Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic studies of mung bean cytokinin-specific binding protein

Grzegorz D. Bujacz,[a,b] Oliwia Pasternak,[a] Yasuyuki Fujimoto,[c] Yuichi Hashimoto,[d] Michal M. Sikorski[a] and Mariusz Jaskolski[a,e]*

[a]Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland, [b]Institute of Technical Biochemistry, Technical University of Łódź, Poland, [c]Faculty of Pharmaceutical Sciences, Teikyo University, Japan, [d]Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan, and [e]Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznan, Poland

Correspondence e-mail: mariuszj@amu.edu.pl

Received 10 October 2002
Accepted 11 December 2002

Cytokinins, or plant growth hormones, bind with very high affinity to cytokinin-specific binding proteins (CSBPs). Recombinant mung bean CSBP has been overexpressed in *Escherichia coli* and crystallized in complex with zeatin, a natural plant growth hormone. The crystals belong to the hexagonal system, space group $P6_2$ or $P6_4$, with unit-cell parameters $a = 113.62$, $c = 86.85$ Å, contain two to five copies of the protein in the asymmetric unit and diffract X-rays to 1.25 Å resolution.

1. Introduction

Cytokinins are plant growth hormones that control the proliferation and differentiation of plants (Skoog & Armstrong, 1970; Hall, 1973). Natural cytokinins are purine analogues, such as zeatin and N^6-(Δ^2-isopentenyl)adenine, which show biological activities at nanomolar concentrations. There are also synthetic urea derivatives with high cytokinin activity, such as *N*-phenyl-*N*′-(4-pyridyl)urea (4PU) and *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU). Both purine-type and urea-type cytokinins seem to share a common site of action (Iwamura *et al.*, 1980, 1985), but the structural basis of their biological activity is not understood. Various cytosolic proteins capable of cytokinin binding have been found in many plant species using purine-type cytokinins as probes (Yoshida & Takegami, 1977; Chen *et al.*, 1980; Hamaguchi *et al.*, 1985; Romanov *et al.*, 1990; Jayabaskaran, 1990; Momotani & Tsuji, 1992; Sakai & Kamei, 1992; Mitsui & Sugiura, 1993; Kulaeva *et al.*, 1990, 1995), but most of these proteins bind cytokinins with association constants of less then $10^8\ M^{-1}$. Nagata *et al.* (1993) detected in the soluble fraction of etiolated mung bean (*Vigna radiata*) seedlings a 17 kDa protein that binds cytokinins with high affinity (10^9–$10^{10}\ M^{-1}$; Fujimoto *et al.*, 1998). The partial amino-acid sequence of this cytokinin-specific binding protein (CSBP) was determined by N-terminal sequencing and the full sequence was deduced from its cDNA. The hydropathy profile of this protein shows that there is no signal sequence or transmembrane domain present. This supports the idea that *V. radiata* CSBP (VrCSBP) is a soluble cytosolic protein.

A database homology search reveals that VrCSBP could be classified as a novel pathogenesis-related class 10 (PR-10) protein, with low sequence identity (about 20%) (Fig. 1). In this family, which is unique to the plant kingdom, not only the amino-acid sequences but also the lengths of the proteins are highly conserved (Van Loon & Van Strien, 1999). Although the structure of the classic PR-10

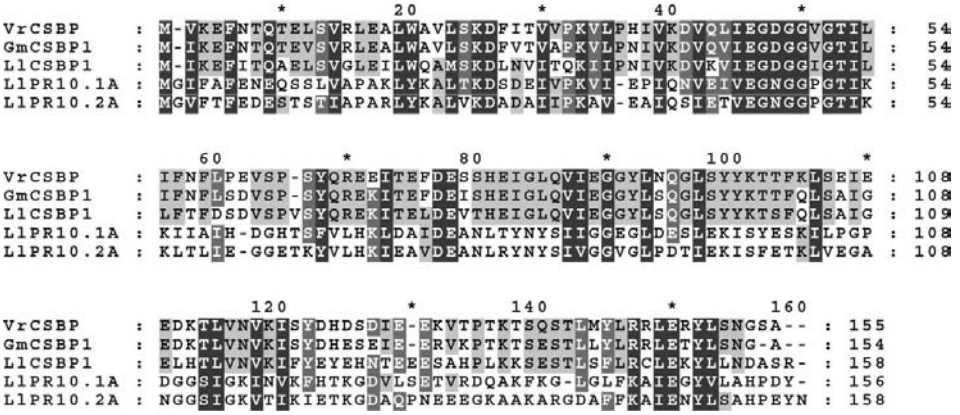

Figure 1
Comparison of selected amino-acid sequences of cytokinin-specific binding proteins (CSBP) from leguminous plants and pathogenesis-related proteins of class 10 (PR-10) (*CLUSTALW*; Thompson *et al.*, 1994). Similar amino acids are highlighted. Note the high degree of conservation in a glycine-rich region extending in both families between residues 38–53, which in PR-10 proteins corresponds to a structurally invariant loop. Species codes: Vr, mung bean (*V. radiata*); Gm, soybean (*Glycine max*); Ll, yellow lupin (*Lupinus luteus*).

proteins has been established (Gajhede *et al.*, 1996; Biesiadka *et al.*, 2002), their physiological role remains unknown. A highly conserved glycine-rich motif in the PR-10 sequences forms a rigid and structurally invariant loop which has been implicated in binding nucleoside-type ligands (Gajhede *et al.*, 1996; Biesiadka *et al.*, 2002). It is of note that this glycine-rich region is also highly conserved in CSBP sequences (Fig. 1).

The main goal of our work is to determine the structure of free and cytokinin-bound CSBP proteins (i) to establish the mode of plant hormone binding, (ii) to verify the classification of CSBP proteins in the PR-10 family and, in the case of a positive result, (iii) to shed more light on the function of the ubiquitous plant PR-10 proteins, the physiological role of which remains a mystery (Sikorski *et al.*, 1999; Biesiadka *et al.*, 2002). As the first step, we have established reproducible crystallization conditions for VrCSBP in complex with zeatin and demonstrate that the crystals diffract X-rays with atomic resolution.

2. Materials and methods

2.1. Protein expression and purification

Recombinant *V. radiata* CSBP was produced in *Escherichia coli* cells, strain BL21(DE3)pLysS, using the T7 promoter/T7RNA polymerase system (Studier *et al.*, 1990). The VrCSBP coding sequence of 465 bp was PCR-amplified from a pGEX-4T3 vector (Fujimoto *et al.*, 1998) and introduced into a pET-3a vector. The recombinant protein appeared in IPTG-induced bacterial cells as described previously for lupin PR-10 proteins (Sikorski, 1997) and was fractionated by 80% ammonium sulfate precipitation. The precipitated protein fraction was dissolved in 20 m*M* sodium phosphate buffer pH 7.5, 5% glycerol, 10 m*M* β-mercaptoethanol and dialyzed against the same buffer. The protein preparation was clarified by centrifugation and passed through a DE52 cellulose column. Fractionation was carried out by stepwise elution with NaCl. The fraction eluted with 200 m*M* NaCl was submitted for further purification on a MonoQ column (FPLC) using a linear NaCl gradient. The fraction eluted with 0.34 *M* NaCl was purified to homogeneity by size-exclusion chromatography on a Superdex 75 HiLoad FPLC column. Finally, the homogenous protein was concentrated to 40 mg ml^{-1} using Centricon 10 filters and the buffer was exchanged for 3 m*M* sodium citrate pH 6.3. The yield of the recombinant protein after purification was 30–40 mg per litre of liquid culture.

2.2. Crystallization of VrCSBP–zeatin complex

Crystallization of VrCSBP was carried out using the hanging-drop vapour-diffusion method. The recombinant VrCSBP yielded diffraction-quality crystals only in the presence of the natural cytokinin zeatin. Several other cytokinins were tried but the results were less satisfactory. The only promising results were obtained with CPPU, but the crystals, which had the form of extremely thin needles, gave very limited diffraction. VrCSBP is very sensitive to organic precipitants such as alcohols, polyalcohols or PEGs, which even as minor additives (below 1%) prevent crystallization. The first crystallization trials failed because of the presence of 2% glycerol in the protein solution, which caused amorphous precipitation after mixing with the salt-containing well solutions. The crystallization drops were set up using a protein concentration of 15 mg ml^{-1} in 3 m*M* sodium citrate buffer pH 6.3, preincubated (in a 1:3 molar ratio) with 100 m*M* zeatin dissolved in 200 m*M* HCl. The high affinity of VrCSBP for cytokinins guarantees that under these conditions the protein should exist practically exclusively as a zeatin complex. The protein–ligand complex crystallized from high-salt precipitants containing 1.3–1.6 *M* sodium citrate/0.1 *M* HEPES buffer pH 7.5. Crystal morphology was improved by mixing the protein with the ligand a few hours prior to setting up the crystallization drops. The initial amorphous precipitate that appeared during the incubation period could be removed by centrifugation. Optimal conditions for growing VrCSBP–zeatin crystals were established in a very narrow salt-concentration range (1.38–1.40 *M*). Drop size was also a significant factor in the crystallization. The initial screening was performed using very small drops (0.8 + 0.8 µl). The final crystallization experiments used 3.0 + 3.0 µl drops and led to single crystals of very good quality. Small crystals appeared after 3–4 d and reached their final size in two weeks. In the initial conditions the crystals grew in the form of hexagonal rods. Fine tuning of the precipitant concentration resulted in clusters of several easy-to-separate crystals (Fig. 2*a*). Finally, removing of the initial precipitate of the VrCSBP–zeatin complex resulted in the formation of single crystals with dimensions of up to 2.0 × 0.3 × 0.3 mm (Fig. 2*b*).

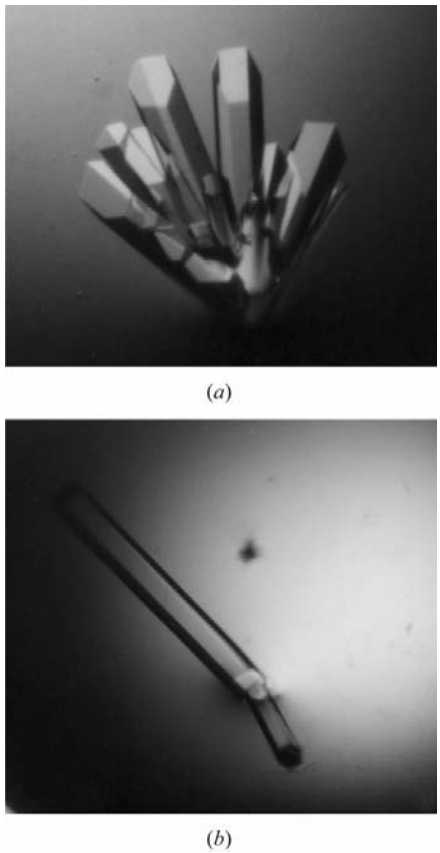

Figure 2
Crystallization of VrCSBP in the presence of the plant growth hormone zeatin. (*a*) Clusters of single crystals obtained after fine-tuning the precipitant (sodium citrate) concentration. (*b*) Isolated single crystals grown in precipitate-free drops; the initial VrCSBP–zeatin precipitate was removed by centrifugation of the solution after incubation.

2.3. Data collection and processing

High-resolution (1.25 Å) diffraction data for the VrCSBP–zeatin complex were measured at the EMBL BW7B beamline at the DORIS ring of the DESY synchrotron using a MAR 345 mm image-plate detector (Fig. 3, Table 1). The data were collected under cryogenic conditions using the flash-freezing method (Teng, 1990). The high concentration of the precipitant (1.4 *M* sodium citrate) provided effective cryoprotection. The crystal used for data collection was an uncut specimen with dimensions of 0.4 × 0.3 × 0.3 mm. Three runs, corresponding to high, medium and low resolution, were collected. The maximum resolution, oscillation range and number of images were as follows: run 1, 1.20 Å, 0.5°, 181; run 2, 1.79 Å, 1.0°, 90; run 3, 2.46 Å, 1.5°, 60. For final scaling, reflections from the following ranges of the high-, medium- and low-resolution runs were used: 2.5–1.25, 4.3–1.79 and 30.0–2.46 Å, respectively.

Table 1
Summary of crystal data and data-collection parameters.

Values in parentheses are for the last resolution shell.

Space group	$P6_2$ ($P6_4$)
Unit-cell parameters (Å)	a = 113.62, c = 86.85
Crystal dimensions (mm)	0.4 × 0.3 × 0.3
Resolution limits (Å)	30–1.25
Radiation source	DESY, BW7B beamline (EMBL)
Wavelength (Å)	0.8441
Temperature (K)	100
No. of measured reflections	1263397
No. of unique reflections	169129
Redundancy	7.47 (5.45)
$R_{\rm int}$	0.081 (0.513)
Completeness (%)	96.4 (94.8)
$\langle I/\sigma(I)\rangle$	20.8 (3.57)

Indexing and integration of the images was performed in *DENZO* and scaling of the intensity data was performed in *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

The recombinant cytokinin-specific binding protein from mung bean could not be crystallized in the absence of its ligands, *i.e.* cytokinins or plant growth hormones. In view of the very high binding constant, it is almost certain that the crystallizing entity is a protein–cytokinin complex, but the ultimate proof will be provided when the crystal structure is solved. In the present experiments, zeatin was used as the ligand in a 3:1 molar excess. Single crystals could be grown only using high but precisely adjusted salt concentrations. Addition of alcohols hindered the crystallization process and resulted in amorphous precipitation. The crystals grow as well shaped hexagonal rods and diffract X-rays to very high resolution. The current data set extends to 1.25 Å and is characterized by very good statistical parameters (Table 1). The crystals belong to the hexagonal system, space group $P6_2$ or $P6_4$, with unit-cell parameters a = 113.62, c = 86.85 Å, and contain multiple copies of the protein in the asymmetric unit. The acceptable range of Matthews volumes (Matthews, 1968), 1.90–4.76 Å^3 Da^{-1}, indicates between five and two molecules, corresponding to 35–74% solvent content. If, as suggested by the limited sequence identity, this CSBP protein does belong to the PR-10 family (Fig. 1), then it is very unlikely that it would form any quaternary aggregates, as all PR-10 proteins that have been characterized structurally exist as monomers (Gajhede *et al.*, 1996; Biesiadka *et al.*, 1999, 2002). This is confirmed by the results of Patterson self-rotation analyses for VrCSBP (not shown), which failed to detect obvious non-crystallographic rotational symmetry. Similarly, a native Patterson map did not provide an indication of non-crystallographic translational symmetry. Our attempts to solve the structure of the VrCSBP–zeatin complex by molecular replacement using the PR-10 structures as molecular probes have so far failed. The fact that the present structure of VrCSBP cannot be solved by molecular replacement using PR-10 probes could indicate one or more of the following possibilities: (i) failure owing to the presence of numerous copies of the protein in the asymmetric unit; (ii) VrCSBP is a member of the PR-10 class but undergoes a significant structural transformation upon ligand binding; (iii) VrCSBP is not structurally similar to PR-10 proteins; (iv) the structural variability visible within the PR-10 class, consisting in a high level of plasticity of the C-terminal helix (Biesiadka *et al.*, 2002), is sufficient to hinder the molecular-replacement approach. Work on solving the structure by the method of isomorphous replacement is in progress.

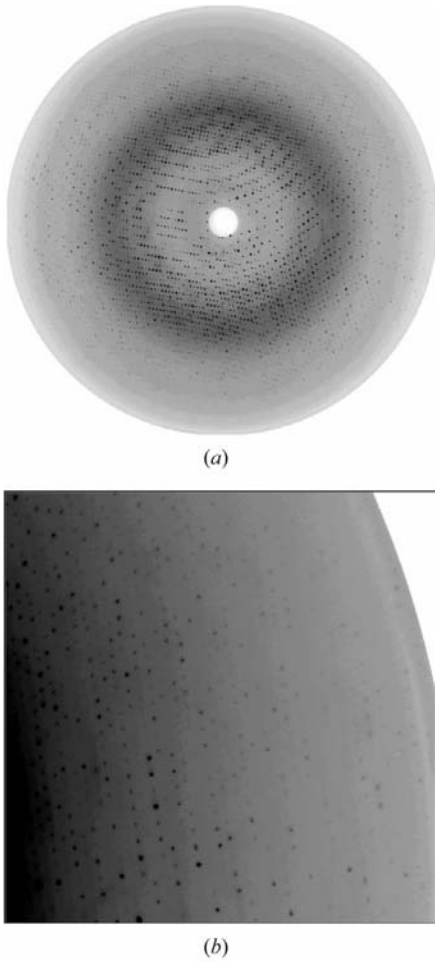

Figure 3
X-ray diffraction images recorded for a single crystal of the VrCSBP–zeatin complex. (*a*) Typical 1° oscillation diffraction image at medium resolution (the edge of the detector corresponds to 1.79 Å *d* spacing). (*b*) Close-up view at the detector edge (1.20 Å *d* spacing) of a high-resolution image (0.5° oscillation).

We wish to thank Alina Kasperska for skilful help with protein expression and purification. This work was supported by grants from the International Centre for Genetic Engineering and Biotechnology (ICGEB) to MJ (CRP/POL00-06) and from the State Committee for Scientific Research (KBN) to MMS (6 P04B 004 21). We acknowledge support from the European Community Access to Research Infrastructure Action of the Improving Human Potential Programme to the EMBL Hamburg Outstation, contract number HPRI-CT-1999-00017. Some calculations were carried out in the Poznan Metropolitan Supercomputing and Networking Center.

References

Biesiadka, J., Bujacz, G., Sikorski, M. M. & Jaskolski, M. (2002). *J. Mol. Biol.* **319**, 1223–1234.
Biesiadka, J., Sikorski, M. M., Bujacz, G. & Jaskolski, M. (1999). *Acta Cryst.* D**55**, 1925–1927.
Chen, C., Melitz, D. K., Petschow, B. & Eckert, R. L. (1980). *Eur. J. Biochem.* **108**, 379–387.
Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., Sugimoto, S. & Shudo, K. (1998). *Eur. J. Biochem.* **258**, 794–802.
Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., van Neerven, R. J. J., Schou, C., Lowenstein, H. & Spangfort, M. D. (1996). *Nature Struct. Biol.* **3**, 1040–1045.
Hall, R. H. (1973). *Annu. Rev. Plant Physiol.* **24**, 415–444.
Hamaguchi, N., Iwamura, H. & Fujita, T. (1985). *Eur. J. Biochem.* **153**, 565–572.
Iwamura, H., Fujita, T., Koyama, S., Koshimizu, K. & Kumazawa, Z. (1980). *Phytochemistry*, **19**, 1309–1319.
Iwamura, H., Murakami, S., Koshimizu, K. & Matsubara, S. (1985). *J. Med. Chem.* **28**, 577–583.
Jayabaskaran, C. (1990). *Plant Growth Reg.* **9**, 9–17.
Kulaeva, O. N., Karavaiko, N. N., Moshkov, I. E., Selivankina, S. Yu. & Novikova, G. V. (1990). *FEBS Lett.* **261**, 410–412.
Kulaeva, O. N., Karavaiko, N. N., Selivankina, S. Yu., Zemlyachenko, Ya. V. & Shipilova, S. V. (1995). *FEBS Lett.* **366**, 26–28.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mitsui, S. & Sugiura, M. (1993). *Plant Cell Physiol.* **34**, 543–547.
Momotani, E. & Tsuji, H. (1992). *Plant Cell Physiol.* **33**, 407–412.
Nagata, R., Kawashi, E., Hashimoto, Y. & Shudo, K. (1993). *Biochem. Biophys. Res. Commun.* **191**, 543–549.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–327.

Romanov, G. A., Taran, V. Y. & Venis, M. A. (1990). *J. Plant Physiol.* **136**, 208–212.
Sakai, S. & Kamei, N. (1992). *Biosci. Biotechnol. Biochem.* **56**, 504–507.
Sikorski, M. M. (1997). *Acta Biochim. Pol.* **44**, 565–578.
Sikorski, M. M., Biesiadka, J., Kasperska, A. E., Kopcinska, J., Lotocka, B., Golinowski, W. & Legocki, A. B. (1999). *Plant Sci.* **149**, 125–137.
Skoog, F. & Armstrong, D. J. (1970). *Annu. Rev. Plant Physiol.* **21**, 359–384.
Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorf, J. W. (1990). *Methods Enzymol.* **185**, 60–89.
Teng, T.-Y. (1990). *J. Appl. Cryst.* **23**, 387–391.
Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Nucleic Acid Res.* **22**, 4673–4680.
Van Loon, L. C. & Van Strien, E. A. (1999). *Physiol. Mol. Plant Pathol.* **55**, 85–97.
Yoshida, K. & Takegami, T. (1977). *J. Biochem.* **81**, 791–799.