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CRYSTALLIZATION OF FRUCTOSE 1,6-BISPHOSPHATASE FROM THE HYPERTHERMOPHILIC ARCHAEON *THERMOCOCCUS KODAKARAENSIS*

NAEEM RASHID[1*], MASOOD AHMED SIDDIQUI[2], MUHAMMAD SALEEM HAIDER[1] AND MUHAMMAD ARSHAD JAVED[1]

[1]School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan
[2]Department of Chemistry, Balochistan University, Quetta, Pakistan.

Abstract

The enzyme Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) is one of the key enzymes of the gluconeogenic pathway. It hydrolyses fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate (Pilkis & Claus, 1991). Here we report the crystallization of FBPase from *Thermococcus kodakaraensis*. This FBPase consists of 375 amino acids, with a molecular weight of 42kDa, and was prepared using an *Escherichia coli* expression system. The purified recombinant FBPase was crystallised using the sitting drop vapour-diffusion method at 17°C. Crystals grew tetragonally and measured approximately 0.4 mm in the longest dimension.

Introduction

Hyperthermophiles are able to grow at or above 85°C and most of them belong to archaea. The euryarchaeal order *Thermococcales* is composed of two major genera, *Thermococcus* and *Pyrococcus* (Huber & Stetter, 2001; Itoh, 2003). This is considered the best-studied hyperthermophilic order (Fukui *et al*., 2005). The microorganisms belonging to this order are strictly anaerobic obligate heterotrophs that grow on complex proteinaceous substrates, and their growth is strongly associated with the reduction of elemental sulphur. Alternatively, with a few exceptions, they are capable of gaining energy by fermentation of peptides, amino acids and sugars, forming acids, CO_2, and H_2 in the absence of elemental sulphur (Fukui *et al*., 2005). The hyperthermophilic archaeon *Thermococcus kodakaraensis* was isolated from a solfatara on Kodakara Island, Kagoshima, Japan (Atomi *et al*., 2004; Morikawa *et al*., 1994). The strain is an obligate anaerobe with optimal growth around 85°C. The genome has been fully sequenced (Fukui *et al*., 2005). Although FBPase activity has been detected in several hyperthermophiles, no orthologs corresponding to the classical FBPases from bacteria and eukaryotes have been identified in their genomes. An inositol monophosphatase (IMPase) from *Methanococcus jannaschii* which displayed both FBPase and IMPase activities and a structurally novel FBPase (Fbp*Tk*) from the hyperthermophilic archaeon *T. kodakaraensis* have been proposed as the "missing" FBPase in hyperthermophilic archaea (Rashid *et al*., 2002; Stec *et al*., 2000). The IMPase ortholog, *impTk*, have also been found in *T. kodakaraensis* genome. We have demonstrated, by gene distruption experiments, that *fbpTk* deleted strain could not grow under gluconeogenic conditions while glycolytic growth was unaffected, and the disruption resulted in the complete abolishment of intracellular FBPase activity showing that *fbpTk* is an indispensable gene for gluconeogenesis and is responsible for almost all

*Corresponding author Email: naeemrashid37@hotmail.com
Tel: +92-(0)429923-1534; Fax: +92-(0)429923-0980

intracellular FBPase activity (Sato *et al*., 2004). In contrast, the endogenous *impTk* gene could not complement the defect of the *fbpTk* deletion, and its disruption did not lead to any detectable phenotypic changes under the conditions examined. These findings provided strong evidence that the true FBPase for gluconeogenesis in *T. kodakaraensis* is the Fbp*Tk* ortholog, not the IMPase ortholog. In *Pyrococcus furiosus*, *fbpTk* orthologe (PF0613) was 15-fold up-regulated in peptide-grown cells. The other FBPase candidate (PF2014) was not significantly regulated and is therefore unlikely to play a gluconeogenic role (Schut *et al*., 2003; Verhees *et al*., 2003). Here we report crystallization of Fbp*Tk*.

Materials and Methods

The gene encoding Fbp*Tk* was expressed in *E.coli* BL21 (DE3) using the recombinant pET-*fbp* plasmid (Rashid *et al*., 2002). The heterologous gene expression was induced with IPTG at 37°C. The recombinant protein was purified *via* sonication, a heat treatment at 85°C for 20 min., anion exchange, hydrophobic interaction and gel filteration column chromatographies as described previously (Rashid *et al*., 2002). The purity of the protein was examined by SDS-PAGE. The purified protein was dialyzed against 50 mM Tris-Cl pH 8.0. Protein concentration was determined with Quick Start™ Bradford Dye Reagent (Bio-Rad, Hercules, CA, USA) according to the instructions of the manufacturer using bovine serum albumin as a standard. The dialyzed sample was concentrated using Centricon YM-30 (Millipore, Bedford, MA, USA). Crystallization conditions were screened using the Crystal Screen 2, Grid Screen PEG 6000, Grid Screen A/S, Grid Screen PEG/LiCl, Grid Screen MPD and Grid Screen Sodium Chloride (Hampton Research, Riverside, CA, USA). The protein solution contained 20 mg/ml protein in 100 mM bicine buffer pH 8.0. An equal volume of reservoir solution was added to a droplet of protein solution (3 µl), and the droplet was equilibrated over 1 ml reservoir solution. The crystallization plates were air sealed to avoid evaporation. Incubations were carried out at 17°C.

Results and Discussion

Fbp*Tk* was overexpressed in *E. coli* and purified according to the previously reported method (Rashid *et al*., 2002). Crystals were prepared by the sitting-drop vapour-diffusion method. The protein crystallized at pH 8.5 in the presence of polyethylene glycol monomethyl ether (PEG MME) and nickel chloride. The crystallization conditions were optimized for pH, the concentration and molecular weight of PEG MME and the concentration of nickel chloride. Crystals grew in the tetragonal space group in 0.1 M Tris-Cl pH 8-8.6 in the presence of 20% PEG MME 2000 and 0.01M nickel chloride after four days of incubation at 17°C (Fig. 1). The crystals were measured approximately 0.4 mm in the longest dimension. The crystals were picked using litholoops (Molecular Dimensions), frozen in a cryostream at 100K and stored in liquid nitrogen for structure determination studies.

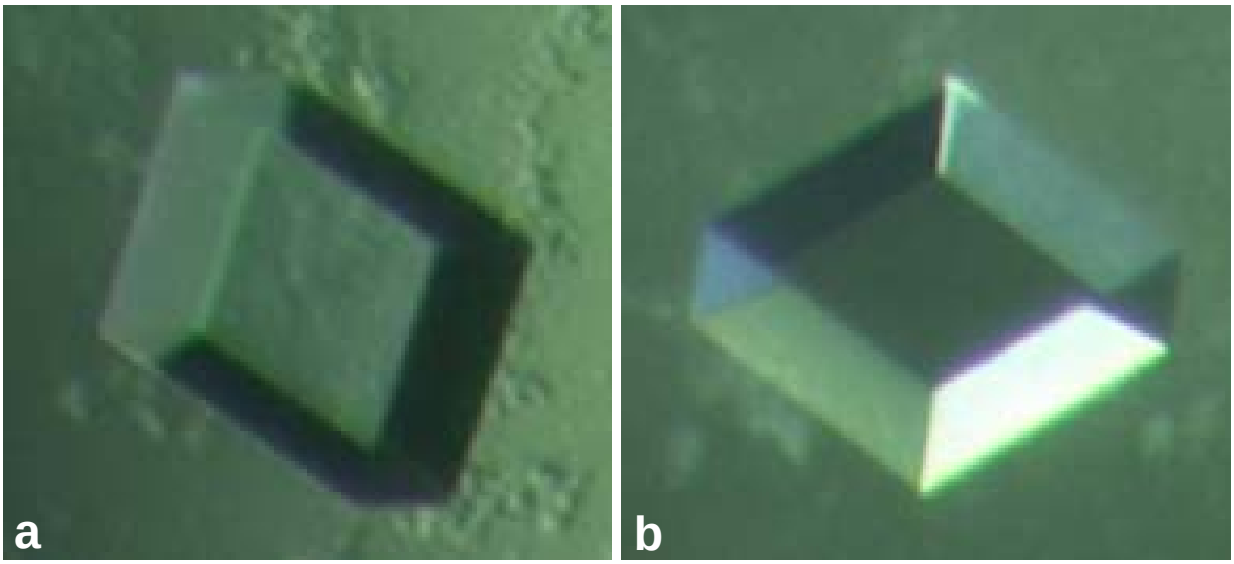

Fig. 1. Crystals of Fbp*Tk* obtained in the presence of 20% PEG MME 2000 and 0.01M nickel chloride at a) pH 8.3 and b) 8.6. Both the crystals were measured approximately 0.4 mm in their longest dimension.

FBPase/IMPase from two archaeal strains, *M. jannaschii* and *Archaeoglobus fulgidus*, have been crystallized. FBPase/IMPase from *M. jannaschii* has been reported to crystallize in the presence of PEG 8000 at pH 7.2 in the space group $P2_12_12_1$ (Stec *et al*., 2000; Johnson *et al*., 2001). The enzyme from *A. fulgidus* crystallized in two distinct crystal forms: $P2_1$ and $P3_2$ in PEG 3350 (Stieglitz *et al*., 2002). These two enzymes exist in solution in a dimeric form (Stec *et al*., 2000). The primary structures of these two proteins are quite different than Fbp*Tk*. Furthermore, the oligomer formation of Fbp*Tk* in solution has been reported. It is suggested by the molecular weight determined by gel-filtration chromatography that Fbp*Tk* exists in solution in an octameric form (Rashid *et al*., 2002). The structure determination of Fbp*Tk* will shed light on the mechanism of novel archaeal type FBPase and its comparison with FBPase/IMPase from archaea. Further crystals of Fbp*Tk* have been grown under similar conditions and structure determination and refinement is in progress.

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