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Crystallization and preliminary X-ray analysis of a Y13S mutant of Spo0F from *Bacillus subtilis*

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Abstract

Spo0F, a member of a superfamily of bacterial response regulatory proteins, is crucial to the regulation of sporulation in *Bacillus subtilis*. As there were difficulties in reproducing crystals of wild-type Spo0F, we report here the crystallization and preliminary studies of a mutant, Y13S protein, which gave well diffracting reproducible crystals. The crystals of the mutant obtained by the hanging-drop method belong to the tetragonal space group $P4_12_12$ ($P4_32_12$) $a = b = 105.1$, $c = 85.9$ Å. Diffraction data were collected at 2.8 Å at the laboratory source and subsequently 2.05 Å data were collected upon flash freezing the crystal at the Stanford Synchrotron Radiation Laboratory. This mutant participates in the phosphorelay in a similar manner to the wild-type protein. The presence of divalent cations are essential for wild-type phosphorylation and the present mutant crystal form is obtained in the presence of calcium.

1. Introduction

Bacteria live in an environment where conditions can change rapidly and unexpectedly. To survive, the cells must constantly monitor the external conditions and adjust their metabolism and behavior accordingly. In recent years it has become apparent that bacteria respond to environmental stress by activating transcription of genes to modify their metabolism for a changed environment through the involvement of 'two component switches'. Bacteria contain multiple switches which allow them to respond to a wide spectrum of stimuli.

Investigations directed towards understanding the structural features of the sporulation-controlling phosphorelay in *Bacillus subtilis* are currently being undertaken to gain insight into the mechanism of sporulation. The phosphorelay is made up of four proteins known as KinA, Spo0F, Spo0B and Spo0A. KinA is a histidine kinase that autophosphorylates using ATP when prompted by some as yet unknown cellular signal. Phosphorylated KinA then passes the phosphate to an aspartate in the protein, Spo0F, that subsequently conveys the phosphate to a histidine in the protein, Spo0B. This phosphorylated protein then acts to transfer phosphate to Spo0A, a two-domain protein with an aspartate as the receptor of phosphate and with transcription regulatory activities (Burbulys, Trach & Hoch, 1991). Spo0F, a low molecular weight protein with 124 amino acids, is a member of the response regulatory group of proteins containing a characteristic triple aspartyl pocket (Parkinson & Kofoid, 1992). So far, most information has been obtained for CheY (Stock, Mottonen, Stock & Schutt, 1989; Volz, 1993; Bruix, Pascual, Santoro, Prieton, Serrano & Rico, 1993), which has provided a comparative standard for other investigations. The complexity of the Spo0F role has been recently emphasized by the report that it is further regulated by phosphatases (Perego, Hanstein, Welsh, Djavakhishvili, Glasa & Hoch, 1994). The remarkable controlling interactions of this small protein have prompted us to pursue its structural evaluation by X-ray crystallographic procedures. The wild-type protein proved to be a fickle source for reproducible crystal formation, however, the Y13S mutant that emerged from the phosphatase investigations (Perego, Hanstein, Welsh, Djavakhishvili, Glasa & Hoch, 1994) proved amenable to crystallization, repeatedly giving crystals which possess good diffraction properties. The Y13S mutant Spo0F is resistant to the rap family of proteins, aspartate phosphatases, presumably because the mutation affects the binding of this class of phosphatases to Spo0F, but the mutation has no discernable effect on the enzymatic functions of Spo0F. This report contains preliminary results from measurements taken with the mutant Spo0F crystals.

1.1. *Experimental methods and results*

The Spo0F Y13S allele was amplified *via* the polymerase chain reaction (PCR) from the chromosome of the *B. subtilis* strain JH12908 (Perego, Hanstein, Welsh, Djavakhishvili, Glasa & Hoch, 1994) using the flanking oligonucleotides described by Feher *et al.* (Feher, Zapf, Hoch, Dahlquist, Whiteley & Cavanagh, 1995). The fragments obtained were cloned in the *Nde*I and *Bam*HI sites of pET-20b to produce plasmid pET0FY13S, which was subsequently transformed into *E. coli* BL21 (DE3) (Studier, Rosenberg, Dunn & Dubendorff, 1990). From the strain obtained, Spo0FY13S was purified to homogeneity by a similar procedure to that described by Zapf *et al.* (Zapf, Hoch & Whiteley, 1996) for the wild-type protein. In this procedure the mutant Spo0F was purified by a three-step procedure entailing cell lysis, DEAE–Trisacryl and hydroxylapatite column chromatography. The purity of the final product was confirmed by sodium dodecyl sulfate (SDS) Tris/Tricine polyacrylamide gel electrophoresis (PAGE) and non-denaturing PAGE. Samples were concentrated after dialysis against 10 m*M* *bis*-Tris, pH 7.3, containing 50 m*M* KCl and 0.02% NaN_3, to approximately 25 mg ml^{-1} prior to crystallization.

Crystals (Fig. 1) were grown by a vapour-diffusion method, using the hanging-drop procedure in Linbro tissue-culture plates at 277 K. The drop was prepared by mixing 3 µl of protein stock (25 mg ml^{-1}) with an equal volume of reservoir solution containing 6–7.5% PEG 3350, 10% glycerol, 200 m*M* $CaCl_2$ in 100 m*M* NaOAc buffer at pH 4.5. Crystals shaped as tetragonal rods appeared in 7 d and grew to a maximum size of 0.2 × 0.2 × 0.6 mm in two to three weeks. The crystals belong to the space group $P4_12_12$ ($P4_32_12$) $a = b = 105.1$, $c = 85.9$ Å and diffracted below 3 Å, however these crystals were moderately sensitive to radiation. The volume of the unit cell suggests that there were three molecules of Spo0F in the asymmetric unit and the value of the Matthews constant (Matthews, 1968) gave $V_m = 2.8$ Å^3 Da^{-1} corresponding to a

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solvent content of approximately 56%. Diffraction data from two native crystals were collected using a Xuong–Hamlin multiwire area-detector system (Xuong, Nielsen, Hamlin & Anderson, 1985) and Cu $K\alpha$ radiation produced by a Rigaku rotating anode Ru 200 operated at 50 kV and 100 mA. The two data sets were merged together. The merged data set is 99.3% complete to 2.8 Å having 12 322 unique reflections and 91 047 observations with an R_{sym} of 10.4% and I/σ is 2.0 at 3.0 Å.

Synchrotron data collection time was secured on the beamline 7-1 at the Stanford Synchrotron Radiation Laboratory. The data were collected using a MAR Research scanner (300 mm image plate) with monochromated radiation ($\lambda = 1.08$ Å) at 90 K. The crystals were 'flash frozen' after dipping into a cryo solvent containing 25% PEG 300 and 25% glycerol in 75 mM sodium acetate at pH = 4.5 with 150 mM calcium chloride. The crystal was mounted on the goniometer head with a nylon loop of about 1 mm diameter attached to a glass capillary. The crystal was initially held inside the loop by the surface tension of the cryo solvent and frozen in a cold stream of nitrogen.

Upon freezing, the unit-cell dimensions changed by more than 2 Å, $a = b = 102.73$ Å (*cf.* 105.1 Å) and $c = 83.1$ Å (*cf.* 85.9 Å) with a resultant solvent content of 52%. The diffraction of the crystal improved dramatically to nearly 2 Å (see Fig. 2). The image plate was kept at a distance of 150 mm from the crystal and the images were collected by rotating the crystal by 1° for 10 s. A total of 80 images were collected from a single crystal and they were processed using the program *MOSFLM* (Leslie, 1991) to a resolution of 2.05 Å. There are 27 222 unique reflections and 129 032 observations with a redundancy of 4.7 and R_{sym} of 5.1%. The statistics of data collection are given in Table 1.

As the crystals diffract well, a structure solution at high resolution is imminent and a search for heavy-atom derivatives is well underway.

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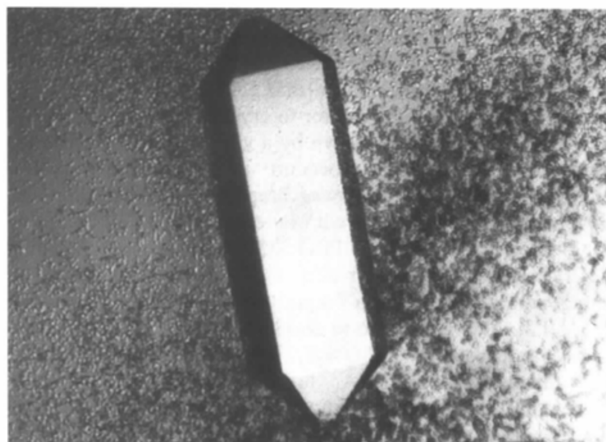

Fig. 1. A crystal of the Y13S mutant Spo0F. Crystals typically grow to a size of 0.2 × 0.2 × 0.6 mm in two to three weeks.

Table 1. *Intensity statistics of data collection for the Y13S crystal at SSRL*

Shell lower limit (Å)	$I/\sigma(I)$	No. reflections	No. observed	R_{sym} (%)*
4.471	13.45	2921	16746	3.73
3.16	14.00	5065	28985	4.56
2.58	10.70	6363	30694	6.45
2.24	7.13	7208	30089	9.55
2.05	4.97	5665	22518	14.37
Totals	10.05	27222	129032	5.10

* R_{sym} is a measure of the agreement among multiple observations and is calculated as $\sum[I_{ave} - I]/\sum I_{ave}$ for all multiple observations.

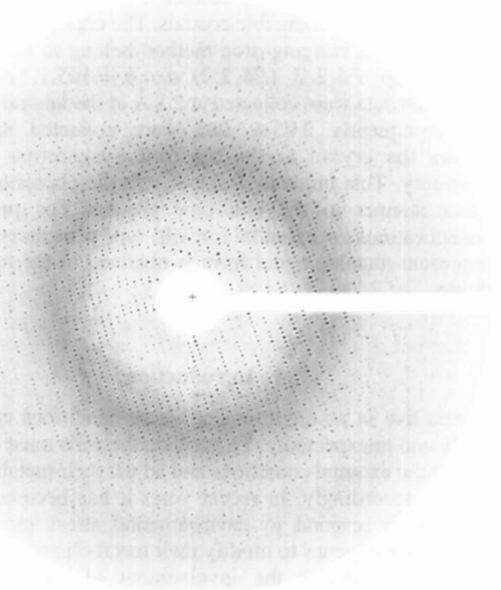

Fig. 2. A diffraction pattern of the crystal taken at 90 K with 1° oscillation on a MAR Research image plate at Stanford Synchrotron Radiation Laboratory.

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