Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression and crystallization of several forms of the *Propionibacterium shermanii* transcarboxylase 5S subunit

Pamela R. Hall,[a] Run Zheng,[b] Marianne Pusztai-Carey,[b] Focco van den Akker,[b] Paul R. Carey[b] and Vivien C. Yee[a,b]*

[a]Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA, and [b]Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

Correspondence e-mail: vivien.yee@case.edu

Received 9 October 2003
Accepted 8 December 2003

The dimeric outer 5S subunit of transcarboxylase has been expressed in three different forms and crystallized: native 5S, 5S-His_6 and selenomethione-5S-His_6. All the crystals have an orthorhombic space group, but while native 5S forms primitive orthorhombic crystals, 5S-His_6 crystals are either *C*-centered or primitive and SeMet-5S-His_6 crystals are *C*-centered. Crystallization of native 5S requires the addition of lithium sulfate, whereas this salt prevented crystallization of 5S-His_6. All 5S crystals diffract to $\sim$2.0 Å resolution with synchrotron radiation. Efforts are under way to solve the structure of SeMet-5S-His_6 using MAD.

1. Introduction

Transcarboxylase (TC) from *Propionibacterium shermanii* is a 1.2×10^6 Da multienzyme complex containing 30 polypeptide chains: a catalytic 336 kDa 12S hexameric core, six catalytic 116 kDa 5S dimers and 12 12 kDa 1.3S biotinylated linkers. Electron-microscopy studies of TC (Wrigley *et al.*, 1977) reveal a central 12S hexamer capped on each end by a ring of three 5S dimers (Fig. 1). 12 1.3S biotinylated subunits link the outer 5S subunits to the 12S core. Structural information is available for both the 12S (Hall *et al.*, 2003) and 1.3S (Reddy *et al.*, 2000) subunits.

The overall transcarboxylation reaction consists of two half-reactions (Wood & Zwolinski, 1976; Wood, 1979; Wood & Kumar, 1985). In the first half-reaction, 12S catalyzes COO^- transfer from methylmalonyl-CoA to the N1′ position of biotin on 1.3S. The second half-reaction, catalyzed by 5S, a cobalt-containing metalloprotein, transfers the COO^- from the 1.3S biotin to pyruvate. The two products of the overall reaction are propionyl-CoA and oxaloacetate.

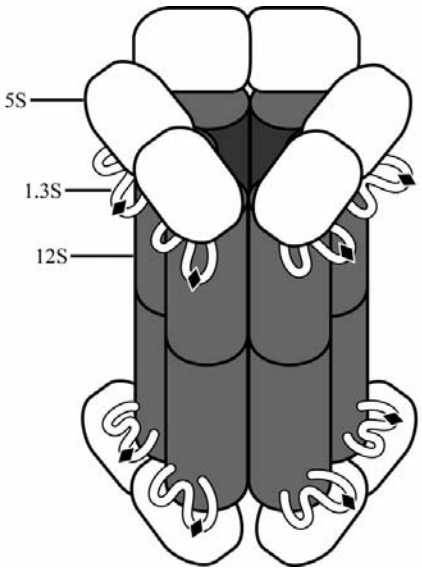

Figure 1
EM-based model (adapted from Wrigley *et al.*, 1977).

TC has historically served as a model system for mammalian enzymes. Human biotin-dependent carboxylases play central roles in such metabolic pathways as oxidation of odd-chain fatty acids, catabolism of branched amino acids and gluconeogenesis. One of these enzymes, pyruvate carboxylase (PC), is the first enzyme in the gluconeogenic pathway and is essential for maintenance of glucose output in the fasting state. Deficiencies of PC, which catalyzes the ATP-driven conversion of pyruvate to oxaloacetate using HCO_3^-, may present as mild lactic academia, developmental delay, severe mental retardation or death by three months of age (Robinson, 1995). TC 5S shares 27% sequence identity and functional homology to the carboxyltransferase region (residues 550–1000) of human PC (EC 6.4.1.1; Wexler *et al.*, 1994; Thorton *et al.*, 1993). Elucidation of the 5S crystal structure may provide mechanistic insight for both TC and related carboxyltransferases such as PC.

2. Material and methods

2.1. Expression and purification

The amino-acid sequence of 5S from the *P. shermanii* strain used here differs from the sequence originally reported (Thorton *et al.*, 1993); the corresponding DNA sequence has been deposited in the EMBL database (ID AJ606310). The 505-residue 5S subunit was expressed and purified as previously described (Xie *et al.*, 1993). 5S-His_6 incorporated into a pETBlue-2 expression plasmid was expressed

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	5S	5S-His_6	5S-His_6	SeMet-5S-His_6
Space group	$P2_12_12_1$	$P2_12_12_1$	$C222_1$	$C222_1$
Source	X25	In-house	19ID	X25
Unit-cell parameters				
a (Å)	79.3	79.4	96.4	96.3
b (Å)	96.3	96.7	146.2	147.2
c (Å)	152.6	152.9	78.7	79.1
Wavelength (Å)	1.10	1.54	1.03	0.98
Resolution (Å)	99–1.95 (2.02–1.95)	30–2.5 (2.59–2.50)	50–2.0 (2.07–2.0)	50–2.0 (2.07–2.0)
No. observations	375655	273048	91956	191798
Unique reflections	78936	39483	29773	66458
$\langle I/\sigma(I)\rangle$	18.4 (6.5)	9.5 (3.5)	19.4 (6.52)	17.1 (3.7)
R_{merge} (%)	6.8 (11.6)	13.5 (28.9)	5.5 (11.8)	5.6 (18.9)
Completeness (%)	91.7 (67.2)	94.7 (87.2)	78 (54.2)	90.2 (77.4)

using Tuner(DE3)pLacI *Escherichia coli* cells (Novagen). SeMet-5S-His_6 was prepared with the same expression system by inhibition of methionine biosynthesis (Van Duyne *et al.*, 1993; M. Simmons personal communication). Freshly transformed single colonies were grown overnight (310 K, 250 rev min^{-1}) in Luria Broth (LB) containing carbenicillin (50 µg ml^{-1}) and chloramphenicol (68 µg ml^{-1}). As 5S-His_6 and SeMet-5S-His_6 expression differ solely in the media utilized (LB *versus* M9 with added amino acids), only the SeMet-5S-His_6 protocol is described here. M9 media for selenomethione inhibition was prepared: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl and 0.5 g NaCl were solubilized in 200 ml water and the pH adjusted to 7.4 with 10 *M* NaOH. Following autoclaving, 800 ml sterile water, 1 ml 1 *M* $MgSO_4$, 10 ml 40%(*w*/*v*) glucose, 100 µl 0.5%(*w*/*v*) thiamine, 1 ml 1 *M* $FeSO_4$, 2.25 ml 100 mg ml^{-1} carbenicillin and 3.06 ml 100 mg ml^{-1} chloramphenicol were added. Cells from 50 ml overnight culture were added to 500 ml of M9 pre-warmed to 310 K. The culture was incubated at 310 K until an OD_{600} of 0.3 was reached. The following were added per 500 ml culture: 50 mg lysine, 50 mg phenylalanine, 50 mg threonine, 25 mg isoleucine, 25 mg leucine, 25 mg valine and 25 mg SeMet. After 15 min incubation, expression was induced with 1 m*M* IPTG, 0.1 m*M* $CoCl_2$ and 0.1 m*M* $ZnCl_2$ and incubation continued for 10–12 h. About 3 g of cell pellet per litre of culture was harvested by centrifugation and suspended in 15 ml of 5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9 (buffer *A*), before lysis by passage through a French press (3×).

The lysate was cleared by centrifugation at 27 000*g* for 35 min, applied to Ni–NTA Superflow beads (Qiagen) pre-equilibrated in buffer *A* and incubated for 1 h at 277 K. Supernatant was removed and the beads were washed, first with buffer *A* and then with 60 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9 (buffer *B*). 5S was eluted with 20 ml 1 *M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9 (buffer *C*). The eluate was concentrated to 10 mg ml^{-1} and buffer-exchanged into 200 m*M* phosphate buffer pH 6.5, 0.1 m*M* PMSF, 0.1 m*M* EDTA, 10 m*M* DTT (buffer *D*) using an Amicon 30 000 molecular-weight cutoff centrifugal filter device (Millipore). Finally, 5 mg of 5S was passed through a Sephadex 200 10/30 size-exclusion column pre-equilibrated in buffer *D*, eluting as a dimer of 120 kDa.

2.2. Crystallization

Prior to crystallization, 5S was dialyzed into 10 m*M* HEPES pH 7.0, 0.1 m*M* PMSF, 0.1 m*M* EDTA, 1 m*M* DTT and concentrated to approximately 10 mg ml^{-1}. Initial crystallization conditions were obtained from commercially available sparse-matrix screening kits (Jancarik & Kim, 1991; Hampton Research, Emerald Biostructures). Optimized crystals of native 5S were grown at room temperature by vapor diffusion at 293 K using equal volumes of the protein sample and well solution containing 18–32% PEG 4K, 0.2 *M* Li_2SO_4, and 0.1 *M* Tris pH 7.0 or 7.3. 5S-His_6 and 5S-His_6-SeMet crystallized under similar conditions, but only in the absence of lithium sulfate. Crystals were cooled by dunking in liquid nitrogen after stabilizing in a cryoprotectant containing 34–35%(*w*/*v*) PEG 4K.

2.3. Data collection and data processing

Initial testing of crystals for diffraction was performed using an in-house rotating-anode generator operating at 50 kV and 100 mA and equipped with Yale mirrors and an R-AXIS IV imaging-plate detector (Rigaku MSC). Higher resolution and more complete data sets were collected in a MAD experiment on NSLS beamline X25 and on beamline 19ID at APS. All data were processed with *HKL* (Otwinowski & Minor, 1997) using a $I > 3\sigma(I)$ cutoff (Table 1).

3. Results and discussion

All 5S crystal forms appear within 7 d (Fig. 2*a*). Assuming one 5S dimer in the asymmetric unit with a molecular weight of 57 456 Da for each chain, a Matthews coefficient V_M (Matthews, 1968) of 2.54 $Å^3$ Da^{-1} was calculated, corresponding to 51.1% solvent content. Since 5S is a cobalt-requiring metalloprotein, a native 5S crystal was soaked for 3 d in mother liquor containing 5 m*M* $CoCl_2$. Data from this crystal allowed location of one Co^{2+} atom per monomer. Phase information from this derivative was insufficient to produce traceable electron-density maps. Numerous further attempts at heavy-atom phasing resulted in non-isomorphous crystals.

To facilitate purification, we prepared 5S-His_6. We found that 5S-His_6 crystallizes under similar conditions to the native protein, except that native 5S requires 0.2 *M* Li_2SO_4 whilst 5S-His_6 crystallizes only in the absence of this salt. As shown in Table 1, 5S-His_6 crystals appear in two forms: $P2_12_12_1$ or $C222_1$. The *C*-centered 5S-His_6 crystals contain only one monomer per asymmetric unit (V_M = 2.33 $Å^3$ Da^{-1}, corresponding to 46.8% solvent content). Both soaking and co-crystallization attempts with both crystal forms failed to yield a useful isomorphous heavy-atom derivative.

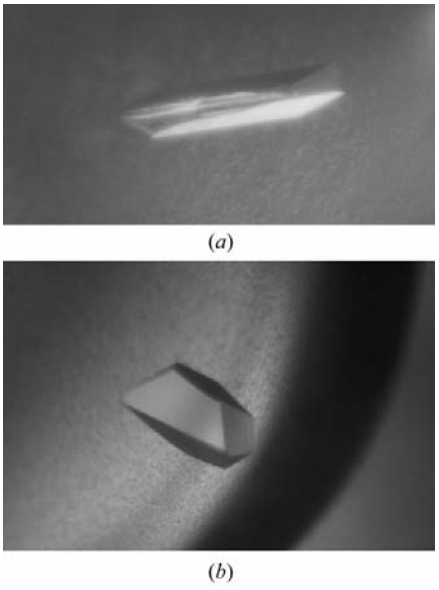

Figure 2
(*a*) Crystal of native 5S measuring 0.3 mm in length. (*b*) Crystal of 5S-His_6-SeMet measuring 0.4 mm in length.

That 5S-His_6 contains 24 methionine residues per monomer and its $C222_1$ crystal form has only a single monomer per asymmetric unit encouraged us to express SeMet-5S-His_6. This crystallized under the 5S-His_6 conditions and exclusively in the $C222_1$ crystal form (Fig. 2*b*). Three-wavelength MAD data have been measured from SeMet-5S-His_6 crystals and phasing efforts are under way.

Diffraction data were measured on beamline 19-ID at the Advanced Photon Source, supported by the US Department of Energy, and at beamline X25 of the National Synchrotron Light Source, Brookhaven National Laboratory, supported by the US Department of Energy and National Institutes of Health. We are grateful to Dr Rune Hartmann for helpful discussions, to Lizamma Antony for expert technical assistance and to Drs Michael Becker and Stephan Ginell for beamline support. PRH is partially supported by NIH training grant GM08803. Research in the VCY and PRC laboratories is funded by NSF MCB0401654 and NIH DK053053, respectively.

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