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Cloning, expression, purification and crystallization of saccharopine reductase from *Magnaporthe grisea*

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The gene coding for saccharopine reductase (E.C. 1.5.1.10), an enzyme of the α-aminoadipic pathway of lysine biosynthesis in the pathogenic fungus *Magnaporthe grisea*, was cloned and expressed in *Escherichia coli*. The purified enzyme was crystallized in space groups $C2$ and $C222_1$ using ammonium sulfate pH 4.8 or PEG 6000 pH 4.1 as precipitants. The unit-cell parameters are a = 115.0, b = 56.6, c = 74.3 Å, β = 111.1° for space group $C2$, and a = 89.3, b = 119.0, c = 195.9 Å for space group $C222_1$. The crystals diffract to resolutions of 2.0 Å ($C2$) and 2.4 Å ($C222_1$) at synchrotron sources.

1. Introduction

There are two completely different metabolic routes for the biosynthesis of lysine: the diaminopimelic acid and the α-aminoadipate pathways. The diaminopimelic acid pathway is present in bacteria, plants and some fungi, while the α-aminoadipate pathway exists in higher fungi and blue-green algae (Vogel, 1960; Umbarger, 1978; Bhattacharjee, 1985). The α-aminoadipate pathway starts from α-ketoglutarate and acetyl-CoA and involves at least seven metabolic intermediates and eight different enzymes (Bhattacharjee, 1985, 1992). Saccharopine reductase (E.C. 1.5.1.10), also referred to as saccharopine dehydrogenase (glutamate-forming) or aminoadipic semialdehyde-glutamate reductase, is the penultimate enzyme of this pathway and catalyses the synthesis of saccharopine from glutamate and α-aminoadipic δ-semialdehyde (Fig. 1). In the last step, saccharopine is converted into lysine and ketoglutaric acid by saccharopine dehydrogenase (lysine-forming).

The genetics and biochemistry of saccharopine reductase have been studied to some extent using the enzyme from *Saccharomyces cerevisiae* (Jones & Broquist, 1966; Borell *et al.*, 1984; Ramos *et al.*, 1988). Early studies suggested a molecular mass of approximately 73 kDa for the yeast enzyme (Jones & Broquist, 1966), whereas the amino-acid sequence derived from the nucleotide sequence (Borell *et al.*, 1984) gives a polypeptide chain of 450 amino acids with a molecular mass of ~48 kDa. Saccharopine reductase utilizes NADPH as cosubstrate, but also has significant affinity for NAD^+ in the reverse reaction (Jones & Broquist, 1966). The enzyme is inhibited by mercurials such as *p*-hydroxymercuribenzoate, suggesting that a reactive cysteine residue might be involved in catalysis.

M. grisea is a pathogenic fungus which causes diseases in grass species, for example rice blast, and is responsible for enormous losses of rice crops all over the world (Talbot, 1995). Since plants use the diaminopimelic acid pathway for synthesis of lysine, the enzymes of the α-aminoadipate pathway represent attractive targets for the design of specific inhibitors towards pathogenic fungi. Here, we report the cloning of the gene coding for saccharopine reductase in *M. grisea*, its expression in *E. coli* and the purification and preliminary crystallographic analysis of the recombinant enzyme.

2. Materials and methods

2.1. Isolation of a cDNA for saccharopine reductase from *M. grisea*

The gene for saccharopine reductase from *S. cerevisiae* was obtained from genomic DNA by the polymerase chain reaction (PCR) with Taq polymerase (Gibco BRL) and the following primers: 5′-CCC GGG TAC CTT AAG CCA CTG TCT TTT CCT T-3′ and 5′-CGC GAT CGA TGG GAA AGA ACG TTT TGT TGC-3′. The PCR product was digested with the restriction enzymes *Kpn*I and *Cla*I and the resulting fragment was ligated into the vector pBluescript II KS+ previously cut with the same enzymes.

Following removal from the vector and end-labelling with ^{32}P, the yeast gene was used as a probe of a bacterial λ-phage cDNA library from *M. grisea*. Positively hybridizing plaques were carried through three cycles of purification and were excised from the phage vector.

2.2. Sequence analysis

The protein sequence derived from the nucleotide sequence for saccharopine reductase from *M. grisea* was used for a search in SWISSPROT, TREMBL and TREMBLNEW protein sequence databases with *FASTA*3 (Pearson & Lipman, 1988).

Table 1
Data-collection statistics for crystals of saccharopine reductase from *M. grisea*.

Values in parentheses are for the highest resolution shell.

Space group	*C*2	$C222_1$
Unit-cell parameters (Å, °)		
a	115.0	89
b	56.6	119.0
c	74.3	195.9
α	90.0	90.0
β	111.1	90.0
γ	90.0	90.0
Resolution (Å)	25.0–2.0 (2.05–2.00)	25.0–2.4 (2.44–2.40)
Number of observations	578167	386685
Unique reflections	29843	40017
Mosaicity (°)	1.9	0.2
$R_{\rm merge}$ (%)	5.3 (33.0)	9.0 (33.5)
Completeness (%)	98.5 (98.3)	97.1 (82.2)
$I/\sigma(I)$	25.6 (3.3)	24.0 (3.3)
Packing density (Å^3 Da^{-1})	2.31	2.66
Beamline/X-ray source	X11, EMBL, Hamburg	711, MAX-Lab, Lund

2.3. Saccharopine reductase assay

Saccharopine reductase was assayed spectrophotometrically at 298 K by following reduction of $NADP^+$ at 340 nm in the presence of saccharopine. The assay mix contained 100 m*M* *N*-(2-hydroxyethyl)-piperazine-*N*′-(-3-propanesulfonic acid) pH 8.5, 0.25 *M* KCl, 2 m*M* saccharopine and 1 m*M* $NADP^+$. One unit (U) is defined as 1 µmol product formed per minute.

2.4. Expression in *E. coli* and enzyme purification

For expression purposes, the cDNA was amplified by PCR using the following primers: 5′-CAT CAT TCA TAT GGC CAC CAA GAG CGT GCT TAT G-3′ and 5′-ATA AGG ATC CGA TCT AGT TTT CAG TCA CGC C-3′. The resulting PCR product was digested with the restriction enzymes *Nde*I and *Bam*HI and ligated into the corresponding sites in the vector pET3a. The over-production strain *E. coli* FM5/pDB45 containing the cloned LYS3 gene for *M. grisea* saccharopine reductase was used to generate enzyme for purification. After growth on LB (Luria–Bertani) broth, cells were harvested and stored at 193 K. The buffer used throughout the purification was 50 m*M* HEPES–KOH pH 7.5. For protein purification, 50 g of cells were thawed and washed twice with buffer and resuspended to about 125 ml final volume in buffer containing 1 m*M* dithiothreitol (DTT). The cells were disrupted by sonication in pulsed mode (50% cycle) for 6 min. After centrifugation to remove cell debris, protamine sulfate (50 mg ml^{-1} stock solution) was added with stirring on ice until it was 15% of the total protein by weight. After 30 min, the solution was centrifuged to remove precipitated nucleic acids and protein. To this supernatant, powdered ammonium sulfate was added to 50% saturation (0.298 g ml^{-1}), the mixture was centrifuged and the clarified solution was brought to 85% saturation (a further 0.234 g ml^{-1}), which precipitated the enzyme. This material was centrifuged and the supernatant discarded. The pellet was redissolved in 30 ml buffer and applied at a flow rate of 1 ml min^{-1} to a Pharmacia Superdex 200 column (6 × 600 cm) equilibrated with buffer plus 1 m*M* DTT. The active fractions were pooled and applied to a Pharmacia Mono Q HR column (1.6 × 10 cm). Enzyme was eluted with a linear salt gradient (0–0.5 *M* KCl) in buffer. The active fractions were collected and concentrated by centrifugation in a PM30 Centricon concentrator. The purity of the saccharopine reductase at this stage was typically greater than 98% as determined by SDS–PAGE. If a greater degree of purity was needed, then the pooled enzyme was desalted using a Pharmacia PD10 column and applied to an Amicon Matrex Gel Blue A column (1.5 × 9 cm) equilibrated with buffer. The enzyme was then eluted with buffer plus 1 *M* KCl after a linear salt gradient (0–0.35 *M* KCl) in buffer. The protein concentration was determined using bicinchoninic acid (Smith *et al.*, 1985).

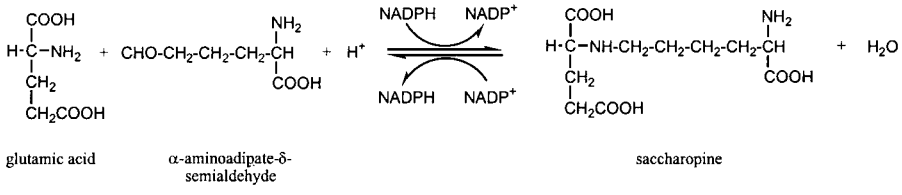

Figure 1
Reaction catalysed by saccharopine reductase.

2.5. Crystallization and crystallographic analysis

Initial crystallization trials were performed with screens from Hampton Research using the hanging-drop vapour-diffusion technique. Two crystal forms were found, which both grew within a few days at 277 K from 3 µl of protein solution (8 mg ml^{-1}) mixed with the same amount of mother liquor. After optimization of the crystallization conditions, the mother liquor for one crystal form consisted of 2.5 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.8 and 1 m*M* DTT. The second crystal form was obtained from a protein solution containing 0.5% β-octylglucoside mixed with a mother liquor including 11–14%(*w*/*w*) PEG 6000, 0.1 *M* citric acid pH 4.1 and 1 m*M* DTT.

2.6. Data collection and processing

For data collection, the crystals were flash-frozen in mother liquor containing an addition of 20% glycerol. For this purpose, crystals were transferred quickly from the mother liquor containing the cryo-solvent into a stream of nitrogen gas at a temperature of 100 K. Data were collected at beamline 711, MAX-Laboratory, Lund, and beamline X11, EMBL Outstation, Hamburg using image-plate detectors from MAR Research. Space-group determination was performed with the autoindex option in *DENZO* (Otwinowski, 1993) and manual inspection of pseudo-precession images produced with the program *PATTERN* (Lu, unpublished work). Data sets were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993).

3. Results and discussion

3.1. Sequence analysis

An open reading frame for saccharopine reductase from *M. grisea* was found using search probes from *S. cerevisiae* saccharopine reductase. The gene was sequenced and Fig. 2 gives the amino-acid sequence as derived from the nucleotide sequence. The nucleotide sequence has been deposited with the GenBank (accession number AF144424).

The amino-acid sequence of saccharopine reductase from *M. grisea* is very similar (63 and 62% identity, respectively) to the sequences of the enzymes from *S. cerevisiae* (Borell *et al.*, 1984) and *Schizosaccharomyces pombe*. The search for other related sequences revealed that the C-terminal part comprising residues 400–800 of lysine-ketoglutarate reductase/saccharopine dehy-

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  1 MATKSVLMLG  SGFVTRPTLD  VLTDSGIKVT  VACRTLESAK
 41 KLSAGVQHST  PISLDVNDDA  ALDAEVAKHD  LVISLIPYTF
 81 HATVIKSAIR  QKKHVVTTSY  VSPAMMELDQ  AAKDAGITVM
121 NEIGLDPGID  HLYAIKTIEG  VHAAGGKIKT  FLSYCGGLPA
161 PESSDNPLGY  KFSWSSRGVL  LALRNAASFY  KDGKVTNVAG
201 PELMATAKPY  FIYPGFAFVA  YPNRDSTPYK  ERYQIPEADN
241 IVRGTLRYQG  FPQFIKVLVD  IGFLSDEEQP  FLKEAIPWKE
281 ATQKIVKASS  ASEQDIVSTI  VSNATFESTE  EQKRIVAGLK
321 WLGIFSDKKI  TPRGNALDTL  CATLEEKMQF  EEGERDLVML
361 QHKFEIENKD  GSRETRTSSL  CEYGAPIGSG  GYSAMAKFVG
401 VPCAVAVKFV  LDGTISDRGV  LAPMNSKIND  PLMKELKEKY
441 GIECKEKVVA
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Figure 2
Amino-acid sequence of saccharopine reductase from *M. grisea*.

drogenase, an enzyme involved in lysine catabolism, is similar to saccharopine reductase. For example, aligning 440 of the 450 amino acids from *M. grisea* saccharopine reductase with lysine-ketoglutarate reductase/saccharopine dehydrogenase from mouse results in 38% sequence identity. No other amino-acid sequences with significant overall similarity (>18%) to saccharopine reductase were found.

3.2. Expression and purification of saccharopine reductase

Using the purification procedure described above, we typically obtained about 400 mg of pure enzyme with a specific activity of 30 U mg^{-1} from 50 g of cell paste. The purified protein had a molecular mass of 50 kDa by SDS–PAGE, which is close to the predicted mass of 48.9 kDa from the gene sequence. On a calibrated Superdex 200 gel-filtration column the enzyme elutes with a mass of 84 kDa, suggesting that the native enzyme is a homodimer.

3.3. Crystallographic analysis

Two crystal forms of saccharopine reductase were found belonging to space groups $C2$ and $C222_1$. Crystals of space group $C2$ grow to approximate dimensions of $0.2 \times 0.2 \times 0.05$ mm from a mother liquor containing ammonium sulfate. The second crystal form, space group $C222_1$, was obtained with PEG 6000 as precipitant and these crystals reach maximum dimensions of $0.1 \times 0.1 \times 0.5$ mm. Diffraction data for the two crystal forms were collected at synchrotron beamlines and Table 1 gives the statistics for the processed data. The screw axis of the $C222_1$ space group is clearly seen as systematic absences in pseudo-precession images of the *kl* and *hl* planes. Assuming one protein molecule in the asymmetric unit in space group $C2$ and two protein molecules in the asymmetric unit in space group $C222_1$ results in reasonable values for the packing densities of 2.3 and 2.7 $Å^3$ Da^{-1}, respectively, corresponding to solvent contents of 47 and 54%, respectively. Both crystal forms diffract to high resolution (2.0 Å for $C2$ and 2.4 Å for $C222_1$) and are therefore suitable for crystallographic structure determination.

In conclusion, well diffracting crystals of saccharopine reductase from *M. grisea* have been obtained. The three-dimensional structure of this enzyme will be useful in the understanding of lysine biosynthesis in fungi and possibly in the design of new fungicides.

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