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Orientation and assignment of the four cytochrome hemes in *Rhodopseudomonas viridis* reaction centers

André Vermeglio, Pierre Richaud* and Jacques Breton+

*ARBS, CEN de Cadarache, 13108 St Paul-lez-Durance Cedex, *Laboratoire de Biochimie Microbienne, UA 1130, CEN de Grenoble 85X, 38041 Grenoble Cedex* and *+Service de Biophysique, CEN de Saclay, 91191 Gif-sur-Yvette Cedex, France*

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Low temperature absorption and linear dichroism measurements on oriented reaction centers of *Rhodopseudomonas viridis* at different redox potentials allow the α-bands of the four hemes to be spectrally resolved. The high-potential heme C_{556} presents an α-band split into two components absorbing at 555 and 551 nm. The corresponding linear dichroism spectrum shows two transitions of opposite sign and equal amplitudes. Comparison of our experimental results with the linear dichroism values calculated from the atomic coordinates leads us to assign unambiguously the high-potential C_{556} cytochrome with heme 2, the third heme away from the primary donor. Taken together with other available spectroscopic observations, this result implies the following sequence for the redox centers in *Rhodopseudomonas viridis*: C_{554} C_{556} C_{552} C_{559} P.

Assignment; Cytochrome heme; Reaction center

1. INTRODUCTION

In photosynthesis, the first steps of the transformation of light into stable chemical compounds occur at the level of a membrane protein called the reaction center. In the case of photosynthetic bacteria, this complex transfers an electron from a primary donor P, a bacteriochlorophyll dimer, to a primary acceptor, a molecule of quinone [1]. Further stabilization of the oxido-reduction products of this photoinduced charge separation involves, in the nanosecond to microsecond time scale, the electron transfer from a secondary donor, a cytochrome *c* molecule, to the photooxidized primary donor P^+, and from the primary acceptor to a secondary quinonic acceptor [2–4].

Since the crystallization of the photosynthetic reaction center of *Rhodopseudomonas (Rps.) viridis* by Michel in 1982 [5], the analysis of X-ray data has provided the complete description of the arrangement of the photosynthetic chromophores and of the four heme groups of the cytochrome subunit [6,7]. Despite this important breakthrough in the issue of understanding the mechanism of electron transfer in a protein, several questions remain open. In particular, the respective assignment of each of the two high and the two low potential hemes in the reaction center structure is still uncertain. Several attempts have been made based upon: (i) kinetics of photoinduced cytochrome oxidation [8,9]; (ii) linear dichroism study of oriented monolayers of reaction centers at room temperature [10]; (iii) nature of the heme ligands [11]; (iv) electrogenicity of the electron transfer from the cytochrome hemes to the primary donor [12] and (v) analysis of ESR spectra of oriented chromatophores (Nitschke, W. and Rutherford, A.W., personal communication).

In the present work, we report on the absorption and linear dichroism spectra of the four hemes of *Rps. viridis* reaction centers at low temperature. Our data unambiguously show that the high-potential heme C_{556} is the third heme away from the primary donor P.

Correspondence address: A. Vermeglio, ARBS, CEN de Cadarache, 13108 St Paul-lez-Durance Cedex, France

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2. MATERIALS AND METHODS

Rps. viridis reaction centers were prepared as described by Clayton and Clayton [13]. Absorption spectra were recorded at low temperature with a Varian 2300 spectrophotometer equipped with a helium gas cryostat (MERIC). The reaction centers were suspended in 10 mM Tris buffer (pH 7), 0.1% LDAO and glycerol (40:60, v/v). Excitation was provided by a 800 W tungsten lamp filtered through a RG 695 filter. The conditions for orientating reaction centers in squeezed polyacrylamide gels and for recording absorption and linear dichroism spectra were as reported in [14].

3. RESULTS AND DISCUSSION

Fig.1 shows the reduced-minus-oxidized difference absorption spectra recorded at 10 K for a suspension of isolated *Rps. viridis* reaction centers in the α-band region. For untreated reaction centers only cytochrome C_{559}, according to the nomenclature of Dracheva et al. [12] established at room temperature, is reduced (fig.1A). At 10 K, its α-band is centered at 557 nm. Upon reduction of the cytochrome C_{556} by addition of sodium ascorbate (1 mM), two additional bands centered at 555 and 551 nm appear in the reduced-minus-oxidized difference spectrum at 10 K (fig.1B). Further reduction of the low potential cytochromes C_{554} and C_{552} by addition of dithionite induces the appearance of two new bands centered at 549.5 and 546 nm in the low temperature absorption spectrum (fig.1C). The wavelength positions of the α-bands at 10 K for each of the four hemes are summarized in table 1. The absorption and the linear dichroism spectra recorded in very similar redox potential and temperature conditions for a suspension of reaction centers oriented in a squeezed gel

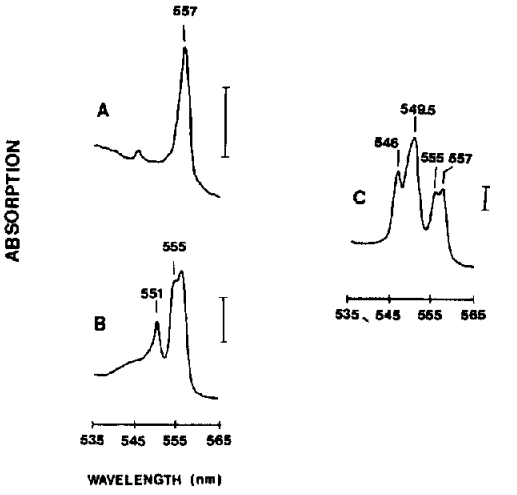

Fig.1. Reduced-minus-oxidized (ferricyanide, 10 mM) difference absorption spectra for *Rhodopseudomonas viridis* reaction centers. All spectra were recorded at 10 K. (A) No addition in the sample. (B) After addition of 1 mM sodium ascorbate. (C) After addition of sodium dithionite. The vertical bars correspond to 0.05 *A* units.

are depicted in fig.2. For an untreated sample (fig.2A, lower part), one notices a strong negative linear dichroism band for the optical transition of the C_{559} cytochrome and a positive linear dichroism band centered at 544 nm attributed to the bacteriopheophytin Q_X transition [14]. With our conventions, a negative value for the linear dichroism means that the angle between the optical transition and the C_2 symmetry axis is smaller than 55°, while a positive value corresponds to an angle greater than 55° [14–16]. The C_2 symmetry axis has been taken as the line joining the Fe^{2+} and the

Table 1

	C_{554}	C_{556}	C_{552}	C_{559}
Wavelength of maximal absorption at 10 K	549.5 nm	555 and 551 nm	546 nm	557 nm
Experimental LD/A values	−0.54 +0.05	−0.60 +0.05 +0.80 +0.05	[a]	−0.60 +0.03

[a] The experimental LD/A value for this heme is difficult to estimate quantitatively because of the contribution of the Bpheo transition in the LD spectrum and the overlap with heme C_{554} in the absorption spectrum. Nevertheless the observed positive LD/A value at 546 nm (fig.3, lower part) is in qualitative agreement with the theoretical prediction

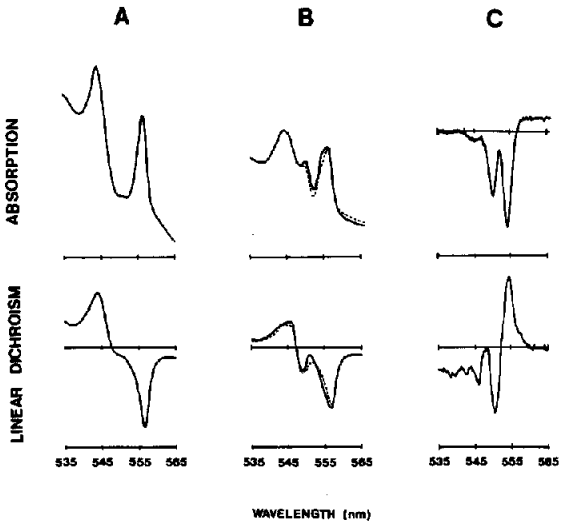

Fig.2. Linear dichroism (lower part) and absorption (upper part) spectra of *Rhodopseudomonas viridis* reaction centers in polyacrylamide gel at 10 K. (A) No addition. (B) After incubation of the gel in 100 mM sodium ascorbate overnight in the dark at 275 K. Spectra were recorded: in the dark (solid line) and after 1 min continuous illumination (50 mW) at 10 K (broken line). (C) Light-minus-dark difference spectra calculated from the spectra in part B.

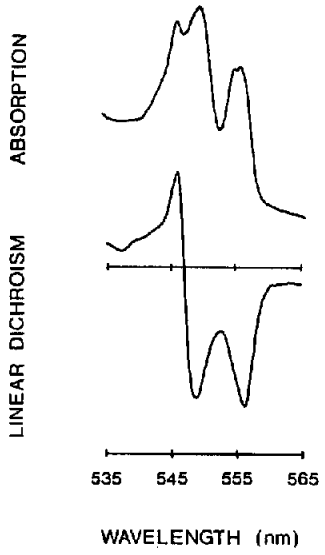

Fig.3. Same as fig.2 but the gel was incubated in the presence of 100 mM sodium dithionite.

middle point of the segment defined by the bacteriochlorophyll's magnesium atoms of the special pair. The reduction of cytochrome C_{556} induces only small changes in the linear dichroism spectrum: the 557-nm band is less symmetrical and a positive band is apparent at 551 nm (fig.2B, lower part, solid line). Upon continuous illumination at low temperature the cytochrome C_{556} can be almost completely oxidized as shown clearly by the absorption spectrum (fig.2B, upper part, broken line) and by the light-minus-dark difference spectrum (fig.2C, upper part). Similar results have been obtained for chromatophores and reaction centers suspended in the glycerol-buffer medium (not shown). The corresponding light-minus-dark linear dichroism spectrum is depicted in fig.2C, lower part. Two bands of nearly equal amplitude and opposite sign are present. Similar observations have been made for chromatophores oriented in squeezed polyacrylamide gel (not shown).

The linear dichroism spectrum recorded under low redox potential condition (fig.3, lower part) shows that the cytochrome absorbing around 550 nm gives rise to a large negative linear dichroism value, while the one absorbing at 546 nm presents a positive band.

The results presented above are in good agreement with the linear dichroism study performed at room temperature by Alegria and Dutton [10] on oriented monolayer reaction center films. Their experiments show that hemes C_{559} and C_{554} present large negative linear dichroism values while hemes C_{556} and C_{552} have less dichroic optical transitions. The same qualitative conclusions have also been derived from ESR studies on oriented chromatophores (Nitschke, W. and Rutherford, A.W., personal communication).

The spectral resolution obtained in our study at very low temperature allows a more quantitative comparison between the linear dichroism data and the reaction center structure obtained by X-ray crystallography [6,7] to be made. In particular, it is clear that the two optical transitions of heme C_{556} are spectrally resolved at 10 K as demonstrated in both the light-minus-dark absorption and linear dichroism difference spectra (fig.2C, upper and lower parts). This is in agreement with the MCD data obtained at room temperature by Dracheva et al. [12] and also with our previous observation of a change in the dichroic ratio within the α-band of this cytochrome C_{556} in oriented cells and

chromatophores [15]. For heme C_{552} we have some indication from light-minus-dark absorbance changes at 77 K of the occurrence of two transitions at 546 and 548 nm (not shown). For both hemes C_{559} and C_{554}, the absorption and linear dichroism spectra exhibit a single band of similar width. Although this suggests degenerate X and Y transitions, we cannot exclude from our data that these transitions are non-degenerate but tilted at the same angle with respect to the C_2 axis.

To compare our linear dichroism data with the heme orientations in the reaction center, we first calculated the angles between the directions joining the nitrogen atoms of opposite pyrroles for each heme and the C_2 symmetry axis, using the atomic coordinates of the X-ray analysis. Hemes are numbered according to [7], i.e. in the order heme 1, heme 2, heme 4, heme 3 and P. These angular values are given in table 2. We then considered two extreme cases for the optical transitions: (i) they are non-degenerate and occur along the X and Y directions; (ii) they are degenerate in the heme plane. The theoretical LD/A values for these two cases are reported in table 2. Experimental LD/A values are given in table 1. It is clear from the comparison of experimental and theoretical LD/A values that heme C_{556}, which presents two resolved optical transitions of nearly identical LD/A magnitudes and of opposite sign, can be unequivocally correlated with heme 2, the only heme presenting these characteristics in the theoretical LD/A values. This assignment leads us to ascribe the short wavelength transition (551 nm) to the molecular axis joining nitrogen A to nitrogen C. Hemes C_{559} and C_{554} can be either attributed to heme 1 or heme 3 as shown by the comparison of experimental and theoretical results in table 1. From our LD experiments there is no way to distinguish between these two possibilities. Several pieces of information have however been used in the literature to ascertain the assignment of the reaction center hemes. The first argument comes from kinetic data [8,9]. Under redox potential conditions where both high potential hemes are reduced, C_{559} is oxidized in about 300 ns and subsequently rereduced in 3 μs by C_{556}. This implies almost certainly that C_{559} is closer to the primary donor P than C_{556} [8,9]. Photoelectric measurements also imply the same type of arrangement [12]. It follows that C_{559} is the heme closest to P, i.e. heme 3, and therefore C_{554} is heme 1. This conclusion agrees with that based on EPR measurements of oriented chromatophores taken together with observations of interheme electron transfer at low temperature (Nitschke, W. and Rutherford, A.W., personal communication). Heme C_{552}, which exhibits a small experimental LD/A value, is then heme 4, in agreement with the fact that the axial ligands of this heme are two histidine imidazoles, as usually observed for low potential cytochromes [17]. From the above considerations, the sequence for the redox centers in *Rps. viridis* is thus: C_{554} C_{556} C_{552} C_{559} P.

The same assignment has also been derived from a recent ESR analysis of the magnetic interactions between high and low potential hemes (Nitschke, W. and Rutherford, A.W., personal communication).

Table 2

	Heme 1	Heme 2	Heme 4	Heme 3
Angle between the X and Y transitions and the C_2 axis	15°	45°	53.5°	28°
	93°	65.5°	57.5°	68°
Theoretical LD/A values for non-degenerate X and Y transitions	−2.70	−0.75	−0.09	−2.00
	+1.48	+0.72	+0.2	+0.86
Theoretical LD/A values for degenerate X and Y transitions	−0.60	−0.012	+0.054	−0.57

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