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New tetragonal form of reaction centers from *Rhodobacter sphaeroides* and the involvement of a manganese ion at a crystal contact point

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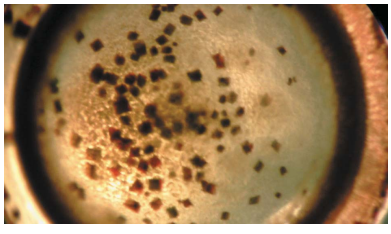

Crystals have been obtained of wild-type reaction centers from *Rhodobacter sphaeroides* using manganese chloride as a precipitating agent. The crystals belong to the tetragonal space group $P4_222$, with unit-cell parameters $a = b = 207.8$, $c = 107.5$ Å. The crystal structure has been determined to a resolution limit of 4.6 Å using a previously determined structure of the reaction center as a molecular-replacement model. The calculated electron-density maps show the presence of a manganese ion at one of the crystal contact points bridging two symmetry-related histidine residues, suggesting that the metal plays a key role in facilitating the crystallization of the protein in this form.

1. Introduction

Despite many technical advances in protein crystallography, our understanding of the functions of an important class of proteins, namely integral membrane proteins, remains limited owing to difficulties in obtaining crystals of these proteins that are suitable for diffraction experiments (Sowadski, 1996; Bowie, 2001). Although the first crystal structure of an integral membrane protein was obtained in 1985 (Deisenhofer *et al.*, 1985), extensive efforts have yielded a relatively limited number of diffraction-quality crystals. In contrast, thousands of structures of soluble proteins are solved every year. The biochemical problems in dealing with these biologically challenging complexes have prevented consideration of membrane proteins for structural studies in proteomic research despite their central importance in many cellular systems.

The limited success in crystallizing integral membrane proteins can be attributed to several factors. Integral membrane proteins are more difficult to isolate than water-soluble proteins, as the native membrane surrounding the protein must be disrupted and replaced with detergent molecules without causing any denaturation. The detergent allows solubilization of membrane proteins into an aqueous environment for crystallization using conventional techniques, such as vapor diffusion. However, the complexity of the conditions that must be searched increases, especially when amphiphiles or mixed detergents are included in the crystallization solutions. In addition to the complexity of possible conditions, a common problem with crystals of membrane proteins is that they are often disordered, as crystal contacts involve interactions between hydrophilic regions, which are quite limited in highly hydrophobic proteins.

One strategy to overcome this problem is to introduce a large hydrophilic domain by the formation of a complex with a monoclonal antibody. This approach lead to the crystallization of cytochrome *c* oxidase from *Paracoccus dentrificans* (Iwata *et al.*, 1995), the cytochrome bc_1 complex from *Saccharomyces cerevisiae* (Hunte *et al.*, 2000) as well as several potassium channels (Zhou *et al.*, 2001; Dutzler *et al.*, 2003; Jiang *et al.*, 2003). Although the success of these efforts demonstrate the feasibility of the approach, the work is difficult and its application has been limited. In principle, proteins can be altered using mutagenesis to improve possible interactions in crystal contact sites, but this approach is only effective when a detailed structural model already exists (Pautsch *et al.*, 1999; Cámara-Artigas *et al.*, 2001). The addition of amphiphiles can play a critical role in facilitating the contacts in the crystal, as found for the light-harvesting complex II from *Rhodospirillum molischianum* which was bridged by

the amphiphile heptanetriol (Koepke *et al.*, 1996), but such outcomes are not predictable. In a few cases, integral membrane proteins have been found to crystallize better when a metal was present in the crystallization solution. For example, the AQP1 water channel and MscL channel were found to yield significantly improved diffraction quality for gold derivatives (Chang *et al.*, 1998; Sui *et al.*, 2001), with the gold compound clearly found to bridge symmetry-related proteins in the crystal structure of MscL. However, metals are usually treated as additional possible additives in crystallization rather than as part of a systematic approach.

Reaction centers are a model integral membrane protein that can be used to develop new methodologies for the crystallization of membrane proteins. The reaction center can be crystallized in several different space groups that have yielded structures with resolution limits ranging from 3.0 to 1.9 Å (reviewed in Fritzsch, 1998; Cámara-Artigas & Allen, 2004). Moreover, the availability of mutants of the reaction center provides the opportunity to investigate the roles of individual amino-acid residues, including the involvement of specific protein interactions at contact regions in crystals (Cámara-Artigas *et al.*, 2001). In this work, we report the importance of manganese ions in the crystallization of the reaction center in a new crystal form.

2. Materials and methods

Wild-type reaction centers were expressed and isolated as previously described by Williams *et al.* (1992). Prior to crystallization, samples were further purified with a tertiary amine column using FPLC (Pharmacia LKB, Uppsala, Sweden). After this chromatography step, the protein was dialyzed against 15 m*M* Tris–HCl pH 8 and 0.025% lauryl dimethylamine oxide. The crystallization conditions followed those used to grow the orthorhombic form (Allen *et al.*, 1987). A protein solution was prepared containing 12% polyethylene glycol 4000, 3.9% heptanetriol, 0.06% lauryl dimethylamine oxide, 15 m*M* Tris–HCl pH 8 and reaction centers at a concentration of 8.3 mg ml^{-1} (A_{802} = 25). Different salts were added to these protein solutions as described in §3. Drops containing 20–50 µl of the protein solution with the added salt were equilibrated by vapour diffusion against a 1 ml reservoir that contained 15% polyethylene glycol 4000. All crystallization was performed at room temperature.

Diffraction data were collected at 295 K on a Rigaku R-AXIS IV^{++} image-plate area detector using Cu $K\alpha$ radiation from a Rigaku RU-200HB rotating-anode X-ray generator (50 kV, 100 mA). The X-ray source was equipped with an Osmic confocal mirror assembly. The data were processed with *MOSFLM* (Leslie, 1999) and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

For the initial trials, the 0.36 *M* sodium chloride used to grow the orthorhombic crystals in the original conditions was replaced with manganese chloride at concentrations varying from 0.05 to 0.3 *M*. The largest crystals were found using a concentration of 0.21 *M* manganese chloride. The addition of 0.04 *M* sodium chloride along with the 0.21 *M* manganese chloride resulted in more reproducible growth of crystals. Under these optimal conditions, the crystals first appeared after 1–2 d and grew slowly until they reached a maximal size of 0.5 mm after two weeks (Fig. 1). The morphology of the crystals was different to the orthorhombic form on which the conditions were based. No crystals were observed when these conditions were performed with a different salt, such as calcium chloride, magnesium chloride or zinc chloride, in the absence of either sodium chloride or manganese chloride.

Table 1
Data and model statistics of the tetragonal crystals.

Values in parentheses are for the last 0.2 Å shell.

Space group	$P4_222$
Unit-cell parameters (Å)	
a	207.8
b	207.8
c	107.5
Resolution limits (Å)	25–4.6
No. of observations	32336
No. of unique reflections	12646
Completeness (%)	94.2 (91.0)
$R_{\rm merge}$† (%)	18.2 (39.6)
$I/\sigma(I)$	5.3 (1.0)
R factor	0.34 (0.34)
$R_{\rm free}$	0.33 (0.39)
R.m.s. deviations, bonds (Å)	0.11
R.m.s. deviations, angles (°)	1.60

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$.

A native data set was measured with a resolution limit of 4.6 Å (Table 1). The reaction-center crystals were found to belong to the tetragonal space group $P4_222$, with unit-cell parameters $a = b = 207.8$, $c = 107.5$ Å. The space-group assignment was made from the systematic absences. The use of 0.36 *M* sodium chloride yields a $P2_12_12_1$ orthorhombic space group with unit-cell parameters $a = 138$, $b = 78$, $c = 142$ Å, rather than the tetragonal form observed with manganese chloride. Two tetragonal forms have been previously reported, but the space groups, $P4_32_12$ and $P4_22_12$ (Katona *et al.*, 2003; Allen, 1994), differ from those reported here. Thus, the crystals are a crystal form of the protein that has not previously been observed, presumably owing to the inclusion of manganese chloride in the crystallization solution.

The structure was solved using molecular replacement and the program *CNS* (Brünger *et al.*, 1998). The starting model was the wild-type reaction center from *Rhodobacter sphaeroides* (PDB file 1m3x) solved in the trigonal form (Cámara-Artigas *et al.*, 2002) with the water and lipid molecules removed. Following clear identification of the orientation and position, the structure was refined using the *CNS* package. Rigid-body refinement was performed before positional refinement using data with $F_o > 2\sigma$ in the resolution range 25–4.6 Å. Cycles of positional refinement and temperature-factor refinement were alternated with manual building using the resulting σ_A-weighted $(2F_o - F_c)$ and $(F_o - F_c)$ electron-density maps and the program *O* (Jones *et al.*, 1991). The quality of the resulting structure was checked with *PROCHECK* (Laskowski *et al.*, 1993)

After refinement, several symmetrically located large difference electron-density peaks were present near the 4_2 rotation axis where

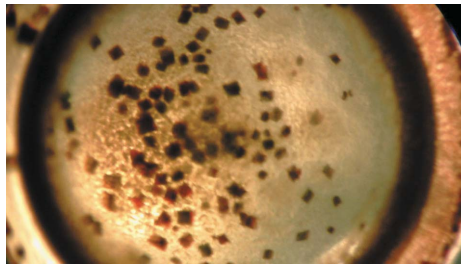

Figure 1
Crystals of the wild-type reaction center from *R. sphaeroides* as grown in a sitting drop by vapor diffusion. The crystals belong to the tetragonal space group $P4_222$, have the characteristic color of the protein and have a maximal length of 0.5 mm.

several proteins are in contact (Fig. 2*a*). The presence of the peaks did not change as different resolution limits of the data were used or other independent data sets. A close examination of the major peaks showed that they could be modeled as arising from a manganese ion positioned between His128 of the H subunit from two symmetry-related proteins (Fig. 2*b*). The electron density is elongated, suggesting that the coordination of the manganese is completed by the presence of two bound water molecules. In addition to coordinating the manganese, the water molecules would be hydrogen bonded to the carbonyl groups of HisH126. The identity of the ion cannot be uniquely determined based upon the current diffraction data without the availability of multiple-wavelength data sets. However, the electron density is tentatively assigned as arising from the presence of manganese based upon the requirement of manganese chloride for growth of the form. Matching the ionic strength but using only sodium chloride resulted only in the orthorhombic form.

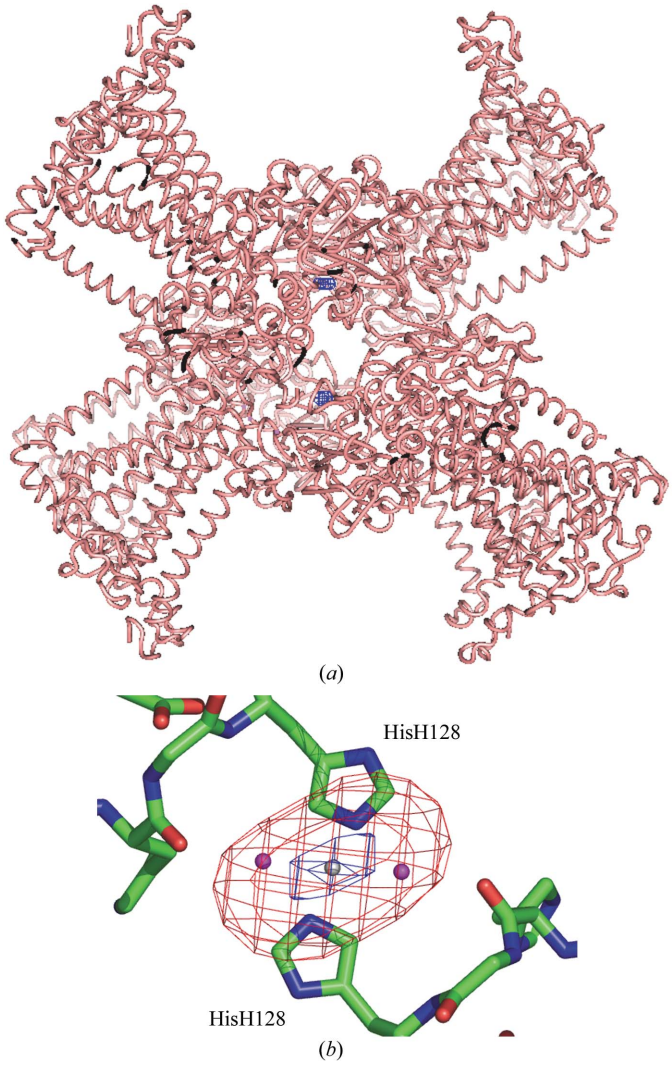

Figure 2
(*a*) The packing of the reaction center (light red) around the 4_2 screw axis. The $F_o - F_c$ electron-density map shown at a contour level of 5σ (blue) shows the presence of an atom that is not part of the protein structure. The peak is located at the contact point between two proteins; two symmetry-related peaks are shown. (*b*) A close-up view of the difference peak showing His128 of the H subunit from two proteins in the unit cell coordinating an atom identified as manganese (silver sphere). Also shown are two possible bound water molecules (purple spheres). The electron density is shown at 6σ (blue) and 3σ (red).

When other salts were present in place of manganese chloride, no crystals were observed. The optimal conditions required a relatively small amount of sodium chloride, probably to help minimize binding of manganese to secondary sites. Divalent ions that bridge symmetry-related proteins have been found for crystals of water-soluble proteins (Iyer *et al.*, 2000). For example, wild-type lysozyme has been found to bind nickel at such a special position (Wray *et al.*, 2000).

The binding site of the manganese requires a contribution from two proteins and so does not represent any physiologically relevant binding site. However, HisH128 is one of the amino-acid residues that can bind zinc at a nearby site on the protein (Axelrod *et al.*, 2000). HisH128 is also one of the amino-acid residues that are part of the contact sites for the tetragonal and trigonal forms but not the orthorhombic form (Cámara-Artigas & Allen, 2004). The unit cell of the new tetragonal form has a large solvent content of 80% with very limited interactions between proteins. One region of the periplasmic side of the protein has several contacts, but on the cytoplasmic side the contacts are limited to an interaction between ThrH63 and ThrH74 and the interactions involving the manganese. Thus, the contribution of the manganese site appears to be critical in establishing crystalline order.

The new crystal form of the reaction center has been grown by use of a solution containing the precipitation agent polyethylene glycol and the salt manganese chloride. The usefulness of polyethylene glycol has been recognized for many years as a crystallization agent (McPherson, 1999) and the majority of membrane proteins have been crystallized using polyethylene glycol or the monomethyl ether form (Ostermeier & Michel, 1997; Iwata, 2003; Lemieux *et al.*, 2003; Wiener, 2004). These results suggest that the crystallization of membrane proteins may be facilitated by the inclusion of metals in the crystallization solutions, in particular as part of polyethylene glycol solutions, not as additives but rather as primary crystallization components. In addition to the wild-type crystals, crystals of a manganese-binding mutant have been obtained recently as described elsewhere (Thielges *et al.*, 2005). Experiments to yield crystals with an improved diffraction quality as well as obtain crystals of other integral membrane proteins using similar conditions are in progress.

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