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Crystallization and Preliminary Analysis of Crystals of Cytochrome c_2 from *Rhodopseudomonas capsulata*

Two crystal forms of the cytochrome c_2 isolated from *Rhodopseudomonas capsulata* have been obtained. One crystal form (type I), grown from ammonium sulfate solutions at pH 7·5, belongs to the space group $R32$ with unit cell dimensions of $a = b = 100{\cdot}0$ Å, and $c = 162{\cdot}2$ Å in the hexagonal setting. These crystals most likely contain two molecules in the asymmetric unit. The other crystal form (type II) was obtained from polyethylene glycol 6000 solutions at pH 6·5. Type II crystals belong to the space group $P3_121$ or $P3_221$ with one molecule per asymmetric unit and unit cell dimensions of $a = b = 52{\cdot}4$ Å, and $c = 87{\cdot}9$ Å. Both crystal forms diffract to at least 1·8 Å resolution and appear to be resistant to radiation damage.

The *c*-type cytochromes are ubiquitous in nearly all living organisms and are involved in a wide variety of electron transport pathways. The cytochromes c_2, for example, are a family of such heme-containing proteins found in most photosynthetic non-sulfur purple bacteria and some non-phototrophic bacteria (for a review, see Meyer & Kamen, 1982). While structurally similar to the mitochondrial cytochromes *c*, they are functionally distinct in their dual role as carriers in both photosynthesis and respiration (Bartsch, 1978). On the basis of amino acid sequences, the cytochromes c_2 can be subdivided into two major groups (Ambler, 1977). The members of one subgroup have a peptide chain essentially the same size as the mitochondrial cytochromes *c*, while those of the other have at least several small insertions in addition to a single-residue deletion.

The X-ray structure of one cytochrome c_2, isolated from *Rhodospirillum rubrum*, has been solved and refined to a nominal resolution of 1·68 Å (Salemme *et al.*, 1973*a*; Bhatia, 1981). A comparative study of the *R. rubrum* cytochrome c_2 structure with that of horse cytochrome *c* demonstrated considerable similarity between these two proteins with respect to their three-dimensional fold (Salemme *et al.*, 1973*b*). Recently, crystals of the cytochrome c_2 from *Rhodopseudomonas viridis* have been obtained (Miki *et al.*, 1986). Unlike the protein isolated from *R. rubrum*, the *Rps. viridis* cytochrome c_2 belongs to the first subdivision in that its amino acid sequence is closer to the mitochondrial cytochromes *c* than to other bacterial cytochromes c_2 in terms of the number of amino acids (Ambler *et al.*, 1979).

We describe here the crystallization and preliminary X-ray crystallographic investigation of another cytochrome c_2, this one isolated from *Rhodopseudomonas capsulata*, strain St Louis. The organism itself has been well studied both biochemically and genetically. The amino acid sequence of the *Rps capsulata* cytochrome c_2 is known (Ambler *et al.*, 1979) and the structural gene for the protein has been cloned (Daldal *et al.*, 1986). It is one of the larger cytochromes c_2 and to align its amino acid sequence with the smaller *R. rubrum* protein, a six-residue insertion near the N terminus and a two-residue deletion near the C terminus are required (Ambler *et al.*, 1979).

The protein used for the studies described here was isolated and purified according to the method of Bartsch (1971). A survey of possible crystallization conditions was conducted at room temperature using either the hanging-drop or sitting-drop method of vapor diffusion (for a review, see McPherson, 1982). The protein concentration was at 30 mg/ml in 10 mM-Na^+/K^+ phosphate, 5 mM-sodium azide (pH 7·0). Various precipitants were tested and it was found that both ammonium sulfate and polyethylene glycol (PEG)† 6000 solutions produced small crystals. Those crystals grown from ammonium sulfate solutions will be referred to as type I; those from PEG solutions as type II.

For X-ray diffraction experiments the crystals were sealed in thin-walled quartz capillary tubes. X-ray diffraction photographs were recorded using nickel-filtered CuKα radiation from an Elliot GX20 rotating anode X-ray generator operated at 35 kV and 39 mA with a 200 μm focal cup. The exposure time was typically 20 h for a 13° precession photograph at a crystal-to-film distance of 100 mm.

Type I crystals were grown first from 3·3 M-ammonium sulfate, 50 mM-succinate, 5 mM-sodium azide (pH 5·5). Subsequently it was found that crystals could be grown from ammonium sulfate solutions anywhere within the pH range of 5·5 to 7·5. Generally the crystals grew overnight but were too small for easy handling and manipulation. Attempts to grow larger crystals included lowering the ammonium sulfate concentration as well as

† Abbreviation used: PEG, polyethylene glycol.

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decreasing the protein concentration. All efforts to grow large crystals failed until the protein was diluted to 10 mg/ml with a 10 mM-phosphate (pH 7·0) solution containing 500 mM-NaCl. Rather than overnight, the crystals appeared only after two weeks and continued to grow slowly for another four weeks. Using the sitting-drop method and a protein concentration of between 10 and 30 mg/ml, crystals are now grown routinely from 3·2 to 3·5 M-ammonium sulfate solutions containing 250 mM-NaCl and buffered to pH 7·5 with 50 mM-Na^+/K^+ phosphate. The crystals appear as huge triangular plates with a typical length of 2 mm, a thickness of 0·5 mm and well-developed [110] and [001] faces. They belong to the space group $R32$, with unit cell dimensions of $a = b = 100{\cdot}0$ Å and $c = 162{\cdot}2$ Å, as expressed in the hexagonal setting. With a molecular weight of approximately 12,900 and the assumption of two molecules in the asymmetric unit, the solvent parameter V_m is 3·03 Å^3/dalton. If there are three molecules per asymmetric unit, V_m is 2·02 Å^3/dalton, which is still within the normal range of 1·68 to 3·53 Å^3/dalton observed for globular proteins (Matthews, 1968). At this time it is not possible to distinguish between these two alternatives on the basis of the solvent parameter. On still setting photographs, the crystals diffract to at least 1·8 Å resolution. In addition, the crystals continue to diffract strongly after 100 hours of X-ray exposure.

Type II crystals have been grown from 13 to 17% PEG 6000 solutions buffered with 50 mM-succinate, 5 mM-sodium azide (pH 6·5) using either the sitting-drop or batch method. The protein first forms oil droplets from which large single rod-shaped crystals eventually grow over a period of six weeks or longer, with approximate dimensions of 2 mm × 1 mm × 0·4 mm. Interestingly, upon setting up the batch experiments, if the protein–PEG solutions are not thoroughly mixed, a shower of small needle-like crystals can be observed almost immediately under the microscope at the protein–PEG interface. Type II crystals belong to the space group $P3_121$ or $P3_221$ with unit cell dimensions of $a = b = 52{\cdot}4$ Å; $c = 87{\cdot}9$ Å. Assuming one molecule per asymmetric unit, the solvent parameter V_m is 2·70 Å^3/dalton. These crystals also diffract to at least 1·8 Å resolution and are stable in the X-ray beam for well over 100 hours. The type II crystals are excellent candidates for a high resolution X-ray crystallographic investigation.

The biological role of the *Rps. capsulata* cytochrome c_2 is complicated by the fact that the bacterium is still capable of functioning without it. Originally it was thought that the *Rps. capsulata* cytochrome c_2, like the cytochromes c_2 from other purple photosynthetic bacteria, acted as an obligatory shuttle transferring electrons from the cytochrome bc_1 complex to the photochemical reaction center. However, Daldal *et al.* (1986) deleted the cytochrome c_2 gene and demonstrated that electron flow from the bc_1 complex to the reaction center still occurs in its absence. Consequently it appears that the cytochrome c_2 in *Rps. capsulata* may be important for efficient photosynthetic growth but is not necessary for the survival of the organism (Prince *et al.*, 1986).

Generally the oxidation–reduction potentials of the cytochromes c_2 are higher and more variable (250 to 470 mV) than the structurally homologous mitochondrial cytochromes c (Meyer & Kamen, 1982). Furthermore, the redox potentials of the bacterial cytochromes c_2 are much more pH sensitive (Meyer & Kamen, 1982). Through numerous studies, it has also been shown that there is a correlation between the electron transfer rate constant and the redox potential of the cytochromes (Meyer *et al.*, 1983, 1984; Tollin *et al.*, 1984). Factors now thought to control the redox potential of these proteins include variations in the sixth ligand to the iron, local dielectric effects arising from the amount of heme exposure to the solvent and the presence of either hydrophobic or hydrophilic residues near the heme, orientation of the fifth and sixth ligands relative to the heme plane, the extent of hydrogen bonding to the ligands, and the ionization of the heme propionates.

It is the goal of this X-ray study to determine the structure of the *Rps. capsulata* cytochrome c_2 to high resolution using standard multiple isomorphous replacement techniques. Subsequently, by using site-directed mutagenesis, we will investigate those factors thought to influence the electron transfer kinetics and redox potential of the *Rps. capsulata* cytochrome c_2 and will correlate changes in the redox properties of the mutant proteins with their three-dimensional structures. The *Rps. capsulata* bacterium was chosen for this work, since it is the only purple phototrophic bacterium for which a genetic system has been developed. This will allow mutant forms of cytochrome c_2 to be expressed in the organism itself rather than in some other host organism such as *Escherichia coli*. Furthermore, because cytochrome c_2 is expendable in *Rps. capsulata*, those mutations potentially lethal to other purple bacteria, will not present a problem in this system.

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