Crystallization and X-ray analysis of the reaction center from the thermophilic green bacterium *Chloroflexus aurantiacus*

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Abstract The photochemical reaction center of *Chloroflexus (Cf.) aurantiacus*, a membrane bound pigment-protein complex, has been crystallized in the presence of monodisperse polyoxyethylene detergents. The crystals possessed a pronounced polymorphism. Three different crystal forms belonging to triclinic, monoclinic and orthorhombic space groups have been characterized by X-ray analysis. The triclinic crystal form, with unit cell dimensions of $a = 88$ Å, $b = 115$ Å and $c = 151$ Å, diffracts up to 3.2 Å in two directions and to 4.0 Å in the third direction.

Key words: Crystallization; Membrane protein; X-ray diffraction; Reaction center; Detergent; *Chloroflexus aurantiacus*

1. Introduction

The initial photochemical electron transfer reactions in photosynthesis occur in membrane bound pigment-protein complexes called reaction centers (RC). Light initiates a series of fast electron transfer steps within the RC which result in an electrogenic charge separation across a cell membrane. The reaction centers of a few photosynthetic bacteria, primarily belonging to the family of *Rhodospirillaceae*, have been biochemically and spectroscopically well characterized [1]. Furthermore, RCs of *Rp. viridis* and *Rb. sphaeroides* have been crystallized and their structures elucidated at atomic resolution [2–6]. Most RCs are comprised of three protein subunits which are called H (heavy), M (medium) and L (light) according to their electrophoretic mobilities in SDS-PAGE. An additional protein subunit, a tetraheme cytochrome, is present in *Rp. viridis* RC. Noncovalently bound to the L-M moiety is a set of cofactors consisting of four bacteriochlorophyll, two bacteriopheophytin, 1–2 quinone molecules and one atom nonheme iron. The cofactors are arranged in two branches with approximate C2 symmetry around the L and M protein subunits. The same approximate two-fold symmetry axis is observed between the two protein entities.

In contrast to the three and four protein subunit-containing RC of purple bacteria, the RC of the thermophilic bacterium *Cf. aurantiacus* is composed of only two integral membrane proteins with almost identical molecular masses of approximately 35 000 Da [7–10]. The two polypeptides possess a moderate identity of 40% each to the respective L and M proteins of *Rb. sphaeroides* and *Rp. viridis* RCs. A characteristic feature of the L subunit of the *Cf. aurantiacus* RC is its extended hydrophilic region at the N-terminus. It was speculated that this domain could be involved in binding the chlorosome antenna structure to the cytoplasmic membrane. Noncovalently bound to these entities are three bacteriochlorophyll, three bacteriopheophytin and 1–2 menaquinone molecules [11,12]. When compared to the amino acid sequences of purple bacteria RCs, some functionally important amino acids are not conserved. These differences have helped to explain certain features such as the presence of a bacteriopheophytin instead of a bacteriochlorophyll and slower charge transfer reactions. Due to the absence of the so-called H subunit, the *Cf. aurantiacus* RC is the smallest functional RC and has an approximate total mass of 75 000–80 000 Da.

About a decade ago the photosynthetic RC of *Rp. viridis* was the first membrane protein to be crystallized and its three-dimensional structure to be solved at atomic resolution [2,13,14]. Subsequently, the structure of three-dimensional crystals of a few other membrane proteins have been determined [2,4–6,15–19]. Despite these achievements, it is still a challenge to obtain crystals of integral membrane proteins which are suitable for subsequent structure analysis.

Initial crystallization attempts with *Cf. aurantiacus* RC yielded crystals that diffracted X-rays to about 10 Å [20]. Here, we report the crystallization of *Cf. aurantiacus* RC in the presence of different detergents and detergent mixtures that resulted in crystals which diffracted to a maximum resolution of 3.2 Å.

2. Materials and methods

Chloroflexus aurantiacus RC was isolated as described previously [7] with the following modifications. Briefly, chromatophores from 140 g wet weight cells were solubilized with 1% LDAO and subjected to DEAE52-cellulose chromatography. The appropriate fractions of RC (about 150 mg RC protein) were pooled, dialyzed against 20 mM Tris-HCl pH 8.0 and subjected to a second DEAE52-cellulose chromatography. Further purification was achieved by chromatofocusing on a 1×40 cm long Polybuffer exchange (PBE-94) column previously equilibrated with 20 mM Tris-HCl, 0.2% LDAO, pH 9.0. The dialyzed RC (up to 100 mg RC protein) was adjusted to 1% LDAO and applied onto the column. After washing with 50 ml equilibration buffer, 200 ml of elution buffer was passed through the column at a flow rate of 30 ml/h. The elution buffer consisted of 30% Polybuffer-96 and 70% Polybuffer-74 as described by the manufacturer and supplemented with 0.2% LDAO. The pH was adjusted with acetic acid to a pH of 6.0. The elution of RC occurred after the column was washed with approximately 170 ml elution buffer which was equivalent to pH 6.4–6.7. The pooled RC fractions were concentrated to about 2–3 ml by ultrafiltration using a Omega 30 K membrane (Pall-Filtron) and adjusted to 1% LDAO (v/v) final concentration. The RC sample was applied onto a 2.5×90 cm Fractogel TSK HW-50 (S) column previously equilibrated with 50 mM Na-phosphate buffer pH 7.5 containing 100 mM NaCl and 0.2% LDAO at a flow rate of 30 ml/h.

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Abbreviations: Rp., Rhodopseudomonas; *Rb., Rhodobacter*; *Cf., Chloroflexus*; RC, reaction center

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2.1. Detergent exchange and sample concentration

The combined RC fractions from the Fractogel column were dialyzed against 20 mM Tris-HCl, pH 8.0, 0.1% LDAO and loaded onto a 1.3×10 cm DEAE52-cellulose column previously equilibrated with 20 mM Tris-HCl pH 8.0 and 0.15% $C_{10}E_8$ or the detergent of choice. After washing with 150 ml equilibration buffer, RC was eluted with 80 mM NaCl in equilibration buffer, resulting in 4–5 ml of RC-containing solution. Further concentration and salt removal was accomplished with a Microsep microconcentrator (Pall-Filtron, cutoff 50 kDa) at 5000×*g* at 4°C. The stock RC solution used for crystallization contained approximately 30–50 mg/ml RC protein, 5 mM NaCl, 0.15% $C_{10}E_8$ (or detergent as stated) in 20 mM Tris-HCl, pH 8.0.

Crystallization experiments were carried out using the sitting drop vapor diffusion technique in Cryschem crystal growth chambers (C. Supper, Boston, MA). If not otherwise stated, droplets of 15 μl, with a final protein concentration of 10–15 mg RC protein per ml, contained 10–12% polyethylene glycol 8000 (PEG 8000, Merck), 5 mM Tris-HCl, pH 8.0, 0.4% *N,N'*-bis(3-D-gluconamidopropyl)amine [21] (a generous gift of W. Welte, University of Konstanz) and the following monodisperse polyoxyethylene detergent(s) (Calbiochem): (A) 0.15% $C_{10}E_8$, (B) 0.15% $C_{10}E_8$, 0.15% $C_{10}E_6$, 0.1% C_8E_5; (C) 0.15% $C_{10}E_8$, 0.15% $C_{10}E_6$, 0.1% C_8E_5, 0.15% $C_{12}E_9$, 0.2%$C_{12}E_8$. This solution was equilibrated against 1 ml of reservoir containing 18–26% PEG 8000 in H_2O at 15°C.

2.2. X-ray analysis

Crystals were analyzed with a Mar-Research imaging plate detector or with a Huber precession camera connected to a Rigaku Rotaflex RU 200 HB rotating anode generator (graphite monochromatized CuKα-radiation). Data were evaluated with the program MOSFLM and the CCP4 program suite.

3. Results and discussion

Only highly purified *Cf. aurantiacus* RC was used for crystallization trials. The purity was assessed by SDS-polyacrylamide gel electrophoresis, by relative absorbance intensities at 865, 813 and 755 nm of 0.95:1.00:0.77 and a A_{280}/A_{813} ratio of 1.3. Attempts to optimize conditions for crystallization included varying the detergents, precipitation agents, addition of different amphiphiles, ionic strength, pH and temperature. Although crystals could be grown under a variety of conditions, the following characteristics for crystal growth were observed: (i) salts as precipitating agents were unsuitable due to their severe denaturing effects; (ii) crystallization with glucoside detergents required the presence of 250–450 mM salts (preferentially NaCl); (iii) the presence of small amphiphilic molecules – except *N,N'*-bis(3-D-gluconamidopropyl)amine – such as triethylammonium phosphate, benzamidine, heptane-1,2,3-triol, piperidine carbonic acid, benzenesulfonic acid and heptyl dimethyl aminoxide did not cause any significant improvement in crystal quality; (iv) when crystallizing with polyoxyethylene detergents, PEG 8000 was the most suitable precipitating agent and the ionic strength had to be kept low.

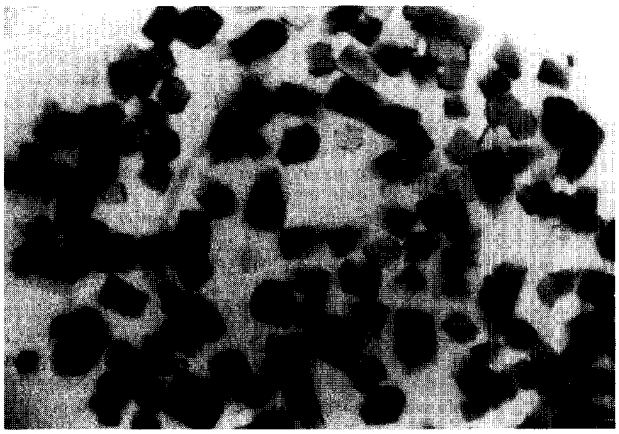

Fig. 1. Crystals of *Cf. aurantiacus* RC grown in the presence of polyoxyethylene detergent mix B.

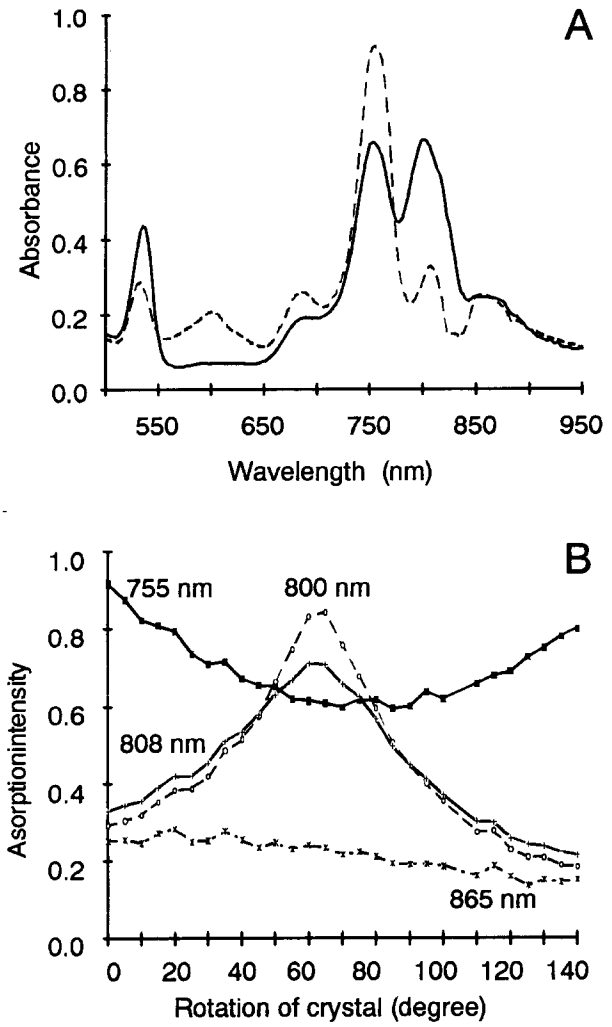

Fig. 2. Polarized absorption spectra of a triclinic *Cf. aurantiacus* RC crystal. A: The direction of the polarized light beam was parallel to the crystal's *c*-axis. The polarization was kept constant while the crystal was rotated around its *c*-axis. Solid line: Crystal at the relative position of 0° (the crystal appeared blue); dashed line: Crystal after a rotation of 70° (crystal appeared pink.) B: Absorption intensities at 755, 800, 808 and 865 nm as a function of crystal rotation.

Under optimal conditions (as described above) crystals with dimensions of approximately 500×400×150 μm^3 grew within 4–6 weeks. There was considerable variety in the crystal morphology (Fig. 1), ranging from twinned and intermeshed platelets to bundles and stacks, although single crystals could be obtained (Fig. 1). Simply changing the protein concentration and/or the volume of the mother liquor did not significantly improve this crystallization behavior. Also the addition of small amphiphilic molecules (see above) was not beneficial for crystallization. Further attempts to obtain larger single crystals by modifying the precipitation agent, e.g. addition

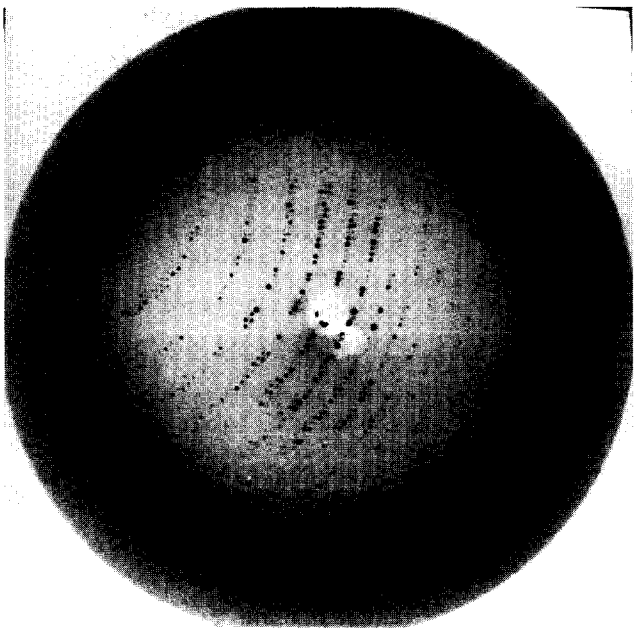

Fig. 3. X-ray diffraction pattern of a single triclinic RC crystal after a 1° degree rotation during a 15 min exposure. The edge of the detector plate is equivalent to 3.0 Å.

of organic solvents such as 1,4-dioxane, methyl-2,4-pentanediol, ethanol, *n*-propanol, and 2-propanol, have thus far not been successful.

Absorption spectra of crystals (Fig. 2) recorded with linear polarized light revealed native absorption properties with characteristic Q_y transitions at 750, 810 and 860 nm and a strong dichroism. Depending on the relative orientation of the crystal to the polarized light, they appeared either blueish with relative absorption intensities at 550, 750, 810 and 860 nm of 0.30, 1, 0.37, 0.30 or, after a 70° degree rotation, pink with intensities of 0.47, 0.71, 0.72, 0.27. Upon strong light exposure, a reversible blue to pink color change indicated photochemically active RC molecules within the crystal.

The morphology of crystals, but not the geometry of the unit cell, and the quality of diffraction pattern were notably influenced by the polyoxyethylene detergent (mixtures) and the amphiphile *N,N'*-bis(3-D-gluconamidopropyl)amine. In the presence of 0.15% $C_{10}E_8$ only, well diffracting crystals (beyond 2.7 Å) but with high mosaic spread were obtained. Upon the addition of 0.4% *N,N'*-bis(3-D-gluconamidopropyl)-amine the mosaicity was almost completely abolished although diffraction resolution decreased to 3.2–3.5 Å (Fig. 3). In general, no crystallization occurred at concentrations higher than 0.6% *N,N'*-bis(3-D-gluconamidopropyl)amine or 1% for other amphiphilic molecules. Crystallization in the presence of the more complex detergent mixtures B and C (including 0.4% *N,N'*-bis[3-D-gluconamidopropyl]amine) benefitted from a diminished amount of noncrystalline precipitation or crystal aggregation and a considerably larger number of single crystals. As a result of preferential growth in two dimensions, platelet shaped crystals were quite often observed. These dimensions might be responsible for the slightly anisotropic diffraction properties of the crystals with approximately 0.8 Å higher resolution (e.g. 3.2 vs. 4.0 Å; see Table 1) when the X-ray beam is oriented parallel to the crystal *c*-axis as compared to a perpendicular exposure.

As indicated by the polymorphous crystallization behavior, we observed at least three different crystal forms: an orthorhombic, a monoclinic and, most frequently, a triclinic form (Table 1). It was not possible to relate a particular crystal form to specific crystallization conditions. Not only was the triclinic geometry most abundant, the cell parameter of these crystals, even when grown within the same droplet, showed considerable variation as well. The best analyzed triclinic crystal diffracted up to 3.2 Å. From precession photographs and from Kabsch's autoindexing program, which was applied to oscillation photographs, we determined unit cell parameters which are given in Table 1. Currently, the best data set of this triclinic crystal has a completeness of 45% at 3.5 Å (R_{sym} 12.5%) and 70% at 5.5 Å (R_{sym} 10%).

The biggest obstacle in recording a complete native data set with a resolution of 3.0–3.5 Å is the limited number of well diffracting crystals combined with the occurrence of varying triclinic crystal geometries. Therefore, we had to record a complete data set with one crystal. Although a fast imaging plate was used as a detector and RC crystals seemed to be reasonably stable against X-ray radiation, the total exposure time required for data collection resulted in significant X-ray damage and, hence, diminished the diffraction properties of the crystal. One suitable approach to overcome this obstacle could be cryocrystallography with flash cooled crystals in the presence of cryoprotectant under conditions that inhibit ice formation and still retain the structural integrity of the crystal. This technique has been shown to minimize radiation damage and thus allow data collection over an extended time period. It has been successfully applied to water soluble and, more recently, to membrane proteins [22].

Although crystallization of *Cf. aurantiacus* RC can be achieved under various conditions in the presence of different detergents, it is still difficult to consistently obtain well-or-

Table 1
Unit cell parameter of three different RC crystals from *Cf. aurantiacus*

Crystal form	Space group	Unit cell *a*, *b*, *c*; α, β, χ			Molecules per asymmetric unit[a] (V_M in Å³/Da)	Resolution *a*-*b*-lattice (*c*-lattice)
T	P1	88 Å	115 Å	151 Å	4–6	3.2 Å
		96°	91°	102°	(4.7; 3.2)	(3.9 Å)
M	$P2_1$	79 Å	212 Å	168 Å	4–6	3.6 Å
		90°	91°	90°	(4.7; 3.1)	(5.0 Å)
O	$P2_x2_x2_x$[b]	115 Å	146 Å	175 Å	2–3	3.8 Å
					(4.8; 3.2)	(5.5 Å)

[a]The volume-to-mass ratios (V_M, Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497) of membrane proteins fall typically in the range of 3–5.5 Å³/Da [2–6]. Consequently, the number of molecules per asymmetric unit cannot be deduced unequivocally.
[b]The screw axes could not be determined reliably because of the crystal lability and the low diffraction quality of crystal form O.

dered and highly diffracting crystals with a constant unit cell geometry. The difficulties in crystallizing the *Cf. aurantiacus* RC might be related to the absence of a H subunit which could form crystal contact along the *c*-lattice by hydrophilic interactions. It is known that in crystals of *Rp. viridis* RC the H protein and, additionally, the cytochrome subunit provide almost exclusively protein-to-protein contact and are responsible for the packing of neighboring molecules in the crystal *c*-lattice [14,23]. Although in the case of *Rb. sphaeroides* RC the crystal packing is completely different, again, the H subunit makes hydrophilic interactions with neighboring RC (detergent) molecules [24]. In light of these results one can speculate whether the extended hydrophilic N-terminus of the L protein subunit of the *Cf. aurantiacus* RC has any impact or is partially responsible for the unfavorable crystallization and if so, can protein chemical modification result in any improvement of crystals.

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