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Crystallization and preliminary crystallographic analysis of the pyruvate–ferredoxin oxidoreductase from *Desulfovibrio africanus*

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For the first time, crystals of a pyruvate–ferredoxin oxidoreductase (PFOR) suitable for X-ray analysis have been obtained. This enzyme catalyzes, in anaerobic organisms, the crucial energy-yielding reaction of pyruvate decarboxylation to acetylCoA. Polyethylene glycol and divalent metal cations have been used to crystallize the PFOR from the sulfate-reducing bacterium *Desulfovibrio africanus*. Two different orthorhombic ($P2_12_12_1$) crystal forms have been grown with unit-cell dimensions a = 86.1, b = 146.7, c = 212.5 Å and a = 84.8, b = 144.9, c = 203.0 Å. Both crystals diffract to 2.3 Å resolution using synchrotron radiation.

1. Introduction

Oxidative decarboxylation of pyruvate is a central step in the energy metabolism of living cells. Most organisms utilize their reducing power for the reduction of a low-potential electron carrier and concomitant formation of an energy-rich thioester linkage between coenzyme A (CoA) and the acetyl group (Kerscher & Oesterhelt, 1982). In most aerobic bacteria and mitochondria-containing eukaryotes, the oxidation of pyruvate is catalyzed by the pyruvate dehydrogenase multienzyme complex (PDH) which has a molecular weight in excess of 5 MDa. PDH consists of a multiplex of at least three different enzymes containing lipoate and flavin and specifically reduces NAD (Patel & Roche, 1990). By contrast, in most anaerobic bacteria, archaea and amitochondriate protozoa, the same reaction,

$$\text{Pyruvate} + \text{CoA} + \text{Ferredoxin}_{ox} \longleftrightarrow \text{AcetylCoA} + \text{CO}_2 + \text{Ferredoxin}_{red},$$

is carried out by a much simpler enzyme, the pyruvate–ferredoxin oxidoreductase.

This opens up the possibility of describing the overall reaction through the determination of a single enzyme structure. The markedly negative potential of this reaction ($E_0' = -540$ mV) allows the reduction of low-potential electron carriers like ferredoxin or flavodoxin. Interestingly, PFORs which are present in human microaerophiles (Hughes *et al.*, 1995; Hoffman *et al.*, 1996) and anaerobic pathogens including protozoan parasites (Muller, 1992) have been recognized to be potential sites for inhibition by specific drugs since they produce reduced ferredoxin from pyruvate oxidation rather than NADH as pyruvate dehydrogenase (Ben Rosenthal *et al.* 1997).

Pyruvate–ferredoxin (or flavodoxin) oxidoreductase has been isolated from several sources and is involved in various physiological functions of many microorganisms (Wahl & Orme-Johnson, 1987; Müller, 1988; Blamey & Adams, 1994; Menon & Ragsdale, 1996; Tersteegen *et al.*, 1997). Three types of PFORs have been described based on different subunit compositions (Kletzin & Adams, 1996). The enzymes isolated from various mesophilic organisms are commonly homodimeric enzymes (A_2 type) with sizes around 250 kDa (Wahl & Orme-Johnson, 1987; Docampo *et al.*, 1987; Brostedt & Nordlund, 1991; Pieulle *et al.*, 1995) whereas PFORs isolated from the archaebacteria typically are heterotetramers ($\alpha\beta\gamma\delta$-type; Blamey & Adams, 1994; Kunow *et al.*, 1995; Bock & Schönheit, 1995); a few exceptions to this include PFORs from aerobic archaea which are composed of only two different subunits with either *ab* or $(ab)_2$ configurations (Kerscher & Oesterhelt, 1981; Zhang *et al.*, 1996). Only the $(ab)_2$ enzyme from *Halobacterium salinarum* has been previously crystallized; however, the needle-shaped crystals were unsuitable for X-ray diffraction studies (Plaga *et al.*, 1992).

In sulfate-reducing bacteria belonging to the genus *Desulfovibrio*, PFOR is involved in the phosphoroclastic reaction allowing energy conservation by substrate-level phosphorylation (Hatchikian & Legall, 1970). *D. africanus* PFOR has been isolated and characterized (Pieulle *et al.*, 1995). It differs from the PFORs isolated from other anaerobic microorganisms in its high stability to oxygen. The enzyme is an homodimer with a subunit molecular mass of 133 kDa and contains one thiamine pyrophosphate (TPP) per subunit as cofactor (Pieulle *et al.*, 1995). Moreover, biochemical characterization indicates the presence of three (4Fe–4S) centres per subunit with midpoint

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potentials of −390, −515 and −540 mV. The primary structure of the enzyme, recently deduced from the nucleotide sequence of the gene, differs from all other known sequences of PFOR owing to the presence of a C-terminal extension which is involved in the stability of the protein towards oxygen (Pieulle *et al.*, 1997). We report here the crystallization and the first preliminary diffraction data analysis of the PFOR from *D. africanus*.

2. Materials and methods

Chemicals were mostly purchased from Fluka (Buchs, Switzerland) including sodium, magnesium, zinc, barium, cobalt and manganese chlorides, ammonium sulfate, glycerol, methylpentanediol and detergents. Calcium chloride was from Merck (Darmstadt, Germany), Tris–HCl was from Sigma–Aldrich Chimie (St Quentin Fallavier, France) and polyethyleneglycol (PEG) 6000 was from LABOSI (Paris). A TSK-DEAE PW5 column was from Toso-Haas (Stuttgart, Deutschland). Sitting-drop supports were from the D.R.O.P. company (St Marcellin, France).

Preparation and standard purification of the enzyme was carried out as previously reported (Pieulle *et al.*, 1995), and a final step was achieved by HPLC on a TSK DEAE PW5 column equilibrated with 10 m*M* Tris-(hydroxymethyl)-aminomethane (Tris–HCl) pH 8.5. PFOR was eluted from the column with 50 m*M* NaCl, 10 m*M* Tris–HCl pH 8.5. Enzyme-containing fractions were dialyzed against 10 m*M* Tris pH 8.5 and concentrated to 10 mg ml^{-1}. The enzyme was then stored in liquid nitrogen in small aliquots.

Crystallization was carried out by the sitting-drop diffusion method (Wlodawer & Hodgson, 1975) at 293 K with Linbro cell-culture plates and special sitting-drop supports designed by our laboratory (Soriano & Fontecilla-Camps, 1993). The 4 µl droplets consisted of 2 µl of protein solution and 2 µl of reservoir solution and were equilibrated against 1 ml of reservoir solution.

Diffraction data were collected at λ = 1.07 Å wavelength on the BM02 beamline at the European Synchrotron Radiation Facility with a CCD detector. To prevent ice formation, crystals were soaked for a few minutes in a 17% PEG 6000 and 25% glycerol buffer just before flash-cooling at 100 K in a nitrogen stream. Collected data were indexed, integrated and reduced using *XDS* (Kabsch, 1993).

3. Results and discussion

Analysis of crystallization data banks (Gilliland & Bickham, 1990, and an in-house database) for large proteins and incomplete factorial design (Carter & Carter, 1979) were used to screen crystallization conditions of *D. africanus* PFOR (266 kDa). Crystallization trials with methylpentanediol or ammonium sulfate were unsuccessful, whereas PEG led to long brown crystals that grew within about one week at 293 K and could reach a size of 2 × 0.2 × 0.2 mm. In this case, crystallization was initiated by mixing 2 µl drops of a 10 mg ml^{-1} protein aqueous solution with the same volume of a reservoir solution containing 12–15% PEG 6000, 100 m*M* $MgCl_2$ (or 10 m*M* $BaCl_2$, $CaCl_2$, $CoCl_2$ or $MnCl_2$) in a 100 m*M* sodium cacodylate pH 6.0–6.2 buffered solution. Thus, although magnesium is essential for the biological activity of the enzyme, the nature of the divalent cation used as an additive to PEG does not appear to be critical for crystallization. This observation can be compared with the fact that other TPP-dependent enzymes can use Mn^{2+}, Co^{2+} or Ca^{2+} instead of Mg^{2+} (Muller & Schultz, 1993; Heinrich *et al.*, 1972). Addition of detergents (octyl-β-glucopyranoside or *N*,*N*-dimethyldodecylamine-*N*-oxide) had no particular effect. Seeding methods increased crystal size and gave more reproducible crystals (Fig. 1).

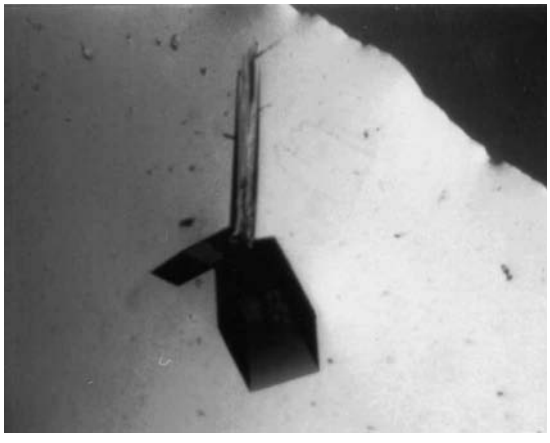

Figure 1
Orthorhombic crystal of pyruvate–ferredoxin oxidoreductase from *D. africanus*. The crystal is approximately 0.3 × 0.3 × 0.4 mm.

The brown colour of the crystals reflects the presence of the (Fe–S) clusters in the crystallized protein. These crystals remain stable for several weeks at room temperature and under aerobic conditions, since *D. africanus* PFOR is particularly stable to oxygen, in contrast to other PFORs.

Analysis of diffraction data indicates that the crystals belong to the orthorhombic space group $P2_12_12_1$ with two unit cells, regardless of the nature of salts or detergents in the crystallization medium; cell parameters are a = 86.2, b = 146.7 and c = 212.5 Å for form 1 and a = 84.8, b = 144.9, c = 203.0 Å for form 2. V_m of the asymmetric unit is 2.67 Å^3 Da^{-1} (Matthews, 1968) corresponding to one dimeric molecule per asymmetric unit (Matthews, 1985). These values correspond to 54%(*v*/*v*) solvent in the crystal.

The diffraction limit was 2.3 Å for both forms and the data reduction yielded a 59% complete data set with 66403 unique reflections and R_{sym} of 12.5% for form 1, and a 68.1% complete data set with 75568 unique reflections and R_{sym} of 8.2% for form 2.

These crystals have been used for heavy-atom soakings and multiwavelength anomalous diffraction (MAD) analysis. This study led to the first determination of a three-dimensional structure of a pyruvate–ferredoxin oxidoreductase, including a detailed description of the active centres, TPP and FeS clusters (Chabrière *et al.*, 1999).

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