Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, co-crystallization and preliminary X-ray analysis of the natural aspartic proteinase inhibitor I^A3 complexed with saccharopepsin from *Saccharomyces cerevisiae*

M. O. Badasso,[a]* J. A. Read,[b] V. Dhanaraj,[c] J. B. Cooper,[d] S. P. Wood,[d] T. L. Blundell,[c] T. Dreyer[e] and J. Winther[e]

[a]Departments of Microbiology and Oral Science, University of Minnesota, 18-246 Moos Tower, 515 Delaware Street SE, Minneapolis, MN 55455, USA, [b]Department of Biochemistry, University of Bristol, Bristol BS8 1TD, England, [c]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB1 1QW, England, [d]Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, England, and [e]Department of Chemistry, Carlsberg Laboratory, Copenhagen, Valby, Denmark

Correspondence e-mail: mohammed@dcmir.med.umn.edu

Received 19 November 1998
Accepted 16 May 2000

The vacuolar aspartic proteinase from baker's yeast, saccharopepsin, has been co-crystallized with its natural inhibitor I^A3, found in the cytosol. The I^A3–saccharopepsin complex crystals belong to the space group $P6_222$, with unit-cell parameters $a = b = 192.1$, $c = 59.80$ Å and one molecule per asymmetric unit. The initial X-ray analysis of the complex indicates that the crystals diffract to 5.0 Å, similar to native saccharopepsin crystals. This is probably a consequence in part of glycosylation of the native saccharopepsin. Full structural analysis of the complex crystal is in progress.

1. Introduction

The main proteinases in *S. cerevisiae* lysate, namely proteinase-A (saccharopepsin) and proteinase-B, have been isolated and characterized (Hata *et al.*, 1967; Lenney & Dalbec, 1967). Saccharopepsin is an aspartic proteinase of 43 kDa molecular weight, whereas proteinase-B is a member of the serine-proteinase family with a molecular weight of 31 kDa (Jones, 1991). Both of these vacuolar proteinases and their natural polypeptide cytosolic inhibitors were further characterized (Saheki *et al.*, 1974; Munez de Castro & Holzer, 1976; Meussdoerffer, 1980; Dreyer *et al.*, 1985, 1986) and it was found that saccharopepsin is inhibited by I^A3 and I^A4, whereas proteinase-B is inhibited by I^A1 and I^A2.

We have reported the co-crystallization of native saccharopepsin with a synthetic peptide renin inhibitor (Badasso *et al.*, 1993). The X-ray structures of both native saccharopepsin and a synthetic peptide inhibitor complex have now been solved (Aguilar *et al.*, 1997). The three-dimensional X-ray structure of saccharopepsin has confirmed the strong structural similarity that had previously been predicted to exist between saccharopepsin and the mammalian aspartic proteinases cathepsin-D, mouse renin and human renin. The structure of saccharopepsin has also confirmed the importance of a unique proline-rich sequence (Pro294–Pro297) that gives rigidity to the enzyme's specificity pockets (for details, see Dhanaraj *et al.*, 1992; Aguilar *et al.*, 1997).

The natural saccharopepsin inhibitor I^A3 investigated in this study comprises 67 amino-acid residues and has a molecular weight of 7700 Da (Beidermann, 1980). It has an isoelectric point of 6.3, the pH at which the inhibitor most effectively inhibits saccharopepsin. The X-ray study of I^A3 complexed with saccharopepsin has been of long-standing interest, as there are no structures available for macromolecular aspartic proteinase inhibitors or their complexes with target enzymes. In view of the important roles played by inhibitors of the serine-proteinase family, this represents a significant gap in our knowledge of the aspartic proteinase enzymes. Previous studies were hindered by the lack of an adequate amount of inhibitor material; the crystals which were obtained yielded poor data for X-ray analysis (Badasso, 1994). The inhibitor used in this study was synthesized as described below and was available in sufficient amounts for crystallography to proceed.

There are several natural specific proteinase inhibitors that have been identified. However, most of these inhibitors are directed towards the serine and cysteine proteinases (Bode & Huber, 1992). Several macromolecular aspartic proteinase inhibitors are known; these include the inhibitors from *Solanum tuberosum* (potato), the inhibitor of cathepsin-D (Mares *et al.*, 1989; Zalatoris *et al.*, 1998) and PI-3 from the pig roundworm *Ascaris suum*, which is a specific inhibitor of pepsin (Martzen *et al.*, 1990; Petersen *et al.*, 1998). In this paper, we report the preliminary analysis of crystals of a complex between the yeast aspartic proteinase inhibitor and saccharopepsin. The complete X-ray analysis of the complex between the inhibitor I^A3 and saccharopepsin is in progress.

2. Methods and materials

2.1. Synthesis and purification of I^A3

The I^A3 peptide was synthesized using Fastmoc (Applied Biosystems, Inc.) chemistry on an applied Biosystems 431A solid-phase peptide synthesizer. The protected peptide was cleaved from the resin and was deprotected

Table 1
Data sets of native saccharopepsin and the inhibitor complex crystals.

CP81,282 and PD130,327 are transition-state analogue inhibitors. Redundancy = 7.7 (6.9) and average $I/\sigma(I)$ = 13.7 (6.37). Values in parentheses are for the outermost resolution shell.

Materials	Resolution (Å)	Total No. of reflections	No. of unique reflections	Completeness	R_{merge} (%)
Native saccharopepsin	3.5	78083	12335	96.0	8.0
Saccharopepsin–CP81,282 complex	2.5	84192	16264	97.0	7.0
Saccharopepsin–PD130,327 complex	2.5	75305	15737	89.0	7.9
Saccharopepsin–I^A3 complex	5.2	22496	2910	94.0	11.0
Overall outer shell	(5.0)			(98.6)	(20.0)

using a mixture of phenol, ethanedithiol, thioanisole, water and trifluoroacetic acid and was precipitated using diethyl ether. The peptide was dried and dissolved in water for reverse-phase HPLC purification. The purity of the product was analyzed on a Fisons Instruments VG platform electrospray mass spectrometer; a single peak was exhibited for the peptide.

2.2. Co-crystallization

The optimum co-crystallization conditions were found after extensive screening of buffer pH and precipitants. Solutions of native saccharopepsin purified from *S. cerevisiae* (Dreyer *et al.*, 1986; Badasso *et al.*, 1993) were initially concentrated to 10 mg ml^{-1} using ultrafiltration (Amicon YM10, Amicon corporation) in 50 m*M* sodium acetate buffer pH 5.5. The enzyme was then mixed with a tenfold molar excess of inhibitor I^A3 and crystals of the complex were obtained using vapour diffusion against 10–35%(*w*/*v*) polyethylene glycol 6000 in the same buffer in the pH range 3.5–5.8. The complex solution produced large crystals (0.35 × 0.36 × 0.25 mm) of the same quality suitable for data collection after several weeks both at room temperature and in the cold room (277 K).

2.3. X-ray data collection

X-ray data from crystals of the complex with I^A3 were initially collected with synchrotron radiation using a MAR Research image plate at station 9.5 of the SRS at Daresbury, England. These data sets are compared with previous data sets of native saccharopepsin and its complexes with synthetic renin inhibitors (Badasso *et al.*, 1993) measured at the Photon Factory, Japan (Table 1).

3. Results and discussion

Baker's yeast produces specific natural inhibitors I^A1 and I^A2 for proteinase-B and I^A3 and I^A4 for saccharopepsin. These inhibitors are localized in the yeast cytosol which surrounds the vacuoles (Meussdoerffer, 1980) and are involved in the regulation of proteolytic activity of their respective proteinases. The proteinases are involved in the protein degradation that takes place when yeast is starved of nitrogen, promoting sporulation. The proteinase inhibitors hence play a significant biological role in the intracellular regulation of these enzymes.

The co-crystallization of inhibitor I^A3 and saccharopepsin produced crystals of different morphology to the native enzyme crystals and to complex crystals obtained with the smaller inhibitors CP21,282 and PD130,327 (Badasso *et al.*, 1993; Aguilar *et al.*, 1997). The native saccharopepsin crystals belonged to the orthorhombic space group $I2_12_12_1$, with unit-cell parameters a = 101.4, b = 128.7, c = 155.4 Å and two molecules per asymmetric unit. The crystals of CP81,282 and PD130,327 complexed with saccharopepsin both belonged to the trigonal space group $P3_221$, with unit-cell parameters $a = b$ = 87.4, c = 110.2 Å and one molecule in the asymmetric unit. The complex crystals of I^A3 and saccharopepsin are hexagonal with point group 622, unit-cell parameters $a = b$ = 192.1, c = 59.8 Å and one molecule in the asymmetric unit. A data set of 98% completeness with an overall R_{merge} of 11.0% was obtained by processing the images with *DENZO* and *SCALEPACK* (Gewirth, 1996; Table 1). Preliminary molecular-replacement calculations on the saccharopepsin–I^A3 complex (in progress) indicate that the space group is $P6_222$.

The I^A3–saccharopepsin complex crystals yielded data roughly comparable in quality to native saccharopepsin. The inhibitor complex crystal had different symmetry to the native enzyme. It is frequently found that co-crystallization of aspartic proteinases with inhibitors yields data of higher quality than those obtained from the native enzyme. The lack of improvement in the data quality with the saccharopepsin complex may be a consequence of the interfering effects of carbohydrates on the protein surface, since saccharopepsin is glycosylated (Aguilar *et al.*, 1997). However, other factors may be implicated, for example the bulkiness of the I^A3 peptide, which is much larger than most of the transition-state analogues studied with other aspartic proteinases. The complex crystals also have similar solvent contents: 60% compared with 59% for the native saccharopepsin crystals, which is unlike other inhibitor-bound aspartic proteinase crystals, where a much greater reduction in solvent content is observed upon inhibitor binding leading to substantial improvements in crystal quality (Badasso *et al.*, 1993).

The natural inhibitor of saccharopepsin I^A3 binds tightly to saccharopepsin with a K_i value of 0.7×10^{-8} *M* (Dreyer *et al.*, 1986). Its selective action for proteinase-A could suggest that the site of interaction of I^A3 with the saccharopepsin enzyme molecule differs from that of the other synthetic inhibitors analysed so far. The present complex crystal structure will define the unique interaction sites specific to I^A3. More importantly, it will provide the first natural inhibitor complex crystal structure in the aspartic proteinase enzyme family.

We acknowledge the financial support of the BBSRC (UK). We thank also Dr C. Aguilar for useful discussions and Dr R. Sarra for help in the synthesis of the I^A3 peptide.

References

Aguilar, C. F., Cronin, N. B., Badasso, M. O., Dreyer, T., Newman, M. P., Cooper, J. B., Hoover, D. J., Wood, S. P., Johnson, M. S. & Blundell, T. L. (1997). *J. Mol. Biol.* **267**, 899–915.

Badasso, M. O. (1994). PhD thesis. University of London, England.

Badasso, M. O., Wood, S. P., Aguilar, C., Cooper, J. B., Blundell, T. L. & Dreyer, T. (1993). *J. Mol. Biol.* **232**, 701–703.

Beidermann, K. (1980). *Carlsberg Res. Commun.* **45**, 225–235.

Bode, W. & Huber, R. (1992). *Eur. J. Biochem.* **204**, 433–451.

Dhanaraj, V., DeAlwis, C., Frazao, C., Badasso, M. O., Sibanda, B. L., Tickle, I. J., Cooper, J. B., Driessen, H. P. C., Newman, M., Aguilar, C., Wood, S. P., Blundell, T. L., Hobart, P. M., Geoghegan, K. F., Ammirati, M. J., Danley, D. E., O'Connor, B. A. & Hoover, D. J. (1992). *Nature (London)*, **357**, 466–472.

Dreyer, T., Halkjaer, B., Svendsen, I. & Ottesen, M. (1986). *Carlsberg Res. Commun.* **51**, 27–41.

Dreyer, T., Valler, M. J., Kay, J., Charlton, P. & Dunn, B. M. (1985). *Biochem. J.* **321**, 777–779.

Gewirth, D. (1996). *The HKL Manual. A Description of the Programs DENZO, XDISPLAY and SCALEPACK*. New Haven: Yale University Press.
Hata, T., Hayashi, R. & Doi, E. (1967). *Agric. Biol. Chem.* **31**, 150–159.
Jones, E. W. (1991). *J. Mol. Chem.* **266**(13), 7963–7966.
Lenney, J. F. & Dalbec, J. M. (1967). *Arch. Biochem. Biophys.* **120**, 42–48.
Mares, M., Meloun, B., Pavlik, M., Kostka, V. & Baudys, M. (1989). *FEBS Lett.* **251**, 94–98.
Martzen, M. R., McMullen, B. A., Smith, N. E., Fujisawa, K. & Peanasky, R. J. (1990). *J. Biochem.* **29**, 7366–7273.
Meussdoerffer, F. (1980). *J. Biol. Chem.* **254**, 423–429.
Munez de Castro, I. & Holzer, H. (1976). *Physiol. Chem.* **357**, 727–734.
Petersen, J. F. W., Chernaia, M. M., Rao-Naik, C., Zalatoris, J. L., Dunn, B. M. & James, M. N. G. (1998). *Adv. Exp. Med. Biol.* **436**, 391–395.
Saheki, T., Matsuda, Y. & Holzer, H. (1974). *Eur. J. Biochem.* **47**, 325–332.
Zalatoris, J., Rao-Naik, C., Fecho, G., Girdwood, K., Kay, J. & Dunn, B. M. (1998). *Adv. Exp. Med. Biol.* **436**, 387–389.