Biochimica et Biophysica Acta, 977 (1989) 157–162
Elsevier

BBABIO 43082

Assignment of cytochrome hemes in crystallized reaction centers from *Rhodopseudomonas viridis*

G. Fritzsch, S. Buchanan and H. Michel

Max-Planck-Institut für Biophysik, Frankfurt (F.R.G.)

(Received 1 May 1989)
(Revised manuscript received 28 July 1989)

Key words: Reaction center; Electron transfer; Heme; Cytochrome *c*; Redox potential; (*Rps. viridis*)

In crystals of reaction centers from the purple bacterium *Rhodopseudomonas viridis*, the four linearly arranged heme groups of the cytochrome-*c* subunit are oriented in a way that the two outer groups lie preferentially in direction parallel to the crystalline z-axis whereas the inner groups lie mainly perpendicular to z. Illumination of redox poised crystals with polarized light show that both the outer and inner groups have each a high- and a low-potential heme. The absorption maxima appear at 552, 552.5, 556 and 558.5 nm. The inspection of difference spectra taken at various redox potentials and the consideration of the individual molecular heme planes in the unit cell lead to the following sequence of heme groups (P-960: special bacteriochlorophyll pair, redox potentials in brackets): P-960, *c*-558.5 (+370 mV), *c*-552 (+10 mV), *c*-556 (+300 mV), *c*-552.5 (−60 mV).

Introduction

The heme molecules in cytochromes play a crucial role in biological electron transfer reactions [1]. The cytochrome subunit in the reaction center (RC) of the purple photosynthetic bacterium *Rhodopseudomonas viridis* contains four *c*-type heme groups. The spatial alignment of the hemes has been revealed by crystallization of the RC [2] and subsequent X-ray structure analysis [3,4]. Although the absorption spectra (e.g., Ref. 5) as well as the redox mid-point potentials are known, there is still a debate on the assignment of the spatial positions of the four hemes and their physical-chemical properties [6–12]. Shopes et al. [6] find by photoinduced cytochrome oxidation an arrangement where the outer hemes are both of high potential and the inner ones of low potential. Alegria and Dutton [7] find the sequence high-low-high-low when titrating monolayer films of RC through a redox potential range of +400 mV to −200 mV. By means of spectroscopic and kinetic studies, Dracheva et al. [8,9] propose the same sequence and also Nitschke and Rutherford [10] come to this conclusion by an ESR analysis with oriented proteins. Vermeglio et al. [11] studied oriented RCs in squeezed polyacrylamide gels with the same findings as in Refs. 7–10. In the most recent paper to date, Hubbard and Evans [12] conclude an arrangement in potential order high-high-low-low. All results are based on the assumption that the heme nearest to the special pair has an absorption peak at about 559 nm and a high redox potential of nearly +400 mV.

The availability of crystallized RCs enables one to study the relation between spatial arrangement and physical-chemical properties by measuring the absorbance of oriented chromophores with polarized light. The heme molecules have a four-fold rotation axis perpendicular to their porphyrin plane [13]. It is known that due to this symmetry axis the electronic transitions are polarized parallel to the porphyrin plane with equal absorption for all directions in this plane [14]. Of course, theoretically possible is also the polarization of electronic transitions perpendicular to the porphyrin plane. According to Antenson, these transitions show little or no intensity [15]. In the following, it is supposed that the electronic transitions are confined mainly to the porphyrin plane. The interaction of the heme molecules with the peptide chain, however, seems to perturb the degeneration of transitions at least in one case as discussed later.

In order to understand the interaction of polarized light with oriented chromophores it is necessary to consider the spatial arrangement of the hemes in detail.

Abbreviations: RC, reaction center; LDAO, lauryldimethylamine *N*-oxide.

Correspondence: G. Fritzsch, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, 6000 Frankfurt/M 71, F.R.G.

The space groups of the crystals is $P4_32_12$. Although the tetragonal unit cell contains eight asymmetric units (1 RC molecule/unit), there exists a preferential alignment of the outer hemes parallel to the crystalline z-axis and an alignment of the inner hemes perpendicular to z. This fact is prerequisite for polarized light spectroscopy. In the following, the numbering of heme groups is defined according to their appearance along the amino-acid chain [16], i.e., HE3 is located next to the special pair (P-960) followed by HE4 and HE2; HE1 is the outermost heme: P-960, HE3, HE4, HE2, HE1.

In Fig. 1 the complete arrangement of chromophore orientations in the crystals is presented. It can be seen from this picture that the absorbance obtained with light parallel or perpendicular to the z-axis should be different. In Fig. 2, two orientations of the four heme groups of one RC are presented. Due to the space group $P\ 4_32_12$ the remaining six orientations yield projections onto the z–x plane that lead to the same absorbances of polarized light as the two types shown (three more for each type).

Material and Methods

RCs from *Rps. viridis* were isolated and purified with 0.1% lauryldimethylamine *N*-oxide, LDAO (Fluka) [2]. The following modification was applied. For molecular sieve chromatography Fractogel TSK HW 55 S (Merck) was used. This step was repeated twice. Fractions with an absorbance ratio 280/830 nm of 1.9–2.1 were pooled and crystallized as in Ref. 2. 5-μl droplets of protein solution containing 1.5 M ammonium sulfate were placed on glass microscope slide covers (10 × 10 × 1 mm) and equilibrated with 1.9–2.1 M ammonium sulfate by vapor diffusion. Thin crystals appeared within 24 h. They grew preferentially in the direction of the crystalline z-axis. Average size: 0.5 mm × 0.1 mm × 0.3 μm. Redox poising was achieved in a buffer in which LDAO was exchanged against 0.3% nonyl β-D-glucopyranoside (Calbiochem), since LDAO is reduced in a reductive environment. The concentration of ammonium sulfate was adjusted to 2.7 M. In order to stabilize redox potentials, eight mediators (at maximally

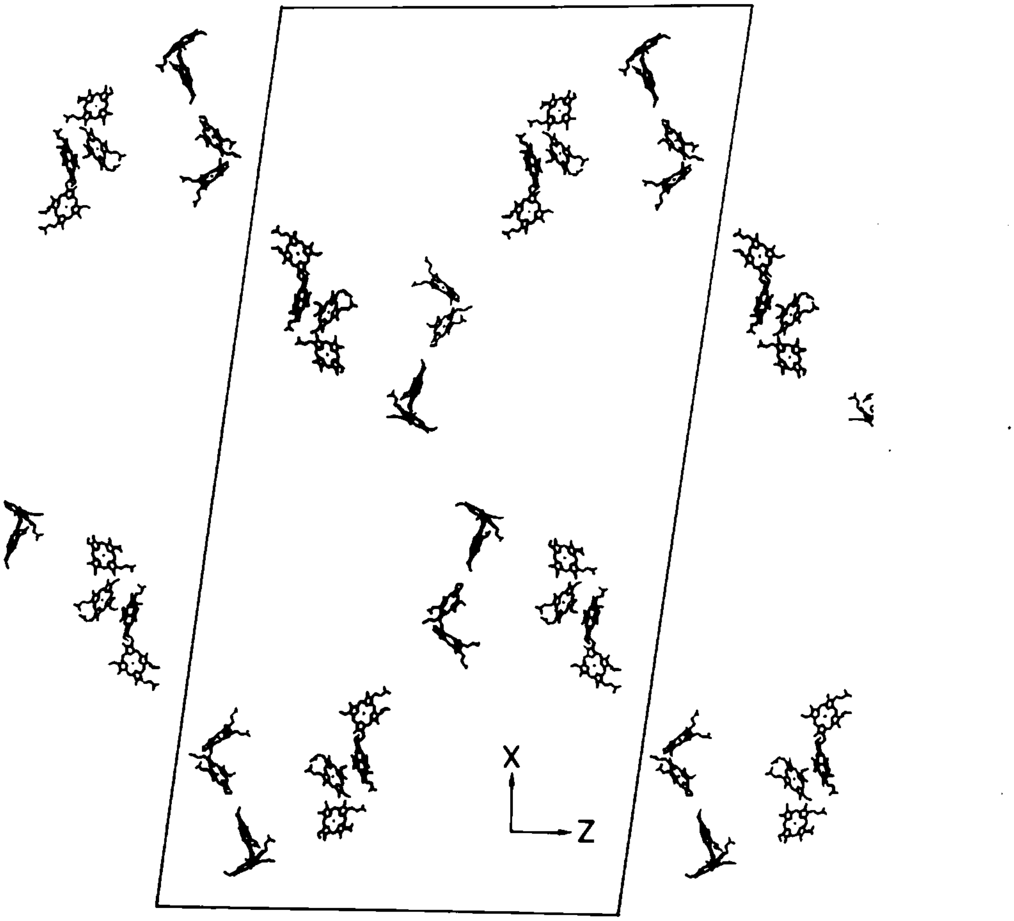

Fig. 1. Projection of the heme groups onto the z–x plane of the crystals. There are eight different orientations in the projected unit cell. It is seen from this representation that the outer hemes (HE3 and HE1) are oriented preferentially along the crystalline z-axis, whereas the inner hemes are oriented perpendicular to this direction. The box surrounds one set of projected heme groups which are equivalent to an arbitrarily chosen unit cell.

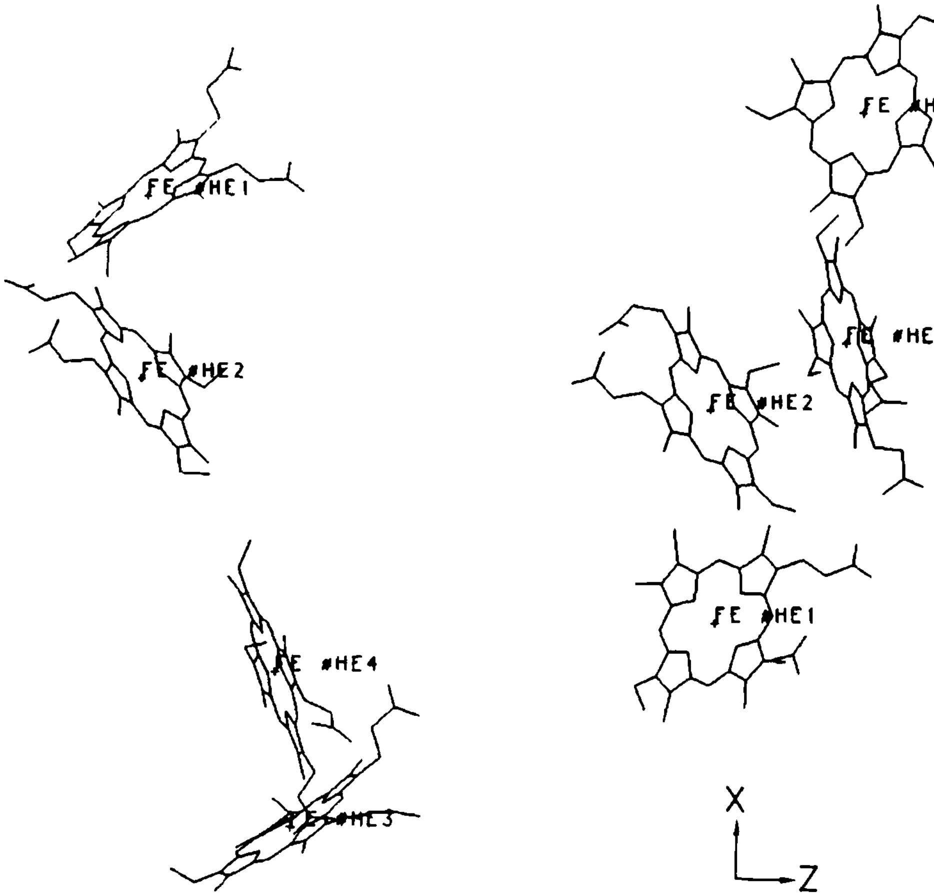

Fig. 2. The two principal projections of heme groups that are significant for polarized light spectroscopy. The remaining six projected configurations can be generated from these two projections by reflections through the x-axis of the z-axis and by a rotation of 180° around the y-axis. It is obvious that HE4 lies more or less perpendicular to z in all configurations implying a strong linear dichroism. HE1, on the other hand, does not fit very well in either of the polarization directions; for this reason it is less dichroitic.

50 μM) have been applied (mid-point potentials in brackets): diphenylaminosulfonic acid from K&K Labs (+400 mV); 1,4-benzoquinone from Sigma (+280 mV); 1,2-naphthoquinone-4-sulfonic acid from Fluka (+217 mV); 4-amino-4-methoxydiphenylamine from Sigma (+150 mV); anthraquinone (−100 mV); anthraquinone 2,6-disulfonate (−184 mV); anthraquinone 2-sulfonate (−225 mV), last three from Fluka. The protein crystals were mounted between glass plates in a home-built plexiglass chamber which was filled with the buffer solution. In a second chamber, the mixing chamber, the solution was titrated with ferricyanide (Sigma, +430 mV) for oxidation or with ascorbic acid (Fluka, +58 mV) and dithionite (Sigma, −460 mV) for reduction. An argon stream (1 bubble/s) was led into the mixing chamber during reduction to ensure anaerobic conditions. The mixing chamber also contained the electrode (AgAgCl, Ingold), the voltage was measured by an HP 3456 Digital Voltmeter. The buffer was gently circulated from the mixing to the measuring chamber. Poising of redox potentials was achieved by adding small aliquots (1 mM to several 100 mM) of the oxidizing or reducing chemicals into the mixing chamber. Avoidance of hysteresis effects within the titration curves required a sufficiently slow equilibration of potential (max. 50 mV/h).

The absorption spectra were taken with a microscope-photometer (Zeiss Microscope UEM, Photometer 03) interfaced with a computer (HP 300 series) and controlled by λ-Scan software package (Zeiss). Slit-width and step-width were 1 nm each. The light beam was polarized in three directions: parallel and perpendicular to the z-axis, and, in addition, at a 45° angle to z. As a criterion for the intact crystalline order, the strong polarization of the bacteriochlorophyll-*b* Q_y absorption band at 830 nm was checked [17].

Results and Discussion

Crystals growing in their x–y plane (quadratic platelets) show indistinguishable absorption spectra for all polarization directions perpendicular to x–y. This is in agreement with the space group $P4_32_12$. Most of the crystals obtained, however, are rectangular platelets with z as the long axis. The positions of the x- and y-axes are unimportant for the following provided one of them

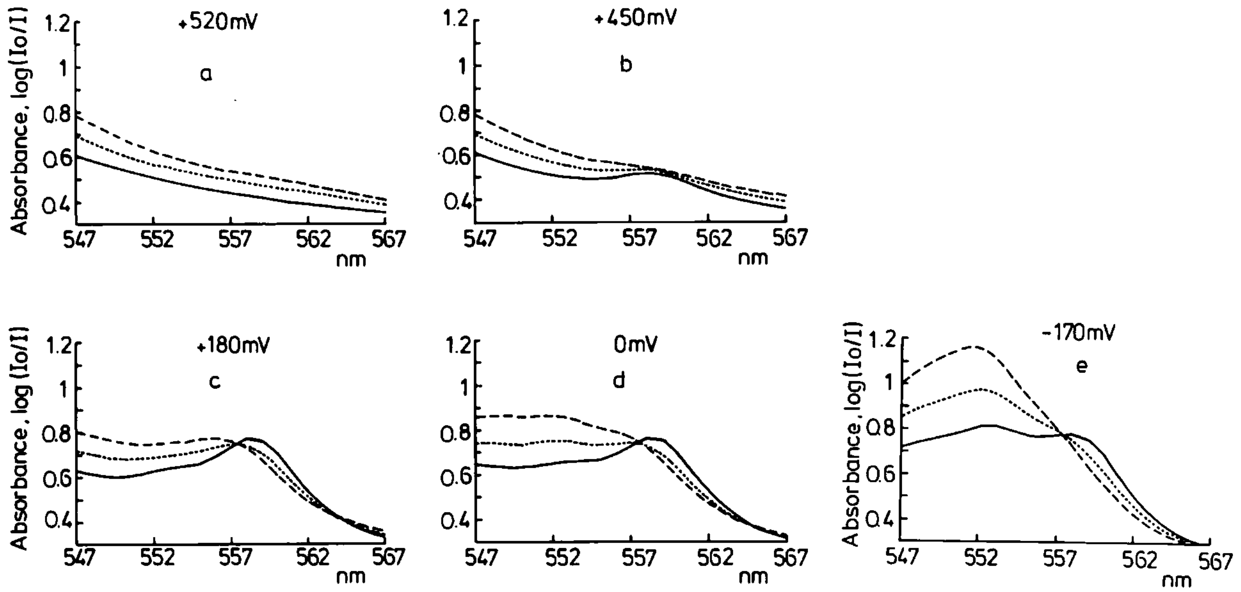

Fig. 3. Absorbance spectra at selected redox potentials taken with plane-polarized light. ———, parallel to *z*; ······, 45° to *z*; — — —, perpendicular to *z*. The step-width of the spectra is 1 nm. The direction 45° to *z* has been observed in order to check the stability of the crystals during measurement (isosbestic point at 557.5 nm).

lies like *z* in the plane of the (thin) crystals. A splitting of the spectra for the three polarization directions is found even in the case of 'electron-empty' heme groups (+520 mV, fully oxidized), (Fig. 3a). In Fig. 3b, the spectra after reduction to +450 mV are shown where the upper of the high-potential hemes is partially reduced. Obviously, a heme group parallel to *z* (an outer heme) absorbs at about 558.5 nm. This is in agreement with the common conception (see Introduction). Further reduction to +180 mV reveals an absorption perpendicular to *z* at 556 nm (Fig. 3c). The potential of +180 mV has been chosen, as at this value both of the high-potential hemes are fully reduced whereas the low-potential hemes are fully oxidized. Two distinct

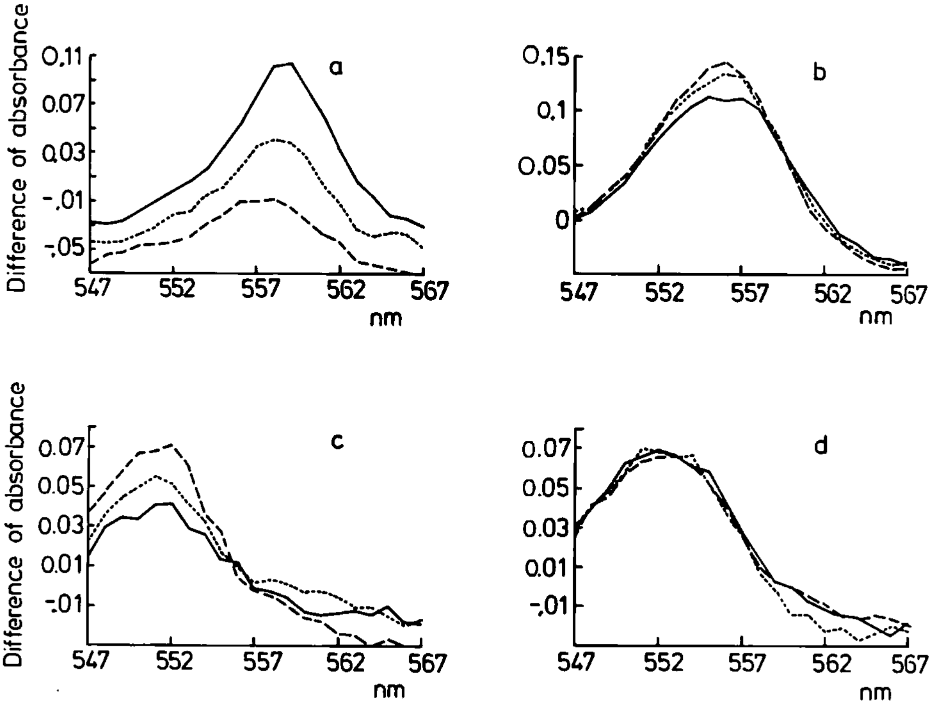

Fig. 4. Difference spectra for the four absorbance peaks. Notation as in Fig. 3. (a) +380 mV minus +520 mV; (b) +150 mV minus +290 mV; (c) +20 mV minus +60 mV; (d) −140 mV minus −70 mV. The special values of redox potential for these difference spectra have been selected in order to show characteristic changes in absorbance.

peaks are detected, one in the *z* direction, the other perpendicular to *z*. The latter must be an inner heme, HE2 or HE4. Between the high and low potentials there is a period of about 150 mV in which the spectra remain unchanged. A strong increase of absorbance occurs between +100 mV and 0 mV at 552 nm perpendicular to *z*. Fig. 3d shows the polarized spectra at 0 mV where the upper of the lower hemes is partially reduced. So far, two absorptions perpendicular and one parallel to *z* have appeared, so that a last one parallel to *z* must occur. In fact, Fig. 3e shows a small peak parallel to *z* at 552.5 nm and at a potential where all hemes are fully reduced (−170 mV). To elucidate the true relations, four characteristic difference spectra throughout the entire potential range should be considered. In Fig. 4a, the difference spectrum of +380 mV minus +520 mV is shown. It contains the spectral information for the upper of the high-potential hemes. The absorption is oriented preferentially in the *z* direction. In Fig. 4b the difference spectrum of +150 mV minus +290 mV has been chosen. In this range, the upper of the high-potential hemes is permanently reduced and it does not contribute to the difference spectrum. (It must be stressed that the rather weak peak at 556 nm perpendicular to *z* is reproducibly found only when the crystalline order is maintained. Mistreatment or aging causes disordering of crystals which leads to a collapse of the split polarization curves, i.e., to an upward shifting of the curve parallel to *z*. Sometimes the peak at 556 nm indeed favored this direction, but this was exclusively the case when the RCs in the crystals were disordered, as indicated by the loss of the 830 nm absorption polarization.) In Fig. 4c, the upper flank of the low-potential hemes is shown by choosing the absorbance difference of +20 mV minus +60 mV. The absorption is significantly strongest in the direction perpendicular to *z*. The relations at the very reductive flank are ambiguous (Fig. 4d); no direction seems to be preferred. For an interpretation one must consider the individual geometrical positions of all hemes within the eight asymmetric units (Fig. 1). None of the 32 planes of heme molecules in the crystal (four hemes each in eight asymmetric units) fits perfectly in either of the planes parallel or perpendicular to *z*. It is the mean contribution of each of the projections of the four heme planes to both polarizations that must be considered. Due to the symmetry relations of space group $P4_32_12$ it suffices to consider the projections of two chromophore configurations (see Introduction and Fig. 2). From Fig. 2 it is obvious that HE4 fits best in one of the polarization directions (perpendicular to *z*). Therefore, this heme must show maximal absorption perpendicular to *z*. The largest absorption occurs at 552 nm and is indeed perpendicular to *z*. Consequently, HE4 is assigned to the 552 nm peak. The other (small) contribution in this direction must be assigned to HE2 and 556 nm. The strong absorption at 558.5 nm and the very high potential is most probably caused by HE3, the one nearest to the special pair; evidence for this assignment has been provided by many independent observations [2–10]. The assignment of HE1 is less straightforward. It competes with the strong absorption of HE4 as it absorbs at very similar wavelength and potential and, more incisive, its averaged molecular plane disagrees considerably with the direction perpendicular to *z* (Fig. 2). For these reasons, it is not surprising that the difference spectrum in Fig. 4d fails to show a clear-cut peak for He1. But there is a significant peak parallel to *z* at 552.5 nm in the original spectrum (Fig. 3e). Thus, the fact that the planes of the four hemes unequally deviate from the planes of the polarized light makes it possible to distinguish not only between pairs of hemes but also between the individuals of both pairs.

The mid-point potentials have been determined quantitatively by fitting the absorbances at 552, 553, 556 and 559 nm to functions with one and two exponential terms. In agreement with Dracheva et al. [9] the fits with one exponential are significantly worse than those with two exponentials. The fitted potentials are +370 mV, +300 mV, +10 mV and −60 mV with an error of 20 mV in each case.

Thus, the microspectroscopic observations with oriented RCs of *Rps. viridis* in crystals imply the following assignment of the heme groups:

HE1	*c*-552.5 nm	−60 mV
HE2	*c*-556 nm	+300 mV
HE4	*c*-552 nm	+10 mV
HE3	*c*-558.5 nm	+370 mV
	P-960	

The absorbance at high potentials shows a shoulder at about 552 nm (see Fig. 3c). The orientation of this shoulder seems to be more or less parallel to *z*, but it is not completely parallel to the orientation of the upper of the high-potential hemes (data not shown). Dracheva et al. [9] found by magnetic circular dichroism that this shoulder is due to non-degenerated Q_x and Q_y transitions similar to the mitochondrial cytochrome *b* heme. Our result is not at variance with this finding. Probably this shoulder indicates that at least one of the high-potential hemes exhibits electronic transitions that are not completely degenerate within the heme plane due to interactions of the heme with the protein environment. The proposed scheme high-high-low-low of Hubbard and Evans [12] with adjacent high-potential hemes next to the special pair seems to be most suitable for a rapid electron transfer from the cytochrome subunit to the bacteriochlorophyll dimer. These authors found that, during reduction, the EPR signal related to the upper of the high-potential hemes decreases as the signal related to the lower of the high-potential hemes increases. It is rational to conclude that electron transfer occurs between both hemes; the statement in Ref. 12, however, that both hemes are therefore adjacent is not very conclusive. Obviously, nature has chosen a more complicated way of electron transport. To date, the function of the low-potential HE4 between the high-potential hemes remains unclear.

Acknowledgements

We thank the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft (Leibniz-Programm), and the Fonds der Chemischen Industrie for financial support.

References

1 Mathews, F.S. (1985) Prog. Biophys. Mol. Biol. 45, 1-56.
2 Michel, H. (1982) J. Mol. Biol. 158, 567-572.
3 Deisenhofer, J., Epp. O., Miki, K., Huber, R. and Michel, H. (1984) J. Mol. Biol. 180, 385-389.
4 Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618-624.
5 Clayton, R.K. and Clayton, B.J. (1978) Biochim. Biophys. Acta 501, 478-487.
6 Shopes, R.J., Levine, L.M.A., Holten, D. and Wraight, C.A. (1987) Photosynth. Res. 12, 165-180.
7 Alegria, G. and Dutton, L. (1987) in Cytochrome Systems (Papa, S., Chance, B. and Ernster, L., eds.), pp. 601-608, Plenum, New York.
8 Dracheva, S.M., Drachev, L.A., Zaberezhnaya, S.M., Konstantinov, A.A., Semenov, A.Y. and Skulachev, V.P. (1986) FEBS Lett. 205, 41-45.
9 Dracheva, S.M., Drachev, L.A., Konstantinov, A.A., Semenov, A.Y., Skulachev, V.P., Arutjunjan, A.M., Shuvalov, V.A. and Zaberezhnaya, S.M. (1988) Eur. J. Biochem. 171, 253-264.
10 Nitschke, W. and Rutherford, A.W. (1988) Proc. VI Int. Symp. Photosynth. Prokaryotes, 35.
11 Vermeglio, A., Richaud, P. and Breton, J. (1989) FEBS Lett. 243, 259-263.
12 Hubbard, J.A.M. and Evans, M.C.W. (1989) FEBS Lett. 244, 71-75.
13 Hoard, J.L. (1971) Science 174, 1295-1302.
14 Hofrichter, J. and Eaton, W. (1976) Annu. Rev. Biophys. Bioeng. 5, 511-560.
15 Antenson, J.E. (1969) PhD thesis, University of Pennsylvania, PA.
16 Weyer, K.A., Lottspeich, F., Gruenberg, H., Lang, F., Oesterhelt, D. and Michel, H. (1987) EMBO J. 6, 2197-2202.
17 Zinth, W., Kaiser, W. and Michel, H. (1983) Biochim. Biophys. Acta 723, 128-131.