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Crystallization and preliminary crystallographic studies of an active-site mutant hydantoin racemase from *Sinorhizobium meliloti* CECT4114

Sergio Martínez-Rodríguez,[a,b] Luis Antonio González-Ramírez,[c] Josefa María Clemente-Jiménez,[d] Felipe Rodríguez-Vico,[d] Francisco Javier Las Heras-Vázquez,[d] Jose Antonio Gavira[c]* and Juan Ma. García-Ruiz[c]

[a]Department of Cellular and Molecular Interactions, Vrije Universiteit Brussel, 1050 Brussels, Belgium, [b]Ultrastructure Laboratory, Vrije Universiteit Brussel, 1050 Brussels, Belgium, [c]Laboratorio de Estudios Cristalográficos, IACT (CSIC–U. Granada), P. T. Ciencias de la Salud, Granada 18100, Spain, and [d]Departamento de Química Física, Bioquímica y Química Inorgánica, Área de Bioquímica y Biología Molecular, Edf. CITE I, Universidad de Almería, Spain

Correspondence e-mail: jgavira@ugr.es

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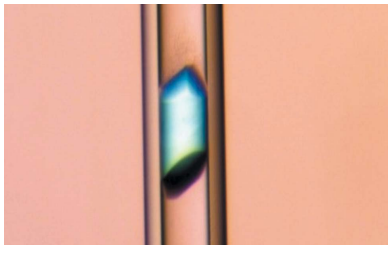

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A recombinant active-site mutant of hydantoin racemase (C76A) from *Sinorhizobium meliloti* CECT 4114 (SmeHyuA) has been crystallized in the presence and absence of the substrate D,L-5-isopropyl hydantoin. Crystals of the SmeHyuA mutant suitable for data collection and structure determination were grown using the counter-diffusion method. X-ray data were collected to resolutions of 2.17 and 1.85 Å for the free and bound enzymes, respectively. Both crystals belong to space group *R*3 and contain two molecules of SmeHyuA per asymmetric unit. The crystals of the free and complexed SmeHyuA have unit-cell parameters $a = b = 85.43$, $c = 152.37$ Å and $a = b = 85.69$, $c = 154.38$ Å, crystal volumes per protein weight (V_M) of 1.94 and 1.98 Å^3 Da^{-1} and solvent contents of 36.7 and 37.9%, respectively.

1. Introduction

Hydantoin racemases have been reported in several lower organisms (Las Heras-Vazquez *et al.*, 2003; Martinez-Rodriguez, Las Heras-Vazquez, Clemente-Jiminez *et al.*, 2004; Martinez-Rodriguez, Las Heras-Vazquez, Mingorance-Cazorla et al., 2004; Suzuki, Onishi *et al.*, 2005; Watabe *et al.*, 1992*a*; Wiese *et al.*, 2000), but their function is not completely clear. Some hydantoin racemase-encoding genes have been found together with a carbamoylase and a hydantoinase/dihydropyrimidinase including a potential hydantoin transporter enzyme (Hils *et al.*, 2001; Suzuki, Takenaka *et al.*, 2005; Watabe *et al.*, 1992*b*; Wiese *et al.*, 2001); this activity has recently been suggested for the corresponding gene from *Microbacterium liquefaciens* (Suzuki & Henderson, 2006). However, the most common application of these enzymes is the use of the 'hydantoinase process', a cheap and environmentally friendly enzymatic method for the potential production of any optically pure natural or unnatural amino acid from a wide spectrum of D,L-5-monosubstituted hydantoins (Altenbuchner *et al.*, 2001; Martinez-Rodriguez *et al.*, 2002). This process has mainly been used in the production of amino acids for which the hydantoin precursors rapidly racemize, thus limiting its application to the production of amino acids such as D-*para*-hydroxyphenylglycine and D-phenylglycine. The inclusion of a third enzyme (hydantoin racemase) allows precursors with a low rate of racemization to be converted to their corresponding products by permitting the presence of both enantiomers for the continuation of the cascade reaction.

Mutants of the hydantoin racemase enzyme from *Sinorhizhobium meliloti* CECT4114 (SmeHyuA) were obtained in our laboratory and were overexpressed in *Escherichia coli* (Andujar-Sanchez *et al.*, 2006), enabling us to demonstrate the importance of two cysteines in the recognition and isomerization of 5-monosubstituted hydantoins (Martinez-Rodriguez *et al.*, 2006). Here, we report for the first time the crystallization and preliminary crystallographic studies of an active-site mutant hydantoin racemase in its free and complexed forms. The three-dimensional structures will provide meaningful insights into the binding properties and specificity of the enzyme towards its substrates.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

	C76A SmeHyuA + D,L-IPH	C76A SmeHyuA
Wavelength (Å)	0.9776	0.9776
Space group	*R*3	*R*3
Unit-cell parameters (Å)	$a = b = 85.69$, $c = 154.38$	$a = b = 85.43$, $c = 152.37$
Resolution range (Å)	20.0–1.85 (1.92–1.85)	20.0–2.10 (2.17–2.10)
Observed reflections	163581	71037
Independent reflections	34860 (3561)	20636 (1753)
Data completeness (%)	98.0 (100.0)	85.5 (72.0)
$R_{\rm merge}$† (%)	9.5 (36.7)	9.2 (22.4)
Average $I/\sigma(I)$	13.9 (4.5)	12.2 (6.6)
Redundancy	4.7 (4.7)	3.4 (3.1)
Molecules per ASU	2	2
Matthews coefficient (Å^3 Da^{-1})	1.98	1.94
Solvent content (%)	37.9	36.7

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and $\overline{I(hkl)}$ is the weighted mean of all measurements.

2. Materials and methods

2.1. Purification and crystallization of C76A SmeHyuA

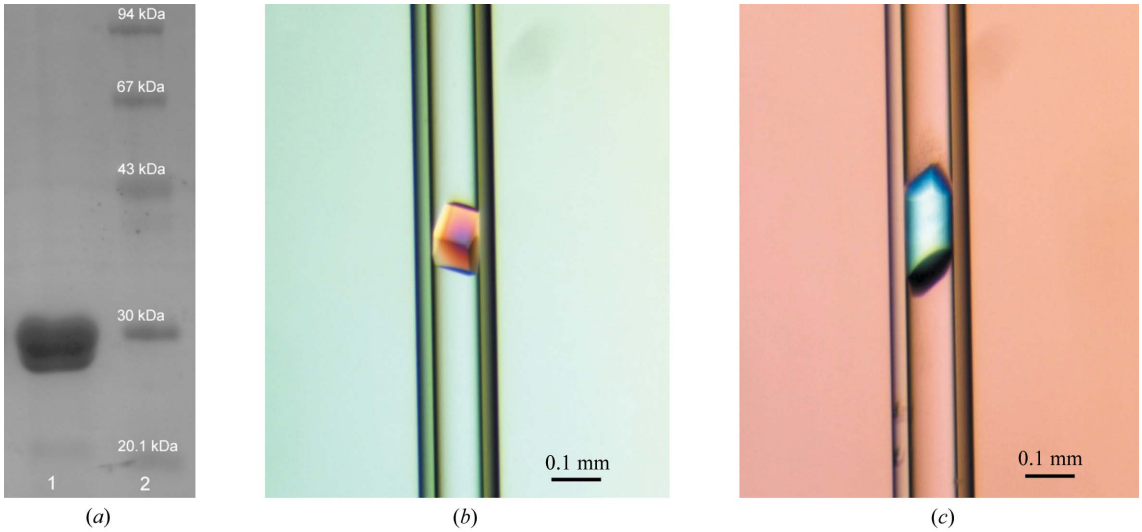
The recombinant active-site mutant (C76A) of SmeHyuA (241 amino acids) was overexpressed using the previously described construct (Andujar-Sanchez *et al.*, 2006). The His_6-tag fused protein was purified by immobilized cobalt-affinity chromatography followed by an additional gel-filtration chromatography step, carried out using a Superdex 200 gel-filtration column (Amersham Biosciences), to eliminate any DNA co-eluting with the protein.

The protein was extensively dialysed in 20 m*M* Tris–HCl and 2 m*M* DTT at pH 8 and concentrated to 6 mg ml^{-1} using an Amicon ultrafiltration system with Amicon YM-3 membranes. Enzyme concentrations were measured using a variation of the Lowry method (Rodriguez-Vico *et al.*, 1989). D,L-5-Isopropyl hydantoin (D,L-IPH) was synthesized as described previously (Martinez-Rodriguez *et al.*, 2007). A stock solution of 100 m*M* D,L-IPH was prepared in the last dialysis buffer of the C76A SmeHyuA.

Initial screening was performed using Hampton Research Crystal Screens I and II (based on the sparse-matrix method; Jancarik & Kim, 1991) at 277 and 293 K for the free enzyme. The hanging-drop vapour-diffusion method was used. The drops were made by mixing equal volumes (2 µl) of enzyme solution and reservoir solution and were suspended over 1.0 ml reservoir solution. Crystals appeared at 277 K using a screening condition containing 0.8 *M* potassium/sodium tartrate and 0.1 *M* HEPES pH 7.5. Improvement of the crystals was achieved by the counter-diffusion technique (García-Ruiz, 2003) using capillaries of 0.1 mm inner diameter pre-filled with different concentrations of potassium/sodium tartrate at various pH values from a custom-made GCB-Domino 'Capillary Counterdiffusion Kit' (Triana Science and Technology, Granada, Spain). The capillaries were further filled with 6 mg ml^{-1} protein solution by capillary force. The upper ends of the capillaries were then sealed with the supplied putty, while the unsealed ends were dipped into the GCB-Domino by punching them across the gel located on top of the precipitant. To obtain D,L-IPH–C76A SmeHyuA crystals, the counter-diffusion method described above was used with samples where 6 mg ml^{-1} protein and 1 m*M* of the substrate had been pre-incubated for 30 min.

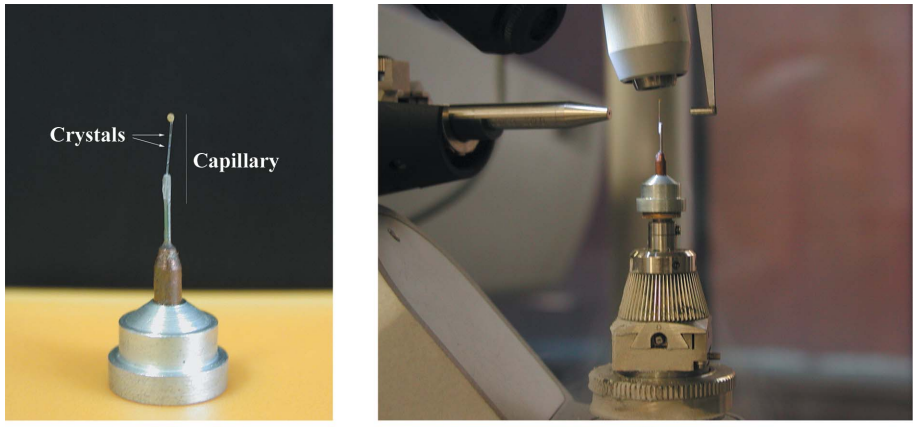
2.2. Data collection and processing

Crystals of free and D,L-IPH-bound recombinant C76A SmeHyuA were located along the capillary under a microscope using polarized light and their positions were marked on the capillary wall. The capillaries were cut into portions of approximately 1.5 cm with a Capillary Cutting Stone (Hampton Research, Laguna Niguel, California, USA). Each portion, containing one or two crystals, was inserted into a PCR tube containing 50 µl 2.0 *M* potassium/sodium tartrate, 10%(*v*/*v*) glycerol and 0.1 *M* Tris–HCl pH 6.0. These fragments were incubated for several days in order to equilibrate the crystals in the cryoprotectant solution. At the synchrotron, the capillary portions were inspected with a microscope and the position of the crystal was re-marked on the capillary wall if necessary. The capillary fragments were then glued against a CrystalCap Copper pin (Hampton Research, Laguna Niguel, California, USA) and directly cryocooled in a cold nitrogen stream (Kryoflex) maintained at 110 K (Ng *et al.*, 2003). In our case, the end of the capillary was not sealed at any step, but sealing is necessary if several CrystalCaps are mounted in advance.



(*a*) (*b*) (*c*)

Figure 1
(*a*) 14% SDS–PAGE. Lane 1, purified C76A SmeHyuA (20 µg). Lane 2, standard molecular-weight markers. (*b*), (*c*) Crystals of recombinant free C76S SmeHyuA (*b*) and D,L-IPH–C76S SmeHyuA (*c*) grown in 0.1 mm inner diameter capillaries by the counter-diffusion technique.



(*a*) (*b*)

Figure 2
(*a*) An example of a capillary glued to a CrystalCap and (*b*) the final setup mounted in a goniometer head. Notice that in this example the sample was sealed with beeswax at both ends.

X-ray intensity data were collected at the BM-16 station of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using a wavelength of 0.978 Å and an ADSC Quantum 4R detector at a crystal-to-detector distance of 150 mm for the free form and 175 mm for the complexed form. An oscillation angle of 0.5° and an exposure time of 15 s were used in both cases. Data were indexed, integrated and scaled with the *HKL*-2000 suite (Otwinowski & Minor, 1997).

3. Results

Sufficient C76A SmeHyuA was overexpressed and highly purified (Fig. 1*a*) in soluble form for crystallization experiments. Aggregation of the protein occurred at concentrations over 2 mg ml^{-1} and therefore the use of DTT was checked for its ability to avoid this phenomenon. This compound not only prevented protein aggregation, but also resuspended aggregated proteins after incubation for 30 min, thus suggesting that the observed aggregation of this protein may arise from the formation of disulfide bonds. A 6 mg ml^{-1} protein sample buffered in 20.0 m*M* Tris–HCl and 2 m*M* DTT pH 8.0 was used to determine crystallization conditions using Hampton Research Crystal Screen I and II with the vapour-diffusion method. Crystals were obtained from drops made up of 2 µl protein solution and 2 µl 0.8 *M* potassium/sodium tartrate, 0.1 *M* HEPES pH 7.5 at 277 K. Optimization was set up using the counter-diffusion method. With this configuration, well faceted crystals with maximum dimensions of 0.3 × 0.1 × 0.1 mm appeared within one week (Figs. 1*b* and 1*c*). X-ray diffraction data were collected at 110 K from crystals in the capillary. To avoid any crystal manipulation, the portion of capillary containing the selected crystals (usually no more than two) was pre-equilibrated with glycerol. The capillary portion was removed from the cryosolution, with the position of the crystal being marked if necessary, glued to a typical copper CrystalCap (Fig. 2*a*) and transferred into the nitrogen stream (Fig. 2*b*).

The crystal of the free form of C76A SmeHyuA belongs to the trigonal space group *R*3 and diffracted to at least 2.17 Å resolution with an $R_{\rm merge}$ of 9.2% overall and of 22.4% for the 2.17–2.10 Å high-resolution shell (Table 1). The unit-cell parameters were a = 85.43, c = 152.37 Å. The asymmetric unit contains two molecules, with a corresponding crystal volume per protein weight ($V_{\rm M}$) of 1.94 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 36.7% by volume (Westbrook, 1985). However, the statistics for the ligand-bound D,L-IPH–C76A SmeHyuA proved to be better than those of the unbound form and these crystals diffracted to 1.85 Å resolution (Fig. 3). It has previously been shown that the presence of the ligand stabilizes active-site mutants of SmeHyuA (Martinez-Rodriguez *et al.*, 2006), which could explain the improved diffraction data obtained for the complex. The crystal belongs to the same space group as the unbound form and has similar unit-cell parameters (a = 85.69, c = 154.38 Å). It also contains two molecules of protein per asymmetric unit and has a $V_{\rm M}$ of 1.98 Å^3 Da^{-1} and a solvent content of 37.9%. During the process of submission of this manuscript, a new structure of the proposed enzyme hydantoin racemase from *Pyrococcus horikoshii* OT3 was deposited in the PDB (code 2eq5). This enzyme shares 30% identity with the hydantoin racemase from *S. meliloti* CECT4114 and we will therefore make use of it as a model for molecular replacement.

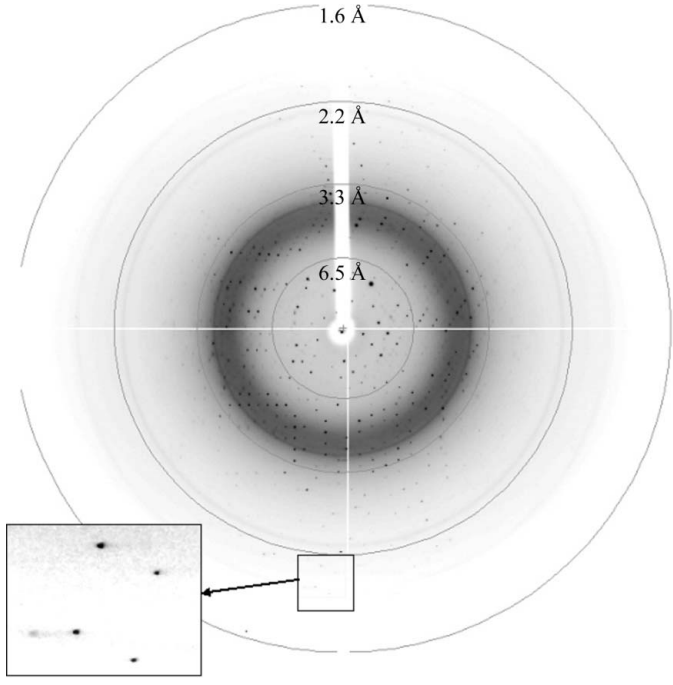


Figure 3
An X-ray diffraction image of a cryocooled crystal of D,L-IPH–C76A SmeHyuA. The crystal diffracted beyond 2.0 Å.

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