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Octomeric pyruvate-ferredoxin oxidoreductase from *Desulfovibrio vulgaris*

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Abstract

Pyruvate-ferredoxin oxidoreductatse (PFOR) carries out the central step in oxidative decarboxylation of pyruvate to acetyl-CoA. We have purified this enzyme from *Desulfovibrio vulgaris* Hildenborough (*Dv*H) as part of a systematic characterization of as many multi-protein complexes as possible for this organism, and the three-dimensional structure of this enzyme has been determined by a combination of electron microscopy (EM), single particle image analysis, homology modeling and computational molecular docking. Our results show that the 1 MDa *Dv*H PFOR complex is a homo-octomer, or more precisely, a tetramer of the dimeric form of the related enzyme found in *Desulfovibrio africanus* (*Da*), with which it shares a sequence identity of 69%. Our homology model of the *Dv*H PFOR dimer is based on the *Da* PFOR X-ray structure. Docking of this model into our 17 Å resolution EM-reconstruction of negatively stained *Dv*H PFOR octomers strongly suggests that the difference in oligomerization state for the two species is due to the insertion of a single valine residue (Val383) within a surface loop of the *Dv*H enzyme. This study demonstrates that the strategy of intermediate resolution EM reconstruction coupled to homology modeling and docking can be powerful enough to infer the functionality of single amino acid residues.

Keywords: Pyruvate-ferredoxin oxidoreductase; *Desulfovibrio vulgaris*; Electron microscopy; Single particle reconstruction; Oligomerization; Homology modeling; Molecular docking

1. Introduction

The oxidative decarboxylation of pyruvate to produce acetyl-CoA is the gateway to the tricarboxylic acid (TCA) cycle. In aerobic bacteria, this central step in energy metabolism is catalyzed by the pyruvate dehydrogenase multi-enzyme complex. In most anaerobic bacteria, archaea, and eukaryotes that lack mitochondria, however, this step is catalyzed by the enzyme pyruvate-ferredoxin oxidoreductase (PFOR) (Charon et al., 1999).

The subunit composition of PFOR varies in different organisms. In some species PFOR occurs as a dimer of a single polypeptide (Brostedt and Nordlund, 1991). In others, four or five different genes encode smaller polypeptides that assemble to form the functional PFOR (Brostedt and Nordlund, 1991; Ikeda et al., 2006). These genes are homologous to different parts of the larger single-polypeptide PFORs, and it is thought that the single polypeptide PFORs arose by operon rearrangement and fusion of smaller common ancestor genes (Kletzin and Adams, 1996).

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Previously, the only structure reported for a PFOR was the X-ray crystal structure of a 266 kDa homodimeric enzyme from the sulfate reducing bacterium *Desulfovibrio africanus* (*Da*). The structure without bound substrate, as well as a complex with pyruvate and that of a reaction intermediate, have been solved (Chabriere et al., 1999; Chabriere et al., 2001).

Here, we report the structure of a PFOR enzyme isolated from a closely related species, *Desulfovibrio vulgaris* Hildenborough (*Dv*H). We show that the enzyme, which purifies as a 1056 kDa oligomer, exists as a stable tetramer of dimers, and we propose that a single residue insertion plays a significant role in this unusual oligomerization state. Our work demonstrates how extremely powerful negative stain electron microscopy can be in the context of proteomic comparisons between related microbes, or between different physiological states of a single microbe, when there is already at least one homolog X-ray structure and the genome for the new organism of interest available.

2. Materials and methods

2.1. Biomass production

Desulfovibrio vulgaris Hildenborough (ATCC 29579) was obtained from the American Type Culture Collection (Manassas, VA). A defined lactate–sulfate medium, LS4D, is used in all cultures. All media preparation details and reagents are as before (Mukhopadhyay et al., 2006). All reagents and preparation protocols are tracked for each media preparation and recorded in the Biofiles database (http://vimss.lbl.gov/perl/biofiles). Media are autoclaved and then filter sterilized, after which phosphate, vitamins and reducing agent (titanium citrate) are added. Stock cultures of *D. vulgaris* were prepared by growing the ATCC culture to log phase, and storing at −80 °C. The starter culture is prepared inside an anaerobic chamber using stock culture at a ratio of 1 ml stock/100 ml LS4D. The starter culture is incubated at 30 °C and allowed to grow for 48 h to log phase (optical density at 600 nm of ~0.3–0.4; $\sim 3 \times 10^8$ cells/ml). From the starter culture, a 10% subculture for inoculating the production culture is made in LS4D, in the anaerobic chamber, and incubated at 30 °C until log phase growth is reached (around 15 h). The generation time for *D. vulgaris* on this medium is 5 h. To minimize repetitive culturing "phenotypic drift" all experiments are started from fresh frozen stock. All experiments were run on cells that are less then three subcultures from the original ATCC culture. All inoculations and transfers were done in a Coy anaerobic glove box chamber (Coy Laboratory Products Inc., Grass Lake, MI) with an atmosphere of 5% CO_2, 5% H_2 and 90% N_2. The production culture is grown in batch, using a 5 L flask. The flask is autoclaved with 5 L LS4D media and cooled on the bench in the anaerobic chamber. Immediately before inoculation, vitamins, phosphate, and reducing agent are injected into the flask. Samples are periodically taken from the fermenter to monitor growth from OD measurement. Once log phase growth is reached, the cells are harvested. To minimize sample variability due to processing time, samples were pulled from the production cultures by peristaltic pump through 7 m of capillary tubing in an ice bath. This was found to drop the temperature of the sample to less then 4 °C in less then 15 s. The samples are directly put into 500 mL centrifuge bottles, which are centrifuged at 6000*g* for 10 min, with refrigeration at 4 °C (Beckman Coulter, Avanti J-25). The supernatant is discarded; the pellets are washed with degassed phosphate buffered saline solution, and pooled for a second spin. The bottles are flash-frozen in liquid nitrogen and stored at −80 °C until further processing.

2.2. Purification and biochemical characterization of PFOR

Cell extracts were produced by thawing 10 g of pelleted cells in 10 ml of 0.1 M Tris–HCl pH 7.5 containing 80 mM KCl, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF). Cells were broken by treatment with 0.5 mg/ml lysozyme for 2 h followed by sonication. The cytosolic portion of the cell extract was separated by centrifugation at 200,000*g* for 90 min and the supernatant was retained for purification of PFOR (Yu et al., 2001). All purification steps were performed at 4 °C using columns and an AKTA FPLC from GE Healthcare. Different mixtures of two buffers were used for column fractionation: Buffer A contained 25 mM Hepes pH 7.6, 10% (v/v) glycerol, 2 mM DTT, 0.01% (v/v) NP 40 and Buffer B contained Buffer A plus 1 M NaCl. The NaCl concentration of the mixture of the two buffers is given by the % Buffer B.

The cell extract supernatant was loaded on a 1.6 × 20 cm Q Sepharose Fast Flow column equilibrated with 5% buffer B. After the column had been washed with two column volumes of equilibration buffer, the bound proteins were eluted with 50% buffer B. All fractions containing significant amounts of protein were pooled and buffer exchanged to 5% buffer B with a 2.6 × 20 cm G-25 desalting column. The eluate was loaded to a 1.6 × 10 cm MonoQ column equilibrated with 5% buffer B. Then the MonoQ column was developed with a linear gradient from 5% buffer B to 50% buffer B in 25 column volumes and 4 ml fractions were collected. The fractions subsequently determined to contain PFOR were pooled and loaded onto a 1.6 × 60 cm Superdex 200 column equilibrated with 5% buffer B. Fractions eluted from that column in 5% buffer B were frozen in liquid nitrogen and stored at −70 °C. Purified Superdex 200 fractions were buffer exchanged using a PD-10 desalting column equilibrated with 10 mM Hepes, pH 7.6, 2 mM DTT, 0.01% NP40. The buffer-exchanged protein was concentrated with Amicon and Microcon centrifugal filters (Millipore) to a concentration of 0.2–0.6 mg/ml. The protein concentration was then determined with Coomassie Plus Bradford Assay Reagent (Pierce) using BSA as the standard.