (Scherz et al.), FTIR- (Buchanan & Michel, Mäntele et al., Thibodeau et al.) and Stark-spectroscopy [14], together with the diversity of magnetic single- and multiple resonance methods (Angerhofer et al., Lendzian et al., Lous et al., Morris et al.). As, for instance, changes in the absorption spectrum of the pigments may occur, modified reaction centers could favour probing of selected states. Pigment exchange, like the replacement of the 3-acetyl- by a vinyl-group in BChl has already proven its potential towards disentanglement of congested spectral absorption features (Struck et al.). Modification induced spectral changes could also lead to the revelance of spectroscopic features which in native reaction centers are buried below congested absorption bands. In this context, a long-hunted state is the radical pair P^+BChl^- at the site B_A.

On the basis of such spectroscopic data, electron transfer dynamics and energetics of intermediate charge-transfer states can be investigated. This is achieved by the appplication of a large variety of methods working in the frequency- or time domain (hole-burning experiments (Small et al.), femto- to millisecond-time-resolved pump-probe absorption (Breton et al., Dressler et al., Finkele et al., Kirmaier & Holten), Raman- (Atkinson et al.) and fluorescence measurements (Müller et al.), including absorption experiments in static magnetic [15] and microwave fields [16]. The congested absorption bands of the different neutral (ground and excited state) and charged species in the reaction center and the necessity to avoid multiple excitation, have initiated considerable improvements in the sensitivity of detection ($\Delta OD < 10^{-3}$) (Finkele et al.), a condition which is especially difficult to meet in experiments with high temporal resolution (femto- to nanoseconds).

Apart from these well-established techniques, the knowledge of the three-dimensional structure of the reaction center stimulated the development of an experimental approach [17] which yields the orientation of the dipole moment of the radical pair formed in competition to the prompt fluorescence from the excited primary donor state, $^1P^*$. The method is based on the measurement of the anisotropy of electric field induced change of the primary charge separation rate, as reflected in the steady state fluorescence yield (Boxer et al., Ogrodnik et al.). Especially when applied to time-resolved fluorescence measurements, the technique carries high potential for identifying the mechanism of primary charge separation; i.e. to infer on the relative contributions of a two-step, sequential vs. an unistep, superexchange mechanism (Bixon et al.). The electric field induced energy shift of radical pairs carrying a large dipole moment is also reflected in field dependent absorption transients in the picosecond- [18] and nanosecond time domain [19] and might be very useful in mapping out energetics in reaction centers.

Data on electron transfer dynamics are interpreted within the conventional nonadiabatic approach [20,21] which provides the conceptual framework for the description of electron transfer processes in the reaction center [22](Bixon et al.). The analysis of the rates in terms of the electronic coupling matrix element and a Franck-Condon factor requires the independent determination of energy parameters, such as the free energy gap between equilibrium nuclear configurations of initial and final states. With the assumption of sufficiently fast protein relaxation times, it is then possible to extract from the rate and its temperature dependence information on the effects a given modification has on the structure, thereby affecting the electronic couplings and the energetics, changing free energy differences and protein reorganization energies, and thus the Franck-Condon factor. Such a treatment cannot, however, account for possible thermal contraction (which might be even different in modified reaction centers) as well as for energetic and structural inhomogeneities.

Changes of electronic couplings can be induced by a variety of effects such as (i) differences in the arrangement of the cofactors, either following from changes in the binding site or in the nature of the cofactors, or both; (ii) differences in the charge distribution at the cofactors in the ground, excited or charged states due to specific interactions with the environment, e.g. with polar amino acids, and (iii) differences in superexchange mediated electronic interactions between cofactors which rely on details of the structure (and energetics) of cofactors and protein. In this context, the mutagenic replacements Trp-M250-Phe,Leu in *Rb. capsulatus* (Coleman et al.) and Trp-M252-Phe,Tyr in *Rb. sphaeroides* (Stilz et al.) were investigated.

Changes in the nuclear Franck-Condon factor may be encountered when modifications influence energetics via a change in the free energy (ΔG) or the protein reorganization energy (λ) which is related to relaxation of the amino acid environment upon electron transfer. Such changes can be either induced by changes in the nature of the protein or of the cofactors. It is one of the characteristic features of native reaction centers that the membrane-spanning, charge separation rates are temperature-independent, i.e. they proceed under conditions where ΔG roughly equals the protein reorganization energy λ. The investigation of reaction centers with modified energetics is very attractive, since these may constitute "new species" where this "optimization principle" is violated.

Moreover, the still controversially debated question of the mechanism of primary charge separation (Dressler et al., Finkele et al., Kirmaier & Holten) is directly related to the energy of the radical pair P^+BChl^- at the site B_A, in that it determines the relative contributions of sequential and superexchange mechanisms (Bixon et al.). In this context, the first explicit studies addressed the role of Tyr-M208 in reaction centers of *Rb. capsulatus* (Parson et al.) and the respective Tyr-M210 in *Rb. sphaeroides* (Gray et al.) in femtosecond time-resolved

experiments and electrostatic calculations (Parson et al.). The phenomenology of the primary electron transfer rate and its temperature dependence can be modeled within the frame of the nonadiabatic theory and is consistent with the notion of a smaller free energy of the state P^+BChl^- at the site B_A (Bixon et al.). A controlled free energy shift of the state P^+BChl^- at the site B_A is also the goal of the more recent pigment exchange experiments (Struck et al.).

Apart from its role in determining both, the free energy differences within the electron transfer cascade and the reorganization energy related to the structural relaxation of the amino acid residues upon electron transfer, the protein might even undergo larger structural changes upon electron transfer and do so in a complex time-dependent pattern (Knapp & Nilsson). This might be influenced also by modifications, leading ultimately to changes in both, the Franck-Condon factor and electronic couplings.

In view of this diversity of complex effects possibly exerted by the cofactors and the protein environment on electron transfer dynamics, the spectroscopic characterization of modified, *de facto* new reaction centers can only aim at a crude differentiation between effects on structure and energetics. Such a global characterization should enable us, however, to screen those modifications for which special efforts towards crystallization and X-ray structure analysis appear rewarding.

OUTLOOK

On the basis of the strategies outlined above, a critical evaluation of the goals, of unwanted side-effects, and of the design of experiments on modified reaction centers is useful. Some immediate goals, already addressed before and especially emphasized in Parts I,II and IV of this volume are related to basic questions of the electron transfer mechanism in reaction centers, e.g.: (1) Although the most recent, highly sensitive femtosecond data (Dressler et al., Finkele et al.) can be readily explained by a sequential electron transfer mechanism, the extent of a parallel "superexchange" unistep contribution to the primary charge separation is not yet clear (Bixon et al.). (2) Although the structural symmetry in terms of cofactor arrangement and thereby electronic couplings favours electron transfer along the active branch, the contribution of asymmetry in energetics to unidirectionality is not understood (Parson et al.). (3) While the present X-ray analysis gives only a static structure, structural or energetic changes due to protein relaxation (Knapp & Nilsson) related to electron transfer may be functionally important.

Addressing these questions requires criteria for the selection of suitable modifications, and an open mind for possible interferences. In a naive expectation, all modifications should show

the largest influence in their immediate environment. However, more far-reaching effects have to be envisaged, even upon seemingly "minor" modifications, and are expected to become more pronounced with "major" ones. For example, a change of the polar tyrosine versus phenylalanin (Tyr-L222-Phe in *Rp.viridis*) at roughly equal molar volume exerts *"a dramatic effect on the structure"* of this reaction center (Sinning et al.), and the Tyr-M208(M210)-Phe mutation close to P leads to a loss of Q_A which is located more than $20\overset{\circ}{A}$ away "on the other side" of the reaction center (Parson et al., Gray et al.). To define and eventually forecast just what may constitute a "minor" or "major" modification and its possible side effects will be one of the most challenging problems of the future. The design of changes introduced into the reaction center by replacing amino-acids or pigments has to account for steric factors, such as e.g. molar volume and specific geometry (Fajer et al., Yang et al.), and interactions as e.g. electrostatic, dipolar and hydrophobic ones, and polarizabilities. It will require all possible techniques including optical and magnetic spectroscopies, X-ray crystallography and ultimately spectroscopic structure-determining techniques (applicable now only to much smaller proteins), which allow to identify the implanted changes on a microscopic scale. Moreover, the design of modified reaction centers as well as the exprimental efforts thereupon have to be accompanied by quantum-chemical and improved molecular dynamics calculations, specifically parametrized for reaction centers (Scherer; Parson et al., Knapp & Nilsson).

While these questions address mainly the reaction center as a system by itself, its relations to other photosynthetic functional units is becoming more and more important. These include the interaction with the antennas (Freiberg & Pullerits), in particular the core ones, and the interactions with secondary electron donors, the cytochromes (Vermeglio et al.) and possibly also the acceptors (cyt b/c complexes). As a transducer between excitation energy and membrane potential, the function of the reaction center depends on its co-action with the antennas on one side, and electron donors and acceptors on the other. Since the short time-scale of excitation energy transfer prohibits any diffusional collision process to be involved, the antenna probably has to be tightly associated with the reaction center. There are several lines of biochemical evidence to this, but the site(s) and nature of the interactions can currently only be speculated on. It is also unknown, if certain pigment(s) present in the reaction center, act(s) as the preferred acceptor(s) of excitation energy, and if there are similarly preferred energy donors in the antennae. In view of the rapid equilibration of excitation energy within the antenna, the transfer to the reaction center is a critical step (Müller et al.). The same reasoning applies to electron transfer to and from the reaction center. Although it occurs on a much slower time-scale, its distance dependence is much more critical. Any modification, pigment or polypeptide, should therefore not only be scrutinized regarding its effect within the isolated reaction center. In view of this, it appears mandatory (i) to characterize modified reaction centers as extensively as possible, and (ii) to go beyond

isolated reaction centers and include their natural environment and functional partners.

Last, but not least, there is an abundance of questions which are not related to the function of the reaction center, but rather to its being the first member of a large and important class of membrane proteins. Such questions address the folding of the individual protein subunits, the incorporation of the pigments and other cofactors, the assembly of the subunits with each other and with neighbouring complexes and the mechanisms regulating these processes. There is finally the challenging problem, whether significant molecular properties relate DNA sequences to the respective translation products (Yang et al.). The reaction center is a fortunate case in this respect, because it has a variety of intrinsic probes which can be used in following organization and turn-over without distorting the system.

By the same token, however, these processes are also complicated in reaction centers because they are heavily loaded with cofactors, which are likely to exert a considerable influence on the reaction center structure. The effects of the tetrapyrrolic cofactors will be even more pronounced in antenna complexes, where the pigments constitute an even larger proportion. A satisfactory modeling of the organisation principle of the reaction center is therefore expected to give valuable insight, too, into the construction principles of antennae.

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APPENDIX

The many modifications now possible pose some nomenclature problems which are currently unsettled. For bacterial reaction centers we suggest to use the following self-consistent approach.

Native reaction centers. *Protein subunits* are labeled L, M and H. *Amino acid residues* are named either by three or one letter code and labeled by the respective subunit and their position with respect to the N-terminus. The two *pigment branches* are labeled by A (active) and B (inactive), as suggested by George Feher. The *cofactor binding sites* are labelled by $P_{A,B}$ for the primary donor, $B_{A,B}$ for the neighbouring monomeric pigments, $H_{A,B}$ for the subsequent ones, $Q_{A,B}$ for the two quinones, and MBS for the metal binding site.

As long as the P- and B- sites contain only bacteriochlorophyll a or b, and H contains bacteriopheophytins a and b, the binding sites are equivalent to the *pigments*, BChla, BChlb and BPha, BPhb in the nomenclature. If this is not the case, as e.g. in the reaction centers of *Cf. aurantiacus* the pigments have to be used together with the binding site. Hence, B_B (= BChla-B_B) in the purple bacterium *Rb. sphaeroides* becomes BPha-B_B in *Cf. aurantiacus* .

Modified reaction centers: *Amino acid exchanges* are characterized in the conventional way. Native amino acid residue, followed by protein subunit and position, followed by the new amino acid residue. As an example, E-M158-Q = Glu-M158-Gln denotes mutation of glutamate at position 158 on the M-subunit to glutamin. *Pigment exchanges* are treated in a similar way. As an example, BChla-B_A-13^2OH-BChla denotes an exchange of BChla at the site B_A against 13^2-OH-BChla.