Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of carboxypeptidase Y inhibitor I^C complexed with the cognate proteinase

Joji Mima,[a] Minoru Hayashida,[b] Tomomi Fujii,[b] Yasuo Hata,[a] Rikimaru Hayashi[c] and Mitsuyoshi Ueda[a]*

[a]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, [b]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan, and [c]College of Bioresourse Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan

Correspondence e-mail: miueda@kais.kyoto-u.ac.jp

Received 11 June 2002
Accepted 21 June 2004

Carboxypeptidase Y (CPY) inhibitor I^C is a naturally occurring serine carboxypeptidase inhibitor from *Saccharomyces cerevisiae*, the sequence of which is not homologous with any other known proteinase inhibitor and is classified as the phosphatidylethanolamine-binding protein (PEBP). I^C has been crystallized in complex with the deglycosylated form of CPY by the hanging-drop vapour-diffusion technique with ammonium sulfate as a precipitant. The crystals of the complex belong to space group $P2_12_12_1$, with unit-cell parameters a = 81.13, b = 186.6, c = 65.14 Å. Diffraction data were collected to 2.7 Å resolution. Structure determination of the complex is in progress by the molecular-replacement method using the structure of CPY as a search model.

1. Introduction

Lysosomal/vacuolar proteinases of eukaryotic cells are specifically inhibited by endogenous cytoplasmic proteinase inhibitors *in vitro*. A number of these inhibitors have been isolated and characterized: cystatins and stefins from mammals (Turk & Bode, 1991), and proteinase A inhibitor I_3^A, proteinase B inhibitor I_2^B and carboxypeptidase Y (CPY) inhibitor I^C from *Saccharomyces cerevisiae* (Schu & Wolf, 1991; Schu *et al.*, 1991; Bruun *et al.*, 1998; Mima, Suzuki *et al.*, 2002). Although some of their three-dimensional structures have been determined in complex with the cognate proteinases, cathepsins and proteinase A (Jenko *et al.*, 2003; Li *et al.*, 2000), their physiological functions and participation in the regulation of lysosomal/vacuolar proteinase activities remain unclear to date.

I^C, a natural serine carboxypeptidase inhibitor composed of 219 amino acid residues, has no significant homology with any other known proteinase inhibitor and can be classified as a member of the phosphatidylethanolamine-binding protein (PEBP) family (Bruun *et al.*, 1998). It has previously been reported, on the basis of the putative biological functions of the mammalian PEBP, that, in addition to phospholipid binding, the protein is an inhibitor of each of serine proteinases (Hengst *et al.*, 2001), Raf-1 kinase (Yeung *et al.*, 1999) and G-protein-coupled receptor kinase 2 (Lorenz *et al.*, 2003), and is a precursor for the hippocampal cholinergic neurostimulatory peptide (Tohdoh *et al.*, 1995). The crystal structures of human and bovine PEBPs reveal that the proteins are mainly composed of a β-sheet structure and contain a binding site for the polar head group of phospholipid (Banfield *et al.*, 1998; Serre *et al.*, 1998). It was recently demonstrated that I^C contains the CPY-binding site as well as the inhibitory reactive site including the N-terminal acetyl group (Mima *et al.*, 2003; Mima, Kondo & Hayashi, 2002). It was also reported that I^C inhibits a Ras GTPase-activating protein from *S. cerevisiae*, Ira2p (Chautard *et al.*, 2004). However, these findings do not clarify the specific cellular functions of the PEBP family including I^C and their physiological roles continue to be obscure.

To solve the first three-dimensional structure of a PEBP family member, in complex with its macromolecular binding partner, and also that of a serine carboxypeptidase inhibitor complexed with the cognate proteinase, we crystallized I^C complexed with CPY and performed a preliminary diffraction study of the complex. The X-ray analysis of the complex structure should provide further insights into the biological functions of PEBPs as well as cytoplasmic proteinase inhibitors.

2. Materials and methods

2.1. Preparation of I^C and the deglycosylated form of CPY

I^C was produced using the yeast *S. cerevisiae* expression system with the pYTF1 expression vector and the vacuolar proteinases-deficient strain BJ2168, and purified as previously described (Mima, Suzuki *et al.*, 2002). Wild type CPY was purified from bakers' yeast and deglycosylated using endoglycosidase H (Roche, Basel, Switzerland) as previously described (Hayashi, 1976; Endrizzi *et al.*, 1994). After deglycosylation, the resulting solution was applied to a Mono Q column (5 × 50 mm,

Amersham Biosciences, Piscataway, USA) equilibrated with 10 m*M* sodium phosphate pH 7.0 containing 0.2 *M* NaCl, and the deglycosylated form of CPY (degCPY) was eluted with a linear gradient using an NaCl concentration from 0.2 to 0.5 *M*.

2.2. Crystallization

The purified I^C and degCPY solutions were buffer-exchanged to 10 m*M* bis-Tris pH 6.5 containing 0.1 *M* NaCl, 1 m*M* DTT and 2 µg ml^{-1} of pepstatin A, and concentrated to 15 mg ml^{-1} and 20 mg ml^{-1}, respectively, using a Centricon YM-30 (Millipore, Bedford, USA). Prior to crystallization, I^C was mixed with equimolar amounts of degCPY to prepare the complex and the complex solution was diluted to a final concentration of 12 mg ml^{-1}. Crystallization was performed using the hanging-drop vapour-diffusion method at 298 K, in which droplets were prepared by mixing 2 µl of the complex solution with 2 µl of a precipitant solution, followed by equilibration against 500 µl of the precipitant solution. Initial screening of the crystallization conditions using Crystal Screen I (Hampton Research, Aliso Viejo, USA) showed that crystals grew when ammonium sulfate was used as a precipitant. The crystallization conditions were further optimized by varying the concentration of the precipitant and the pH of the buffer system.

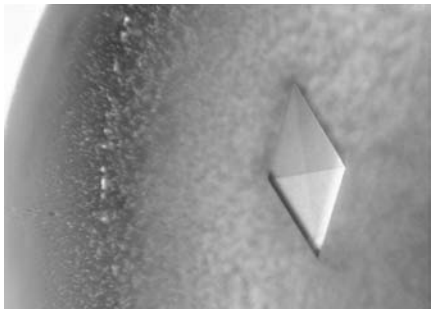

Figure 1
Crystal of I^C complexed with the deglycosylated form of CPY. The dimensions of the crystal are approximately 0.2 × 0.2 × 0.4 mm.

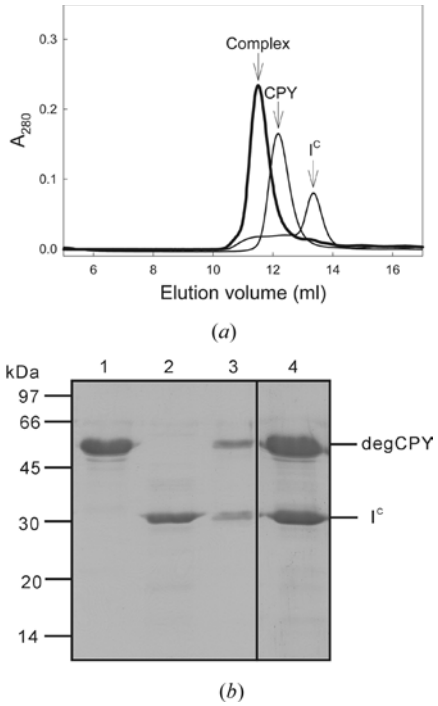

Figure 2
Analysis of the complex formation of I^C with the deglycosylated form of CPY (degCPY) under the crystallization conditions used. (*a*) Gel-filtration analysis. The I^C–degCPY complex was applied to a Superdex 75 column (10 × 300 mm) pre-equilibrated with 0.1 *M* sodium acetate (pH 4.7), and was eluted with the same buffer. (*b*) SDS–PAGE analysis. Lane 1, degCPY; lane 2, I^C; lane 3, a single peak obtained by gel filtration of the complex; lane 4, crystals of the complex.

2.3. Chemical analysis

Gel-filtration analysis was carried out using a Superdex 75 column (10 × 300 mm, Amersham Biosciences) equilibrated with the buffer used for a reservoir solution of the crystallization of the I^C–degCPY complex (0.1 *M* sodium acetate pH 4.7). An equimolecular mixture of I^C (67 µg) and degCPY (145 µg) was applied to the column, elution was performed with the equilibration buffer and the eluant was monitored by measuring the absorbance at 280 nm. Fractions containing a single peak were pooled and subjected to SDS–PAGE analysis.

The inhibitory constant, K_i, of I^C was determined using the method described by Mima, Suzuki *et al.* (2002), with a minor modification, in which various concentrations of I^C were mixed with degCPY in 0.1 *M* sodium acetate pH 4.7 containing 0.3 m*M* *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) and 10%(*v*/*v*) *N*,*N*-dimethylformamide. The K_m value of 0.57 m*M* for BTPNA was used to calculate the inhibitory constant.

2.4. Data collection and processing

X-ray diffraction data were collected from the I^C–degCPY complex crystals at 298 K using an R-AXIS IV image-plate area detector (Rigaku, Tokyo, Japan) and Cu $K\alpha$ radiation from a Rigaku RU-300 rotating-anode X-ray generator equipped with a confocal mirror system operating at 40 kV and 100 mA. The crystal-to-detector distance was set to 170 mm, and each oscillation frame was taken with a 1.5° oscillation range. The diffraction data were processed and scaled using the *HKL*2000 program package (Otwinowski & Minor, 1997).

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest-resolution shell (2.80–2.70 Å).

Wavelength (Å)	1.542
Resolution limit (Å)	2.70
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 81.13, b = 186.6, c = 65.14
Observed reflections	99 650
Unique reflections	28 017
Redundancy	3.6
Completeness (%)	99.3 (100)
R_{merge}† (%)	7.0 (34.9)
$I/\sigma(I)$	15.8 (2.9)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of symmetry-related reflections and $\langle I(hkl)\rangle$ is the average intensity over all symmetry equivalents.

3. Results and discussion

Wild type CPY was efficiently deglycosylated by endoglycosidase H, as evidenced by a decrease in molecular mass from 61 000 to 53 000, and the deglycosylated form of CPY (degCPY) was subsequently repurified by anion-exchange column chromatography. The activity of CPY was not affected by the deglycosylation, as has been reported previously (Endrizzi *et al.*, 1994). The purity of I^C and degCPY in the preparation for crystallization was confirmed by SDS–PAGE analysis.

Prismatic crystals of I^C in complex with degCPY were first obtained from condition No. 46 of Hampton Research Crystal Screen I (2.0 *M* ammonium sulfate, 0.1 *M* sodium phosphate pH 4.6) within three weeks. After optimization of the crystallization conditions, suitable crystals with typical dimensions of 0.2 × 0.2 × 0.4 mm (Fig. 1) were prepared using a reservoir solution containing 1.95 *M* ammonium sulfate and 0.1 *M* sodium phosphate pH 4.7. They diffracted to 2.7 Å resolution.

To confirm complex formation of I^C with degCPY under the crystallization conditions used, gel-filtration, kinetic and SDS–PAGE analyses were carried out. Gel filtration at pH 4.7 of an equimolecular mixture of I^C and degCPY showed a single peak corresponding to the I^C–degCPY complex (Fig. 2*a* and lane 3 in Fig. 2*b*). The intrinsic inhibitory constant, K_i, at pH 4.7 of I^C was calculated to be 21.0 n*M* for the BTPNA hydrolysis of degCPY. SDS–PAGE of the dissolved crystals indicated that the crystals contained both I^C and degCPY (lane 4 in Fig. 2*b*). These results demonstrate that I^C specifically inhibits and interacts with degCPY under the crystallization conditions.

Preliminary analysis of the X-ray diffraction data shows that the complex crystals

belong to space group $P2_12_12_1$, with unit-cell parameters $a = 81.13$, $b = 186.6$, $c = 65.14$ Å. The 2.7 Å data set had an $R_{\rm merge}$ of 7.0% and a completeness of 99.3%. Details of the crystal parameters and data-collection statistics are summarized in Table 1. Assuming that the asymmetric unit of the crystal contains one molecule of the complex, the Matthews (1968) coefficient ($V_{\rm M}$) is 3.16 Å^3 Da^{-1} with a solvent content of 61%. Molecular replacement with the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) was performed using the coordinates of CPY (Endrizzi *et al.*, 1994; PDB code 1ysc) as a search model. The calculation gave a reasonable solution for CPY. In an initial electron-density map, clear densities corresponding to the I^C molecule are observed beyond the boundary of the CPY molecule. The structure determination of the complex is in progress.

References

Banfield, M. J., Barker, J. J., Perry, A. C. F. & Brady, R. L. (1998). *Structure*, **6**, 1245–1254.

Bruun, A. W., Svendsen, I., Sorensen, S. O., Kielland-Brandt, M. C. & Winther, J. R. (1998). *Biochemistry*, **37**, 3351–3357.

Chautard, H., Jacquet, M., Schoentgen, F., Bureaud, N. & Benedetti, H. (2004). *Eukaryot. Cell*, **3**, 459–470.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Endrizzi, J. A., Breddam, K. & Remington, S. J. (1994). *Biochemistry*, **33**, 11106–11120.

Hayashi, R. (1976). *Methods Enzymol.* **45**, 568–572.

Hengst, U., Albrecht, H., Hess, D. & Monard, D. (2001). *J. Biol. Chem.* **276**, 535–540.

Jenko, S., Dolenc, I., Guncar, G., Dobersek, A., Podobnik, M. & Turk, D. (2003). *J. Mol. Biol.* **326**, 875–885.

Li, M., Phylip, L. H., Lees, W. E., Winther, J. R., Dunn, B. M., Wlodawer, A., Kay, J. & Gustchina, A. (2000). *Nature Struct. Biol.* **7**, 113–117.

Lorenz, K., Lohse, M. J. & Quitterer, U. (2003). *Nature* (*London*), **426**, 574–579.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Mima, J., Kondo, T. & Hayashi, R. (2002). *FEBS Lett.* **532**, 207–210.

Mima, J., Narita, Y., Chiba, H. & Hayashi, R. (2003). *J. Biol. Chem.* **278**, 29792–29798.

Mima, J., Suzuki, H., Takahashi, M. & Hayashi, R. (2002). *J. Biochem.* **132**, 967–973.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Schu, P., Suarez Rendueles, P. & Wolf, D. H. (1991). *Eur. J. Biochem.* **197**, 1–7.

Schu, P. & Wolf, D. H. (1991). *FEBS Lett.* **283**, 78–84.

Serre, L., Vallee, B., Bureaud, N., Schoentgen, F. & Zelwer, C. (1998). *Structure*, **6**, 1255–1265.

Tohdoh, N., Tojo, S., Agui, H. & Ojika, K. (1995). *Brain Res. Mol. Brain Res.* **30**, 381–384.

Turk, V. & Bode, W. (1991). *FEBS Lett.* **285**, 213–219.

Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K. D., Rose, D. W., Mischak, H., Sedivy, J. M. & Kolch, W. (1999). *Nature* (*London*), **401**, 173–177.