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Crystallization and aldo–keto reductase activity of Gcy1p from *Saccharomyces cerevisiae*

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Crystallization and preliminary X-ray diffraction studies of Gcy1p, an aldo–keto reductase from *Saccharomyces cerevisiae*, have been performed. Both the wild type and a double-mutant form of Gcy1p were crystallized using the hanging-drop method at 298 K; however, only the double-mutant form has so far yielded crystals suitable for X-ray diffraction analysis. These crystals belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 50.94, b = 65.64, c = 86.23 Å, β = 92.64°. Diffraction data were collected to 2.5 Å. Assuming two 35 kDa subunits in the asymmetric unit yielded a V_m of 2.06 Å^3 Da^{-1}. Additionally, a kinetic study performed by measuring the rate of oxidation of NADPH in the presence of several substrates indicates that both wild-type and double-mutant proteins are enzymes possessing NADPH-dependent reductase activity.

1. Introduction

Gcy1p, also known as AKR3A, is a protein from *S. cerevisiae* which is a member of the aldo–keto reductase family of enzymes, a group of several score proteins spanning eukaryotes and prokaryotes (Jez *et al.*, 1996). In most cases, they fulfill poorly defined physiological roles, catalyzing the NADPH-dependent reduction of a carbonyl-containing compound to the corresponding alcohol. Although the sequences are divergent, there is substantial overlap of substrate specificity between members. Furthermore, each individual protein is usually able to catalyze the reduction of a broad range of substrates, often ranging in size and hydrophobicity from DL-glyceraldehyde to steroids. Despite this lack of specificity, the aldo–keto reductases have been studied *in vivo*, kinetically and structurally because of the apparent involvement of aldose reductase in the development of often severe diabetic complications in humans, such as retinopathy, nephropathy and neuropathy (Kinoshita & Nishimura, 1988). Inhibitors of aldose reductase have been useful in treating these complications, but serious side effects have arisen in humans, presumably owing to these compounds inhibiting non-target family members. Based upon the sequences, AKRs diverge significantly in substrate-binding sites, with insertions and deletions of loops. Knowledge of various enzyme active-site architectures will be useful in the development of more specific inhibitors.

The structure of aldose reductase (Rondeau *et al.*, 1992; Wilson *et al.*, 1992) defined the protein fold for the family as a $(\beta/\alpha)_8$ barrel, the most common motif found for enzymes. Besides aldose reductase, structures of other family members, aldehyde reductase (El-Kabbani *et al.*, 1995), 3α-hydroxysteroid dehydrogenase (Hoog *et al.*, 1994), FR-1 (Wilson *et al.*, 1995), 2,5-diketo-D-gluconic acid reductase (Khurana *et al.*, 1998) and a K^+ channel β subunit (Gulbis *et al.*, 1999), have also been determined. Inhibitor binding to both target and some non-target proteins have also been structurally characterized (Wilson *et al.*, 1993, 1995; Urzhumtsev *et al.*, 1997).

GCY1 was a gene initially identified as a yeast homolog of the eye-lens protein ρ-crystallin from the European common frog (Oechsner *et al.*, 1988). It is a dispensable protein, which may be a consequence of the presence of at least four other potentially redundant aldo–keto reductases in yeast. It is transcriptionally induced by galactose, a candidate substrate for the protein, implicating it in carbohydrate metabolism (Magdolen *et al.*, 1990). Although it had not been clearly shown to be an enzyme, Gcy1p is 61% homologous and 44% identical to human aldose reductase. More importantly, some of the NADPH-binding residues and all of the catalytic residues are conserved, suggesting that it possesses reductase activity.

2. Materials and methods

The intron-free *GCY1* gene was PCR amplified from genomic DNA isolated from the CRY1 strain of *S. cerevisiae* with the 5′ primer 5′-CCCATATGCCTGCTACTTTACATGAT and

Table 1
Kinetic constants for Gcy1p substrates show activity which is comparable to that found in other aldo–keto reductases.

Constants of the double-mutant enzyme (Pro268→Leu; Ser281→Phe) indicate that the catalytic properties are not significantly changed from the wild-type enzyme. ND, not determined.

	Wild type		Double mutant	
	K_m	k_{cat} (s^{-1})	K_m	k_{cat} (s^{-1})
DL-Glyceraldehyde	11.3 m*M*	3.83	8.4 m*M*	4.4
Glucuronate	ND	ND	20 m*M*	1.5
Propionaldehyde	ND	ND	144 m*M*	2.3
NADPH	28.5 µ*M*	4.57	24 µ*M*	3.7

Table 2
Crystal parameters and data-collection statistics for Gcy1p.

Values for the high-resolution shell are given in parentheses.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 50.94, b = 65.64, c = 86.23, β = 92.64
V_m (assuming two molecules per asymmetric unit) (Å^3 Da^{-1})	2.06
Resolution (Å)	100–2.49 (2.59–2.49)
Unique reflections/observations	19639/59321
Multiplicity	3.02
Completeness (%)	97.1 (80.7)
$I/\sigma(I)$	22.4 (6.0)
R_{merge}	0.054 (0.168)

the 3′ primer 5′-TCCCCCGGGCTTGAA-TACTTCGAAAGGAGACCA. The resulting fragment was inserted into a blunt-ended cloning vector and then transferred to the expression vector pTYB2 (New England Biolabs) and used to transform the *Escherichia coli* strain BL21(DE3) for expression. DNA sequence analysis indicated that two point mutations (Pro268→Leu and Ser281→Phe) were introduced by errors in the PCR reaction. A second attempt yielded the wild-type sequence.

The mutant enzyme and subsequently the wild-type enzyme were overexpressed as intein/chitin-binding domain fusion proteins and purified by the same protocol. Cells were grown in LB medium and were induced at 288 K with 0.75 m*M* IPTG for 16 h. They were sonicated and the clarified supernatant was passed over a 15 ml chitin column and cleaved and eluted according to the manufacturer's instructions. Further purification was achieved with a CM weak cation-exchange column on a Perseptive Biosystems Biocad Sprint system with a 0–600 m*M* NaCl gradient at pH 7.5. The protein yield was approximately 1.5 mg per liter of culture after this final purification step. The protein was desalted and concentrated to approximately 20 mg ml^{-1} in 10 m*M* HEPES pH 7.4, 25 m*M* NaCl, 60 m*M* β-mercaptoethanol and a tenfold molar excess of NADP$^+$ for crystallization.

For the determination of kinetic parameters of the mutant and wild-type enzymes, initial velocities were measured by following the oxidation of NADPH to NADP$^+$ at 340 nm on either a Shimadzu UV160U or Hewlett Packard 8453 spectrophotometer. Assays were performed by varying substrate over six to eight different concentrations at 298 K in a mixture containing 10 m*M* HEPES pH 7.3, 100 m*M* NaCl, 0.15 m*M* NADPH and 3.5–8 µg ml^{-1} enzyme. Kinetic constants for NADPH were measured under the same conditions, but in the presence of 140 m*M* DL-glyceraldehyde. All reactions were performed in at least duplicate trials, were initiated by the addition of enzyme and were corrected for non-enzymatic rates of reduction. K_m and k_{cat} values were determined by fitting the data directly into the hyperbolic form of the Michaelis–Menten equation with the Levenberg–Marquardt algorithm provided in *KaleidaGraph* v.3.09.

Dynamic light-scattering measurements were made on a Protein Solutions Dynapro-801 molecular-sizing instrument. All crystallizations were carried out by the hanging-drop vapor-diffusion method at 298 K. Both the wild-type and mutant protein were crystallized by suspending 1–1.5 µl protein with 1 µl of 23–25%(*w*/*v*) PEG 8000 and 0.2–0.25 *M* ammonium sulfate. Crystals formed as plates measuring 0.5 × 0.5 × 0.05 mm after approximately one week. Owing to the high degree of twinning in these crystals, they were deemed unsuitable for diffraction analysis. Further trials, however, yielded another crystallization condition for the mutant protein. Suspending 1 µl of protein solution with 1 µl of 28–30% PEG 8000, 0.2 *M* ammonium acetate and 0.1 *M* sodium citrate pH 5.5 gave rod-like crystals measuring 0.3 × 0.1 × 0.1 mm after approximately one month. Mutant crystals obtained from this condition were used in subsequent diffraction experiments. Attempts to crystallize the wild-type protein in this condition are under way.

Crystals were harvested directly into Paratone-N oil, the excess solvent was removed and the crystals were cooled in a 100 K cold stream. Oscillation frames were collected with an R-AXIS IV image plate mounted on a Rigaku RU-300 rotating-anode generator. *DENZO* (Otwinowski & Minor, 1997) was used to determine the unit-cell parameters and space group. Data were reduced with *DENZO* and *SCALEPACK*.

3. Results and discussion

Kinetic results indicate that both wild-type and the mutant Gcy1p possess NADPH-dependent reductase activity when DL-glyceraldehyde is used as a substrate (Table 1). Furthermore, the K_m and k_{cat} values are similar, indicating there is no major perturbation of the substrate-binding site in the mutant enzyme. As with most aldo–keto reductases assayed to date, assays with an NADH cofactor yielded no measurable activity.

Dynamic light-scattering experiments on a 1 mg ml^{-1} protein solution indicated that the protein was monodisperse, with a radius of 28 Å and a polydispersity of 6 Å. This corresponds to a molecular weight of 34 kDa, very close to the expected value of 35 kDa. Since the only available wild-type crystals were highly twinned and unsuitable for diffraction analysis, the rod-like crystals of the mutant protein were used for diffraction experiments. Based upon several oscillation data frames, the unit cell was determined to be primitive monoclinic, with unit-cell parameters a = 50.94, b = 65.64, c = 86.23 Å, β = 92.64° (see Table 2). Systematic extinctions indicated a space group of $P2_1$. A calculation assuming two 35 kDa subunits in the asymmetric unit yields a V_m of 2.06 Å^3 Da^{-1} (Matthews, 1968). Diffraction data have been collected to 2.49 Å with an overall R_{merge} of 5.4%, an R_{merge} of 16.8% in the 2.59–2.49 Å resolution shell and an overall completeness of 97%. Self-rotation functions performed with *AMoRe* (Navaza, 1994) show no evidence of a non-crystallographic twofold rotation axis.

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