Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary crystallographic analysis of *N*-acetyltransferase Mpr1 from *Saccharomyces cerevisiae*

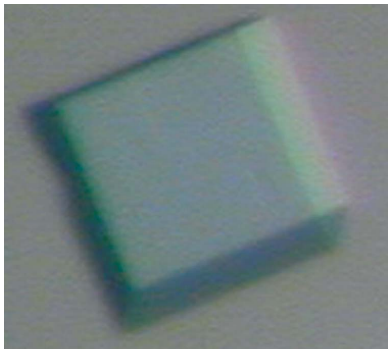
Takao Hibi,[a]* Hiromi Yamamoto,[a] Genichi Nakamura[a]‡ and Hiroshi Takagi[b]

[a]Department of Bioscience, Fukui Prefectural University, Fukui 910-1195, Japan, and [b]Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara 630-0192, Japan

‡ Present address: Department of Haematology, University of Cambridge, Cambridge CB2 0XY, England.

Correspondence e-mail: hibi@fpu.ac.jp

Received 5 December 2008
Accepted 3 January 2009



Mpr1 is an enzyme that catalyzes the *N*-acetylation of the toxic L-azetidine-2-carboxylic acid (AZC). Recently, Mpr1 has been shown to reduce levels of intracellular reactive oxygen species (ROS) under oxidative stress. The natural substrate involved in the ROS elimination *in vivo* is still unknown. Mpr1 has been purified and crystallized in space groups $P1$ and $P3_112$. X-ray data were collected to 1.9 Å resolution from a trigonal crystal soaked with AZC.

1. Introduction

Mpr1 was discovered as an enzyme that is involved in the resistance of *Saccharomyces cerevisiae* Σ1278 to the toxic L-azetidine-2-carboxylic acid (AZC), which is a four-membered ring analogue of L-proline. The incorporation of AZC into a protein causes the synthesis of abnormal misfolded proteins and thereby inhibits cell growth. Primary structure analysis of Mpr1 using *BLAST* suggested that the enzyme was a member of the *N*-acetyltransferase superfamily, which was supported by the results of site-directed mutagenesis of the acetyl-CoA-binding motif that is highly conserved in the superfamily (Shichiri *et al.*, 2001; Kotani & Takagi, 2008). Homologues of the *N*-acetyltransferase specific for AZC acetylation are only present in some yeast strains (Kimura *et al.*, 2002; Nomura *et al.*, 2003; Wada *et al.*, 2008). AZC, a rare toxic amino acid, only occurs in some plant species such as the Liliaceae family and is unlikely to be a natural substrate of Mpr1 (Leete, 1964; Peterson & Fowden, 1963). The Mpr1 enzyme may acetylate another substrate *in vivo* and have a physiologically conserved function in addition to AZC detoxification.

Recently, Mpr1 has been shown to reduce the levels of intracellular reactive oxygen species (ROS) under various types of oxidative stress, including H_2O_2, heat shock, freezing and ethanol treatment (Nomura & Takagi, 2004; Du & Takagi, 2005, 2007). Overexpression of Mpr1 leads to an increase in cell viability and a decrease in the ROS level after oxidative treatments, while *MPR1* gene-disrupted yeast cells are hypersensitive to oxidative stress. Our attention has been drawn to the L-proline catabolism intermediate Δ^1-pyrroline-5-carboxylate (P5C) and its equilibrium compound glutamate-γ-semialdehyde (GSA), as ROS was detected in a yeast mutant lacking P5C dehydrogenase. In *S. cerevisiae*, L-proline is converted to L-glutamate within mitochondria by the action of two mitochondrial enzymes: proline oxidase (the *PUT1* gene product) and P5C dehydrogenase (the *PUT2* gene product). Genetic and enzymatic analyses have shown that Mpr1 probably regulates the ROS level by acetylating P5C or GSA under P5C-induced oxidative stress. Unfortunately, the natural substrate of Mpr1 in the yeast cells has not yet been determined, so that the endogenous role of Mpr1 is still unclear.

There are some other members of the *N*-acetyltransferase superfamily whose functional properties are not fully understood *in vivo*: human arylamine *N*-acetyltransferases (NATs). The NAT enzymes show a relatively broad specificity for heterocyclic amines, including hydrazine drugs and carcinogens (Dalhoff *et al.*, 2005), and are involved in their detoxification or activation. NATs have genetic polymorphisms that produce enzymes that range from slow to fast acetylators and that have also been associated with individual

susceptibilities to various cancers and proposed as a possible biomarker for prostate cancer (Ambrosone & Kadlubar, 1998; Hamasaki *et al.*, 2003; Stacey *et al.*, 1996). NAT and its orthologues are widely present in both prokaryotes and eukaryotes and the essential catalytic triad Cys-His-Asp has been identified in all known NATs (Grant, 2008; Sim *et al.*, 2008; Westwood *et al.*, 2006; Dupret & Rodrigues-Lima, 2005). Mpr1 contains only two cysteine residues, which can be replaced by alanine residues without any loss of catalytic activity (data not shown); this mutagenesis result suggested that Mpr1 requires a different catalytic mechanism from NATs as a heterocyclic amine *N*-acetyltransferase that is involved in resistance towards oxidative stress. In order to reveal the structural fundamentals of substrate recognition and the catalytic mechanism, we have initiated X-ray crystallographic studies of Mpr1.

2. Materials and methods

2.1. Protein expression and purification

The Mpr1 protein was overexpressed according to the method of Shichiri *et al.* (2001) with slight modification. Competent cells of *Escherichia coli* strain SG13009 (Qiagen) were transformed with the expression vector pQE2 bearing the gene for full-length Mpr1 with 14 additional N-terminal amino-acid residues (MKHHHHHHMHAG-AQ) in combination with the pRARE plasmid (Novagen) instead of pPREP4. The transformant cells were grown at 303 K in M9 medium containing 2%(*w*/*v*) casamino acids, 50 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol with constant shaking. When the absorbance of the culture at 660 nm reached 0.3, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.1 m*M* and the culture was grown for an additional 18 h at 291 K.

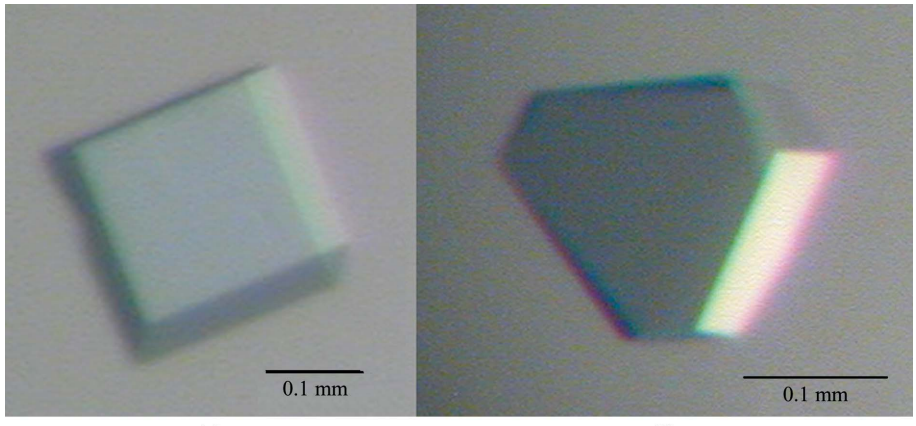
The bacterial cell pellet was resuspended in 50 m*M* sodium phosphate buffer pH 7.8 and 300 m*M* NaCl and then disrupted using a Vibra-Cell VCX500 ultrasonic processor (Sonics) at 277 K. The cell lysate was subjected to centrifugation at 20 000*g* for 20 min to remove the cell debris. The resulting cell-free extract was applied onto an Ni-affinity column (His-Trap FF, GE Healthcare Bioscience) equilibrated with 20 m*M* sodium phosphate buffer pH 7.4, 500 m*M* NaCl, 10 m*M* imidazole and the bound protein was eluted using a linear gradient of 10–500 m*M* imidazole. Fractions containing active Mpr1 were pooled and dialyzed against 50 m*M* Tris–HCl buffer pH 7.5, 1 m*M* EDTA, 10 m*M* imidazole and 10%(*w*/*v*) glycerol (buffer *A*), which was also used to equilibrate an anion-exchange column (DEAE Toyopearl 650M, Tosoh). The applied enzyme passed through the column under isocratic conditions using buffer *A* and a small amount of concomitant proteins were removed thoroughly. This treatment was an essential step in order to achieve sufficient growth of single crystals. The eluted enzyme was concentrated using an Amicon Ultra-15 ultrafiltration device (10 kDa cutoff). The purified enzyme was analyzed by SDS–PAGE, enzyme-activity assay and DLS. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

The acetyltransferase activity was assayed at 303 K by monitoring the increase in 5-thio-2-nitrobenzoic acid (TNB) as described previously with slight modification (Shichiri *et al.*, 2001). The increase in absorbance at 412 nm was measured in the presence of 50 m*M* $H_3BO_3/Na_2B_4O_7$ buffer pH 7.5 instead of ammonium acetate buffer. The reaction rate was calculated using an extinction coefficient for TNB of 15 570 M^{-1} cm^{-1}. One unit is defined as the amount of enzyme that catalyzes the formation of 1 µmol TNB per minute at 303 K.

2.2. Crystallization and X-ray data collection

For crystallization, the purified protein was concentrated to 1.5–2.0 mg ml^{-1} by ultrafiltration (Amicon Ultra-500, 10 kDa cutoff, Millipore). Crystals of Mpr1 were grown by the sitting-drop vapour-diffusion technique using a Cryschem crystallization plate (Hampton Research) at a constant temperature of 293 K. 2 µl protein solution was mixed with an equal volume of reservoir solution and the volume of the reservoir solution in the well was 1000 µl.

X-ray diffraction data were collected from the crystal belonging to space group *P*1 using an ADSC Quantum 4R CCD on synchrotron beamline BL-6A at the Photon Factory, KEK, Japan. The crystal was covered with Paratone-N oil (Hampton Research) and exposed to X-rays at 100 K. Individual frames consisted of a 1° oscillation angle measured for 40 s at a crystal-to-detector distance of 216.1 mm. The $P3_112$ crystals were soaked momentarily in a cryoprotectant solution containing 25% polyethylene glycol (PEG) 400 before cryocooling. In this case, X-ray data were collected using an ADSC Quantum 315 CCD on synchrotron beamline BL-5A and individual frames consisted of a 0.5° oscillation angle measured for 10 s. Intensity data were processed, merged and scaled with *MOSFLM* and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).



(*a*) (*b*)

Figure 1
Crystals of Mpr1 from yeast. (*a*) A single triclinic crystal (form I) obtained by the sitting-drop method. (*b*) A prism-shaped trigonal crystal (form II) obtained in the presence of $MgCl_2$.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

	Form I	Form II	Form II, AZC-soaked
Beamline	Photon Factory BL-6A	Photon Factory BL-5A	Photon Factory BL-5A
Space group	$P1$	$P3_112$	$P3_112$
Unit-cell parameters (Å, °)	a = 84.92, b = 89.93, c = 107.40, α = 93.93, β = 100.45, γ = 90.73	$a = b$ = 84.06, c = 192.34	$a = b$ = 83.87, c = 193.82
Wavelength (Å)	0.9726	1.1405	0.9793
Resolution (Å)	52.1–3.20 (3.37–3.20)	68.1–2.50 (2.64–2.50)	48.3–1.90 (2.00–1.90)
Total No. of reflections	389081	279029	490882
No. of unique reflections	50206	27342	61875
Completeness (%)	97.6 (97.3)	100.0 (99.9)	100.0 (100.0)
$I > 3\sigma(I)$ (%)	85.1	80.9	78.4
$\langle I/\sigma(I)\rangle$	18.4 (9.4)	25.0 (5.7)	26.0 (7.1)
Redundancy	7.7 (7.8)	10.2 (9.5)	7.9 (7.7)
$R_{\rm merge}$†	0.103 (0.222)	0.075 (0.380)	0.060 (0.287)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

3. Results and discussion

Mpr1 was overexpressed and purified with a yield of 2 mg protein from 0.5 l culture. The purity of the obtained enzyme was judged to be >95% from SDS–PAGE analysis and DLS analysis gave a monodisperse size distribution (data not shown). Its activity did not change over two weeks at 293 K. Initial screening for Mpr1 crystallization was carried out using the Crystal Screen HT kit (Hampton Research). A number of tiny plate-shaped or needle-shaped crystals were obtained using condition No. 33 (30% PEG 1500). The addition of 25%(*w*/*v*) glycerol to the protein solution prevented twinning of the crystals and promoted growth of the crystals up to 200 µm. Optimized crystals were obtained within two weeks at 293 K using reservoir solution containing 20%(*w*/*v*) PEG 1500 and 25%(*w*/*v*) glycerol. A typical crystal (form I) is shown in Fig. 1(*a*). Data from the crystal were recorded to 3.2 Å resolution and were indexed in the triclinic space group $P1$, with unit-cell parameters a = 84.92, b = 89.93, c = 107.40 Å, α = 93.93, β = 100.45, γ = 90.73°. Unfortunately, this triclinic crystal was unsuitable for producing ligand-complex crystals; the presence of ligands (substrates, inhibitors or products) either led to the inhibition of crystal growth or disrupted the crystals.

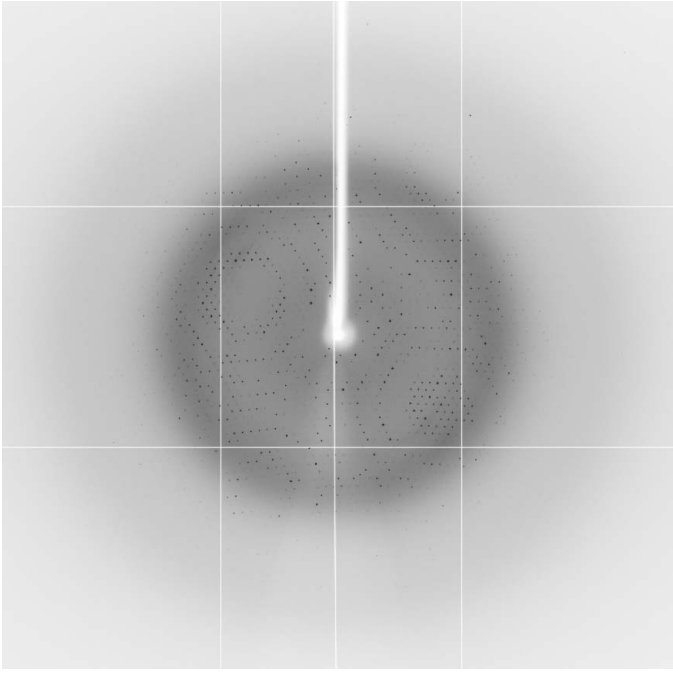


Figure 2
Diffraction image obtained on Photon Factory beamline BL-5A. Diffraction data were collected at a wavelength of 0.9793 Å with a crystal-to-detector distance of 237.8 mm. The resolution at the edges of the image is 1.9 Å.

In order to obtain another crystal form that might be suitable for producing a ligand complex, a second crystallization screening trial was carried out using the Index HT kit (Hampton Research). Prism-shaped crystals (50 µm) were obtained using condition No. 82 [25%(*w*/*v*) PEG 3350, 0.1 *M* bis-tris pH 5.5, 0.2 *M* $MgCl_2$]. Improved crystals were subsequently obtained within the pH range 5.1–5.5 with 21%(*w*/*v*) PEG 3350, 0.1 *M* bis-tris, 0.2 *M* $MgCl_2$. Crystals appeared after 4 d (1–2 d later than for the form I crystals) and grew to maximum dimensions of 0.16 × 0.14 × 0.06 mm within two weeks at 293 K. A typical crystal (form II) is shown in Fig. 1(*b*). The crystal used for X-ray analysis was soaked in its reservoir solution containing 25%(*w*/*v*) PEG 400 as a cryoprotectant and then flash-cooled in a cold nitrogen stream at 100 K. Data for the crystal were collected to a resolution of 2.5 Å at a crystal-to-detector distance of 250 mm. Analysis of merging statistics and systematic absences indicated that the crystals belonged to space group $P3_112$, with unit-cell parameters $a = b$ = 84.06, c = 192.34 Å. Cumulative intensity distributions indicated no twinning. In contrast to the triclinic crystals, soaking crystal form II in reservoir solution containing 50 m*M* AZC and 25%(*w*/*v*) PEG 400 for 1 min at 293 K led to a notable improvement in the diffraction quality of the crystal without a significant change in the unit cell. Data were collected from the AZC-soaked crystal to a resolution of 1.9 Å at a crystal-to-detector distance of 237.8 mm (Fig. 2). This crystal also belonged to space group $P3_112$, with unit-cell parameters $a = b$ = 83.87, c = 193.82 Å. Estimation of the content of the asymmetric unit based on a single Mpr1 subunit gave a Matthews coefficient $V_{\rm M}$ of 2.36 Å^3 Da^{-1} (Matthews, 1968), corresponding to 47.9% solvent content. Data-collection and processing statistics are shown in Table 1.

Structure determination was attempted using the molecular-replacement method as implemented in the program *Phaser* (McCoy *et al.*, 2007), but no solution could be found that correctly placed the template molecules in the crystal unit cells. The failure of molecular replacement is probably a result of the low degree (<15%) of sequence identity between Mpr1 and the search models of other acetyltransferases. A search for heavy-atom derivatives for use in the multi-wavelength anomalous diffraction method is now under way.

This work was supported in part by grants from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and a Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI; 18380062) to HT. The authors would like to thank the staff of the Photon Factory for the provision of synchrotron data-collection facilities.

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