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Expression, purification, crystallization and preliminary X-ray structure analysis of wild-type and L(M196)H-mutant *Rhodobacter sphaeroides* reaction centres

The electron and proton transport mediated by protein-bound cofactors in photosynthesis have been investigated by various methods in order to determine the energetics, the dynamics and the pathway of this process. In purple bacteria, primary photosynthetic charge separation and the build-up of a proton gradient across the periplasmic membrane are catalyzed by the photosynthetic reaction centre (RC). Here, the purification, crystallization and preliminary X-ray analysis of wild-type and L(M196)H-mutant RCs of *Rhodobacter sphaeroides* are presented, enabling study of the influence of the protein environment of the primary electron donor on the spectral properties and photochemical activity of the RC.

1. Introduction

The conversion of light energy to chemical energy in photosynthesis is, at least from an energetic point of view, the most important biological process on earth. The first steps of the photosynthetic conversion of light to energy take place in photosynthetic membranes. Light is first absorbed by light-harvesting antenna complexes and the energy is then transferred to the so-called photosynthetic reaction centres (RCs), in which the primary charge separation and subsequent electron transfer across the photosynthetic membranes occur with a quantum yield of nearly 100%. The most elementary part of this process is implemented at the bacterial photosynthetic reaction centres. Several reviews have described the structure of the bacterial reaction centre in detail (Okamura & Feher, 1992; Deisenhofer & Norris, 1993). The photosynthetic RCs are complexes that contain several integral membrane proteins and a number of cofactors. In general, they consist of three protein subunits which are called H (heavy), M (medium) and L (light) according to their apparent molecular weights as determined by SDS-PAGE. The L and M subunits bind the photosynthetic pigments in a nearly symmetrical manner. The pigments are organized in two membrane-spanning electron-transfer branches called A and B. The bacteriochlorophylls (BChls) P_A and P_B are combined into a dimer to form the special pair P that serves as a primary electron donor. They are followed by two monomeric BChls B_A and B_B, two bacteriopheophytins (BPhe) H_A and H_B, two quinines Q_A and Q_B, one carotenoid molecule and a non-haem Fe atom. X-ray crystal structures of bacterial RCs show a substantial number of bound water molecules, including those that are enclosed in the hydrophobic cores of the complexes. Some of the conserved water molecules not only contribute to the stability of the protein structure but also appear to be directly involved in photosynthetic electron transfer. The axial ligands, hydrogen bonds and nearby residues that constitute the microenvironment of the BChls can greatly affect their photophysical and redox properties and can be altered by site-directed mutagenesis. Comparison of these crystal structures provides information on how protein-cofactor interactions may affect the spectral properties of BChls and the stability of the RC complex assembly.

The substitution of Leu196 in the M subunit by Phe in the bacterial RC from *Rhodobacter sphaeroides* (*Rba. sphaeroides*) has been described previously by Ivancich *et al.* (1998). It was shown that the redox potential of the primary electron donor was altered, while no changes in the hydrogen-bond interactions of the π -conjugated

carbonyl groups of the special pair P occurred. To increase the polarity of the primary electron donor environment, we mutated Leu-M196 to His. This paper describes the crystallization and preliminary structural analysis of the photosynthetic reaction centre from *Rba. sphaeroides* strain RV and its point mutant. Comparison of the wild-type RC with its mutant allows an investigation of the effects of the protein surroundings at the special pair P on the spectral properties and the photochemical activity of bacterial reaction centres.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutations were generated using PCR oligonucleotides as described previously (Khatypov *et al.*, 2005). Nucleotide changes were confirmed by DNA sequencing. Altered *pufL* or *pufM* genes

Figure 1
Crystals of wild-type RCs of the purple bacterium *Rba. sphaeroides* strain RV.

were shuttled into a broad host-range vector: a derivative of pRK415 that contained a 4.2 kb *EcoRI*–*HindIII* restriction fragment and included the *pufLMX* genes (Khatypov *et al.*, 2005). The resulting plasmids were inserted into *Rba. sphaeroides* strain DD13 through conjugative crossing to produce transconjugant strains with RC-only phenotypes (Jones *et al.*, 1992). The control strain comprised DD13 complemented with a pRK415-derived plasmid containing a wild-type copy of the *pufLMX* genes.

2.2. Purification of the bacterial reaction centre

Details of the growth of the wild-type and mutant bacterial strains under dark semiaerobic conditions have been described previously papers (Khatypov *et al.*, 2005; Fufina *et al.*, 2007). The cells were harvested and broken by ultrasonication; membranes for reaction-centre purification were then pelleted by ultracentrifugation. Reaction centres were solubilized from the membranes using lauryldimethylamine oxide (LDAO) and were purified by passage through a DE52 anion-exchange column followed by passage through Fractogel EMD DEAE (S) columns (Merck), as described in detail in Fufina *et al.* (2007). The purity of the reaction centre at each step was estimated by absorbance spectroscopy, measuring the ratio of protein absorbance at 280 nm to bacteriochlorophyll absorbance at 802 nm (A_{280}/A_{802} ; Okamura *et al.*, 1974). When the value of A_{280}/A_{802} falls below 1.4 the reaction centre is sufficiently pure. Such a reaction-centre preparation was used for subsequent crystallization experiments.

2.3. Crystallization

Crystallization of the reaction-centre preparation was performed at 289 K on siliconized glass slides (Hampton Research, USA) in Linbro plates using the hanging-drop vapour-diffusion method as described by Ermler *et al.* (1994) and McAuley-Hecht *et al.* (1998).

Figure 2
X-ray diffraction patterns from crystals of wild-type RC (a) and the L(M196)H mutant of RC (b): concentric rings indicate resolution ranges (labelled in Å) and the high-resolution diffraction pattern is magnified.

Figure 3

Electron-density maps around the special pair P calculated with coefficients $(2|F_{\text{obs}}| - |F_{\text{calc}}|)$ with $\sigma = 1.0$ (a) for wild-type RC and (b) for the L(M196)H mutant of RC.

Table 1

Statistics of data collection and processing.

Values in parentheses are for the last resolution shell.

	Wild-type RC	L(M196)H-mutant RC
Wavelength (Å)	0.91841	0.91841
Temperature (K)	100	100
Oscillation (°)	0.6	0.5
Total rotation range (°)	60	40
Crystal-to-detector distance (mm)	236.4	275.24
No. of crystals	1	1
Exposure time per image (s)	15	20
Space group	$P3_121$ [No. 152]	$P3_121$ [No. 152]
Unit-cell parameters (Å, °)	$a = b = 139.85$, $c = 185.37$	$a = b = 140.11$, $c = 185.8$
Asymmetric unit contents	1 molecule	1 molecule
V_M (Å ³ Da ⁻¹)	4.76	4.87
Solvent content (%)	73.9	74.2
Resolution limits (Å)	43.3–2.3 (2.5–2.3)	19.9–2.4 (2.5–2.4)
Completeness (%)	99.9 (99.9)	98.6 (98.7)
No. of reflections	474727 (76576)	221093 (23075)
No. of unique reflections	93247 (20440)	81691 (9340)
$R_{\text{observed}}(I)$ (%)†	7.2 (44.8)	6.2 (57.5)
Mosaicity (°)	0.14	0.22
Wilson B factor (Å ²)	45.6	59.9
Mean $I/\sigma(I)$	50.07 (3.03)	40.3 (1.71)
Multiplicity	5.09 (3.74)	2.70 (2.47)

$$\dagger R_{\text{observed}}(I) = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

Initial screening was accomplished at 298 K employing crystallization solutions from the MemStart, MemSys (Molecular Dimensions), MembFac, Crystal Screen Lite and MembFac HT (Hampton Research) screens. The final conditions for crystal growth were as follows: the drops consisted of reaction-centre protein (10 mg ml⁻¹), 3.5% 1,2,3-heptanetriol, 2% dioxane, 0.1% LDAO, 1 M potassium phosphate pH 7.4 and the reservoir solution consisted of 1.5 M potassium phosphate pH 7.4. Crystals appeared within 3–4 weeks and grew as prisms of variable size (Fig. 1).

2.4. Data collection

X-ray data sets were collected on beamline BL14.1 at HZB, BESSY II, Berlin, Germany equipped with a fast-scanning MX-225 CCD detector from Rayonics (Evanston, USA) and a Cryojet XL system (Fig. 2) at an X-ray wavelength of 0.9184 Å (Mueller *et al.*, 2012). Crystals were prepared for cryocooling by sequential soaking in mother liquor containing increasing concentrations of ethylene glycol to give a final concentration of 25%. All data were processed and scaled using *XDS* (Kabsch, 2010). All data were indexed, merged and processed using *XDS* with the XYCORR, INIT, COLSPOT, IDXREF, DEFPIX, XPLAN, INTEGRATE and CORRECT

options. The minimal value of the *XDS* quality-of-fit function was used for a semiautomatic determination of the space group. The crystals of wild-type and mutant RC of *Rba. sphaeroides* belonged to space group $P3_121$. Detailed data statistics are presented in Table 1.

2.5. X-ray structure analysis and preliminary X-ray structure analysis

Structures of wild-type and mutant RC were resolved by the molecular-replacement technique using the program *Phaser* with the rigid-body refinement option (McCoy *et al.*, 2007). The X-ray structure of the photosynthetic reaction centre from *Rba. sphaeroides* at 2.0 Å resolution (PDB entry 3i4d; R. Fujii, S. Adachi, A. W. Roszak, A. T. Gardiner, R. J. Cogdell, N. W. Isaacs, S. Koshihara & H. Hashimoto, unpublished work) was utilized as a search model in both cases. Metal ions, water molecules and carbohydrates were excluded from the search model. One molecule was found in the asymmetric unit. The Matthews coefficient (Matthews, 1968) was 4.76 Å³ Da⁻¹ (73.9% solvent content) for wild-type RC and 4.87 Å³ Da⁻¹ (74.2% solvent content) for the mutant RC (Table 1). The top molecular-replacement models were assessed using *Z*-score and log-likelihood gain (LLG) statistics. Before refinement, 5% of the observations were chosen at random and were set aside for cross-validation analysis and to monitor the various refinement strategies. The models were initially subjected to crystallographic refinement using *REFMAC5* (Murshudov *et al.*, 2011) and subsequently using *PHENIX* (Afonine *et al.*, 2012). Manual rebuilding of the models was carried out in *Coot* (Emsley *et al.*, 2010). Figures were prepared using *PyMOL* (<http://www.pymol.org>).

Analysis of difference electron-density maps calculated with $(|F_{\text{obs}}| - |F_{\text{calc}}|)$ coefficients in *Coot* (Emsley *et al.*, 2010) confirms the position of the L(M196)H mutation in RC (Fig. 3). Refinement of both structures is currently in progress and the structures will be published in the future.

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References

- Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2012). *Acta Cryst. D* **68**, 352–367.

- Deisenhofer, J. & Norris, J. R. (1993). *The Photosynthetic Reaction Center*. San Diego: Academic Press.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Ermiler, U., Fritzsche, G., Buchanan, S. K. & Michel, H. (1994). *Structure*, **2**, 925–936.
- Fufina, T. Y., Vasilieva, L. G., Khatypov, R. A., Shkuropatov, A. Y. & Shuvalov, V. A. (2007). *FEBS Lett.* **581**, 5769–5773.
- Ivancich, A., Artz, K., Williams, J. C., Allen, J. P. & Mattioli, T. A. (1998). *Biochemistry*, **37**, 11812–11820.
- Jones, M. R., Visschers, R. W., van Grondelle, R. & Hunter, C. N. (1992). *Biochemistry*, **31**, 4458–4465.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Khatypov, R. A., Vasilieva, L. G., Fufina, T. Y., Bolgarina, T. I. & Shuvalov, V. A. (2005). *Biochemistry*, **70**, 1256–1261.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McAuley-Hecht, K. E., Fyfe, P. K., Ridge, J. P., Prince, S. M., Hunter, C. N., Isaacs, N. W., Cogdell, R. J. & Jones, M. R. (1998). *Biochemistry*, **37**, 4740–4750.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Mueller, U., Darowski, N., Fuchs, M. R., Förster, R., Hellmig, M., Paithankar, K. S., Pühringer, S., Steffien, M., Zocher, G. & Weiss, M. S. (2012). *J. Synchrotron Rad.* **19**, 442–449.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Okamura, M. Y. & Feher, G. (1992). *Annu. Rev. Biochem.* **61**, 861–896.
- Okamura, M. Y., Steiner, L. A. & Feher, G. (1974). *Biochemistry*, **13**, 1394–1403.