Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray crystallographic analysis of p24, a component of the potato nuclear factor PBF-2

Darrell Desveaux, Julie Allard, Normand Brisson and Jurgen Sygusch*

Département de Biochimie, Université de Montréal, CP 6128, Station Centre Ville, Montréal, Québec H3C 3J7, Canada

Correspondence e-mail: jurgen.sygusch@umontreal.ca

Received 27 August 2001
Accepted 5 November 2001

The *Solanum tuberosum* (potato) nuclear factor PBF-2 is implicated in pathogen-induced expression of the pathogenesis-related gene *PR-10a*. Crystals of the DNA-binding component of PBF-2, p24, have been obtained at 277 K in 20 m*M* Tris–HCl pH 8.0. Recombinant protein with a His tag at its C-terminus was overexpressed in *Escherichia coli* in the presence and absence of selenomethionine and was purified using a combination of HiTrap affinity columns and gel-filtration chromatography. Crystals suitable for structural analysis were obtained for both native and selenomethionine-labelled proteins and yielded diffraction data at 100 K that were processed to 2.3 and 2.8 Å resolution, respectively. The p24 protein crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 69.4 (69.1), b = 89.4 (90.5), c = 144.1 (144.3) Å. The asymmetric unit contains four protomers, giving a crystal volume per protein mass (V_M) of 2.23 Å^3 Da^{-1} and a solvent content of 45% by volume.

1. Introduction

Transcriptional regulation of gene expression is a vital component of the plant defence response against invading pathogens (Lamb *et al.*, 1989). The pathogenesis-related (PR) genes of plants induced during pathogen infection represent an excellent system for the study of gene regulation during the defence response. Transcriptional induction of the potato *PR-10a* gene in response to infection by the late blight pathogen *Phytophthora infestans* requires a 25 bp promoter sequence termed the elicitor response element (ERE; Matton & Brisson, 1989; Matton *et al.*, 1993; Després *et al.*, 1995). This element is specifically recognized by the nuclear factor PBF-2, whose activity correlates with *PR-10a* gene expression suggesting that PBF-2 is involved in activation of this gene (Després *et al.*, 1995; Desveaux *et al.*, 2000). Purification of PBF-2 led to the identification and cloning of its DNA-binding component p24, which preferentially binds to single-stranded DNA (Desveaux *et al.*, 2000).

Analysis of the derived amino-acid sequence of p24 (AAF91282) does not reveal any striking similarity to other proteins of known function. However, in the plant kingdom, proteins with strong similarity to p24 are encoded by genes from evolutionary distant plants such as loblolly pine, rice, maize, *Arabidopsis* and tomato (Desveaux *et al.*, 2000). Single-stranded DNA-binding factors (SSBs) that regulate gene expression are found in both prokaryotes and eukaryotes. Although in most cases the mechanistic details of regulation are not known, possible mechanisms have been proposed (Rothman-Denes *et al.*, 1998). We have initiated structural analyses of p24 in order to provide insight into the mechanism of transcriptional regulation and to complement the functional characterization of this ubiquitous class of plant proteins.

2. Materials and methods

2.1. Protein expression and purification

Potato p24 protein was expressed as a 227 amino-acid protein containing an N-terminal T7 tag and a C-terminal His tag in the *E. coli* expression strain BL21 (Novagen; Desveaux *et al.*, 2000). Selenomethionine (SeMet) labelling of the same recombinant protein used methionine-biosynthesis inhibition as described by Doublié (1997). The incorporation of SeMet was confirmed by mass spectrometry of labelled p24, revealing the incorporation of five SeMet molecules per protein subunit. Labelled and unlabelled fusion proteins were purified using a combination of HiTrap affinity columns (Amersham Pharmacia Biotech) as described by Desveaux *et al.* (2000) and gel-filtration chromatography. Following elution from the HiTrap columns, proteins were concentrated using Ultrafree-4 centrifugal filters (Millipore) and loaded onto a Sephadex 200 column (Amersham Pharmacia Biotech) equilibrated with 20 m*M* Na_2HPO_4 pH 7.2 and 250 m*M* NaCl. Proteins were eluted at a flow rate of 1 ml min^{-1} using an FPLC apparatus (Amersham Pharmacia Biotech). Fractions containing p24 were pooled and the buffer was changed to 20 m*M* Tris–HCl pH 8.0

by repeated concentration and dilution using Ultrafree-4 centrifugal filters (Millipore) according to the manufacturer's instructions. For crystallization purposes, the protein was concentrated to a final concentration of 12–15 mg ml^{-1} and stored at 277 K.

2.2. Crystallization and data collection

Crystals of p24 protein were readily obtained upon storage of the concentrated protein solution at 277 K for both unlabelled and selenomethionine-labelled protein. Protein crystals appeared within 24 h which could be solubilized at room temperature and recrystallized at 277 K.

p24 crystals obtained at 277 K were used for X-ray diffraction studies. A crystal measuring about 0.1 × 0.1 × 0.5 mm was mounted in a nylon cryoloop (Hampton Research, USA), immersed in cryoprotectant (20 m*M* Tris–HCl pH 8.0, 20% glycerol) for a few seconds and flash-cooled at 100 K in a nitrogen-gas stream. Diffraction data for the SeMet derivative were collected at 100 K at beamline X8-C of the Brookhaven National Laboratory National Synchrotron Light Source, Brookhaven, USA. Anomalous pairs for the SeMet derivative were collected in a single pass using inverse-beam geometry at three different wavelengths. An X-ray fluorescence spectrum was recorded and used to select the wavelength optima for MAD data collection. Data were collected at 0.9791 Å (the inflection point of the fluorescence spectrum, f' minimum), 0.9790 Å (f'' maximum) and 0.9400 Å (remote high-energy wavelength) with a crystal-to-detector distance of 200 mm, an oscillation range of 0.66° per frame and an exposure time of 70 s for each image. A single native protein crystal was used to collect low- and high-resolution data sets at beamline X25 of the Brookhaven National Laboratory National Synchrotron Light Source at a wavelength of 1.1 Å. The respective data-collection parameters for the low- and high-resolution data sets are crystal-to-detector distances of 300 and 120 mm, an oscillation range of 0.66° per frame and exposure times of 3 and 40 s for each image. All data reduction was performed with the *DENZO* and *SCALEPACK* software (Otwinowski & Minor, 1997).

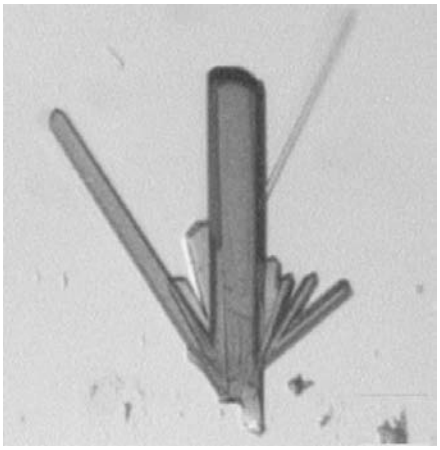

Figure 1
Crystals of selenomethionine-labelled p24 grown at 277 K in 20 m*M* Tris–HCl pH 8.0. The approximate dimensions of the largest crystal are 0.1 × 0.1 × 0.5 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

		Se-Met		
	Native	Inflexion	Peak	Remote
Resolution (Å)	2.3 (2.38–2.30)	2.8 (2.59–2.50)		
Wavelength (Å)	1.10	0.9791	0.9790	0.9400
Observed reflections	306528	118433	118752	117087
Unique reflections	40505	20862	20805	20695
Completeness (%)	98.4 (89.1)	90.5 (100.0)	90.0 (99.9)	89.4 (100.0)
Average $I/\sigma(I)$	31.8 (2.8)	28.4 (5.1)	31.8 (5.6)	32.1 (5.9)
$R_{\rm merge}$†	0.081 (0.37)	0.086 (0.38)	0.073 (0.34)	0.071(0.34)
Space group	$P2_12_12_1$	$P2_12_12_1$		
Unit-cell parameters (Å)	a = 69.9, b = 89.8, c = 144.4	a = 69.1, b = 90.5, c = 144.3		

† $R_{\rm merge} = \sum |I_{\rm obs} - I_{\rm avg}| / \sum I_{\rm obs}$, where $I_{\rm obs}$ is an individual intensity observation, $I_{\rm avg}$ is the mean intensity for that reflection and the summation is over all reflections.

3. Results and discussion

About 20 mg of both labelled and selenomethionine-labelled p24 protein was recovered per litre of culture and purified protein was estimated to be over 90% pure by SDS–PAGE (data not shown). As shown in Fig. 1, rod-shaped crystals with average dimensions of 0.1 × 0.1 × 0.5 mm grew in Eppendorf tubes containing 12–15 mg ml^{-1} p24 protein in 20 m*M* Tris–HCl pH 8.0 at 277 K within 24 h. Selenomethionine labelling of p24 did not observably affect the crystallization conditions of the protein. The crystals grown at 277 K were stable for at least several months and could be solubilized by increasing the temperature or lowering the pH.

Complete data sets were collected from both native and selenomethionine-labelled protein crystals to resolutions of 2.0 and 2.3 Å, respectively. Because of a poor signal-to-noise ratio at higher resolutions, processed data were truncated to 2.3 and 2.8 Å, respectively (Table 1). Both crystals belong to the orthorhombic system, with space group $P2_12_12_1$. The unit-cell parameters (SeMet derivative in parentheses) were determined to be a = 69.4 (69.1), b = 89.4 (90.5), c = 144.1 (144.3) Å. The unit cell contains four molecules per asymmetric unit, corresponding to a calculated $V_{\rm M}$ value of 2.23 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 45%, which is in the range typically found in protein crystals. As a result of the successful crystallization and data collection of selenomethionine-labelled p24 protein, structural determination of p24 has been initiated. Data collected from the native protein will be used to increase the resolution of the structure determined from the selenomethionine-labelled protein.

The tertiary structure will show if p24 possesses structural motifs shared by single-stranded DNA-binding proteins outside the plant kingdom, which may help in understanding its mechanism of transcriptional regulation. Furthermore, knowledge of its three-dimensional structure will allow functional studies to focus on the domains of the protein, which are difficult to predict solely from the primary structure. The occurrence of these domains and the key amino acids in other members of the p24 family may provide clues to their function and mode of action, which will undoubtedly enhance our understanding of the role of these proteins in the plant defence response.

The assistance of Dr Tae-Sung Yoon in data collection and reduction is gratefully acknowledged. This work was supported by a fellowship from the Natural Sciences and Engineering Research Council of Canada (NSERC) to DD, research grants from NSERC and the Fonds pour la formation de Chercheurs et l'Aide à la Recherche, Québec to NB and a Multi-User Maintenance Grant from NSERC and CIHR (Canada) to JS for synchrotron beamline operation. Work was carried out in part at the National Synchrotron Light Source, Brookhaven National Laboratory, which is supported by the US Department of Energy,

Division of Materials Sciences and Division of Chemical Sciences under contract number DE-AC02-98CH10886.

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