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Crystallization of quinoprotein glucose dehydrogenase variants and homologues by microseeding

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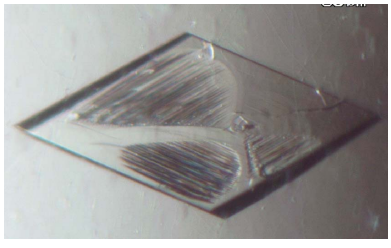

The soluble quinoprotein glucose dehydrogenase oxidizes glucose, maltose and a variety of other monosaccharides and disaccharides to the corresponding lactones. An efficient microseeding protocol is reported to produce crystals of three variants that display reduced activity towards maltose. Similar cross-seeding protocols to grow crystals of homologues from *Escherichia coli* and *Streptomyces coelicolor* are described.

1. Introduction

Quinoproteins constitute a class of proteins that use one of five different quinone-containing cofactors for the two-electron oxidation of alcohols and amines. Quinoproteins containing pyrroloquinoline quinone (PQQ) are present in a variety of different prokaryotes, including Gram-negative and Gram-positive bacteria and archaea (Oubrie, 2003), but the alleged presence (Kasahara & Kato, 2003) of PQQ-dependent dehydrogenases in eukaryotes is controversial (Felton & Anthony, 2005; Kasahara & Kato, 2005; Rucker *et al.*, 2005).

The PQQ-dependent soluble glucose dehydrogenase (sGDH) from the Gram-negative bacterium *Acinetobacter calcoaceticus* is the prototype of a subclass of enzymes with a six-bladed β-propeller structure (Oubrie, 2003) and the only protein of its class for which a detailed biochemical characterization has been carried out. The enzyme functions as a homodimer of 100 kDa (454 amino acids per monomer after cleavage of the signal peptide that directs it to the periplasm) and binds three calcium ions and one PQQ per subunit (Olsthoorn & Duine, 1996; Olsthoorn *et al.*, 1997; Oubrie, Rozeboom, Kalk, Duine *et al.*, 1999; Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999). The sGDH catalyzes the conversion of monosaccharides and disaccharides as well as the trisaccharide maltotriose into their corresponding lactones (Olsthoorn & Duine, 1998; Igarashi *et al.*, 2004). The kinetic mechanism of the reductive half-reaction includes an initial reversible fast step leading to formation of a fluorescing intermediate followed by an irreversible and rate-limiting step (Olsthoorn & Duine, 1998). Crystal structures have been determined of the sGDH in different states, *i.e.* in the apo form (Oubrie, Rozeboom, Kalk, Duine *et al.*, 1999), in complex with PQQ (Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999) and in ternary complexes with PQQ and the substrate glucose (Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999) or the inhibitor methylhydrazine (Oubrie, Rozeboom & Dijkstra, 1999). The combined structural and biochemical data are consistent with a catalytic mechanism involving direct hydride transfer (Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999).

The enzyme has been implemented on test strips that are used by diabetics to monitor their blood glucose levels. Improvement of the substrate specificity of the sGDH could improve the reliability of these strips, as high levels of maltose in blood, only relevant in some specific clinical conditions, can interfere with the glucose diagnostics (Wens *et al.*, 1998). For this purpose, three generations of sGDH variants with successively reduced activity towards maltose have been generated. Here, we describe an efficient and reproducible micro-seeding method that was used to grow crystals of each of the three variants. Using the same method, we also succeeded in growing

crystals of the sGDH homologues from *Escherichia coli* K12 (Gene ID 945467) and *Streptomyces coelicolor* A32(2) (Gene ID 1100948).

2. Material and methods

2.1. Protein expression and purification

Samples of wild-type and three variants of *A. calcoaceticus* sGDH were generously supplied by Roche Diagnostics (Penzberg, Germany) as a freeze-dried powder. The variants are called mutant A (T348G), mutant B (T348G, N428P) and mutant G (Y171G, E245D, M341V, T348G, N428P) (Kratzsch *et al.*, 2002). Before use, samples were resuspended in a buffer solution containing 3 m*M* $CaCl_2$, 120 m*M* NaCl, 10 m*M* HEPES pH 7.5. Methods to clone, express and purify the sGDH homologues from *E. coli* and *S. coelicolor* as well as a biochemical characterization of these proteins will be published elsewhere.

2.2. Production of microseeds containing stock solutions

To obtain microseeds, a single crystal was transferred to an Eppendorf cup containing 5 µl of a stabilizing solution containing 30% PEG 6000, 3 m*M* $CaCl_2$, 120 m*M* NaCl and 50 m*M* CHES pH 9.2. The crystal was crushed manually using a polypropylene pellet pestle (Sigma) while keeping the sample on ice. The sample was then diluted with the stabilizing solution to an end volume of 200 µl to obtain the 1/1 (or 10^0) stock. Serial dilutions up to $1/10^6$ were produced and stored at 277 K. Microseeds of *E. coli* and *S. coelicolor* sGDH homologues were produced in the same way using stabilizing solutions buffered with 100 m*M* of either CHES pH 9.2 or HEPES pH 7.5.

2.3. Microseeding

Crystallization conditions were screened using the hanging-drop vapour-diffusion method (Q Plate II, Hampton Research) at 289 K. A resuspended protein solution (5 mg ml^{-1}) was mixed with an equal volume of precipitant solution (17–22% PEG 6000, 1 m*M* $CaCl_2$, 120 m*M* NaCl, CHES pH 9.2). A total of eight drops of 2 µl of this mixture were placed on a cover slip. To seven of these drops, 1 µl of one of the seven seed-stock solutions (10^0 to 10^6 dilution series) was added, whilst 1 µl stabilizing solution was added to the last drop as a control to check for crystallization in the absence of microseeds. The drops were equilibrated against a 1 ml reservoir of precipitant solution. The sGDH homologues from *E. coli* and *S. coelicolor* were screened using wild-type seeds in the same way, but a precipitant solution buffered at a different pH (17–22% PEG 6000, 1 m*M* $CaCl_2$, 120 m*M* NaCl, 100 m*M* HEPES pH 7.5) was also used.

2.4. Cryoprotection and preliminary X-ray diffraction analysis

Crystals of *A. calcoaceticus* sGDH variants were flash-frozen in a solution containing 20%(*v*/*v*) glycerol or ethylene glycol in addition to 30%(*w*/*v*) PEG 6000, 120 m*M* NaCl, 3 m*M* $CaCl_2$, 100 m*M* CHES pH 9.2. Crystals of the *E. coli* sGDH homologue were cryocooled in a solution containing 20%(*v*/*v*) ethylene glycol in addition to 30%(*w*/*v*) PEG 6000, 120 m*M* NaCl, 3 m*M* $CaCl_2$, 100 m*M* CHES pH 9.2, while crystals of the *S. coelicolor* homologue were frozen in a cryoprotectant containing 20%(*v*/*v*) glycerol in addition to 30%(*w*/*v*) PEG 6000, 120 m*M* NaCl, 3 m*M* $CaCl_2$, 100 m*M* HEPES pH 7.5. Data sets were collected at station XRD1 of the ELETTRA synchrotron-radiation source in Trieste, Italy, at station 14.2 at the Synchrotron Light Source (SLS) in Daresbury, United Kingdom and at station BL-2 of the Protein Structure Factory at BESSY in Berlin, Germany. Data sets were processed and reduced using either the *HKL*2000 package (Otwinowski & Minor, 1997) or *MOSFLM* (Leslie, 1992) and pro-

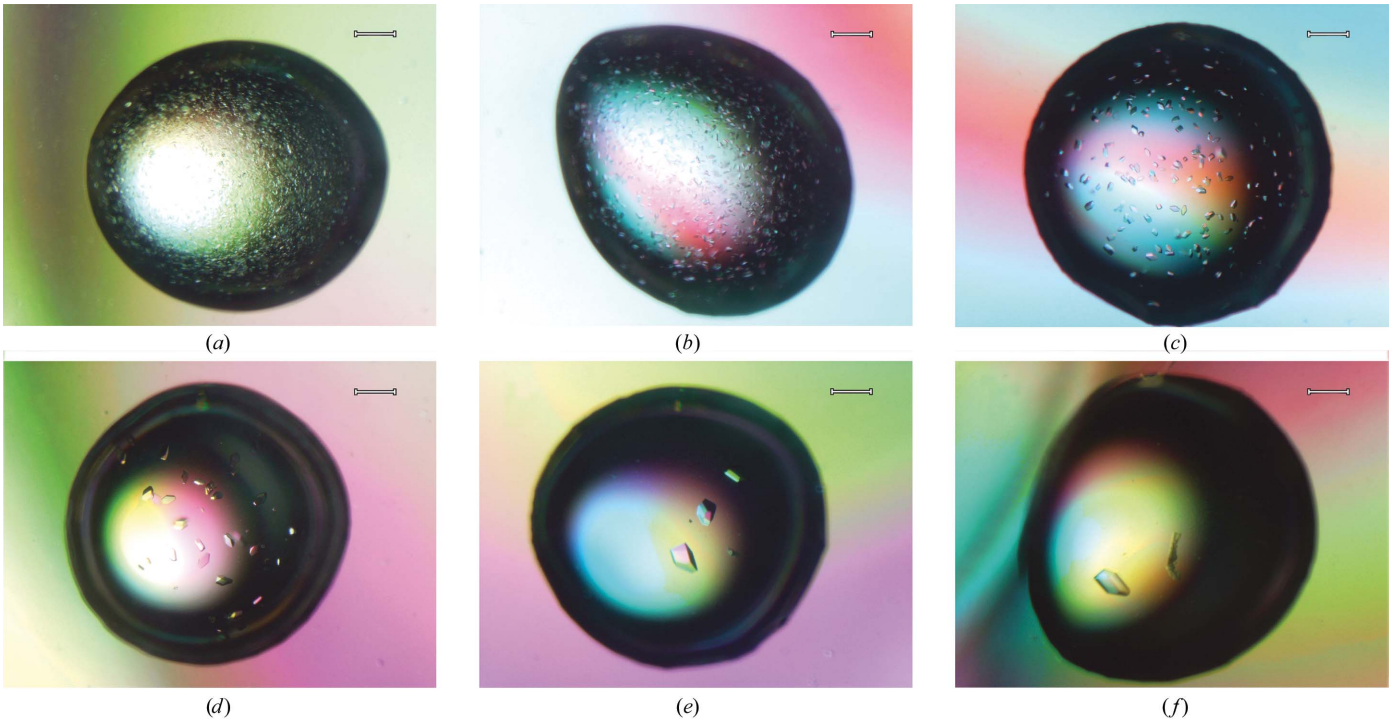

Figure 1
Effect of seed serial dilution (1/10 to $1/10^6$), successively in (*a*) to (*f*), on the crystal growth of the apo-form variant A of sGDH from *A. calcoaceticus*. The scale bar represents 300 µm.

Table 1
Data-collection statistics for sGDH variants A, B and G.

Values in parentheses are for the highest resolution shell.

	Apo A	Apo B	Apo G
Beamline	Elettra XRD1 (Trieste, Italy)	SLS station 14.2 (Daresbury, UK)	SLS station 14.2 (Daresbury, UK)
Wavelength (Å)	1.0	0.9795	0.9795
Temperature (K)	100	100	100
Resolution (Å)	40–2.36	40–2.15	40–2.2
Measured reflections	154723	279025	159358
Unique reflections	34741	58234	45860
Redundancy	4.4	4.79	3.47
Space group	$C222_1$	$C222_1$	$C222_1$
Unit-cell parameters (Å)			
a	59.97	60.27	60.40
b	157.27	158.28	158.09
c	219.88	219.44	219.89
Completeness (%)	80.8 (67.5)	98.8 (99.1)	82.7 (35.4)
Mean $I/\sigma(I)$	25.6 (10.01)	18.92 (3.86)	32.74 (5.99)
R_{merge}	0.049 (0.119)	0.083 (0.417)	0.053 (0.176)

Table 2
Statistics for data sets of apo forms of the *E. coli* and *S. coelicolor* sGDH homologues.

Values in parentheses are for the highest resolution shell.

	E. coli sGDH	*S. coelicolor* sGDH
Beamline	BESSY, PSF BL-2 (Berlin, Germany)	BESSY, PSF BL-2 (Berlin, Germany)
Wavelength (Å)	0.9184	0.9184
Temperature (K)	100	100
Resolution (Å)	2.0	1.8
Measured reflections	100719	122017
Unique reflections	26574	43602
Redundancy	3.8	2.8
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters		
a (Å)	59.22	48.94
b (Å)	73.80	40.67
c (Å)	75.80	86.06
β (°)		93.41
Completeness (%)	97.0 (99.0)	96.5 (97.5)
Mean $I/\sigma(I)$	4.6 (1.2)	10.46 (2.37)
R_{merge}	0.101 (0.32)	0.099 (0.36)

grams from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Crystallization using microseeding

Crystals of wild-type sGDH were previously obtained using the hanging-drop vapour-diffusion method with a reservoir solution containing 16–23% PEG 6000, 3 m*M* $CaCl_2$, 120 m*M* NaCl, 100 m*M* Tris–glycine pH 9.2 (Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999). These crystallization conditions were also screened for the sGDH variants, but results were erratic in terms of reproducibility and crystal morphology. A second drawback of these conditions is the presence of Tris, which for reasons that are only poorly understood prevents reconstitution of the holoenzyme in the crystals upon soaking with the PQQ cofactor. In order to obtain structural information on cofactor- and substrate-bound states, it has therefore been found necessary to transfer the crystals to a buffer containing CHES (Oubrie, Rozeboom, Kalk, Duine *et al.*, 1999; Oubrie, Rozeboom & Dijkstra, 1999). Unfortunately, few crystals survive this treatment and crystallization in a buffer containing CHES has so far been unsuccessful, even with wild-type *A. calcoaceticus* protein.

These drawbacks could be circumvented by using the microseeding method (Fig. 1). Initially, *A. calcoaceticus* wild-type sGDH seeds were used to obtain crystals of all three variants as well as the two homologues. In subsequent crystallization experiments, seeds of the target proteins themselves were used. The microseeding method facilitated efficient and reproducible crystal growth of all sGDH

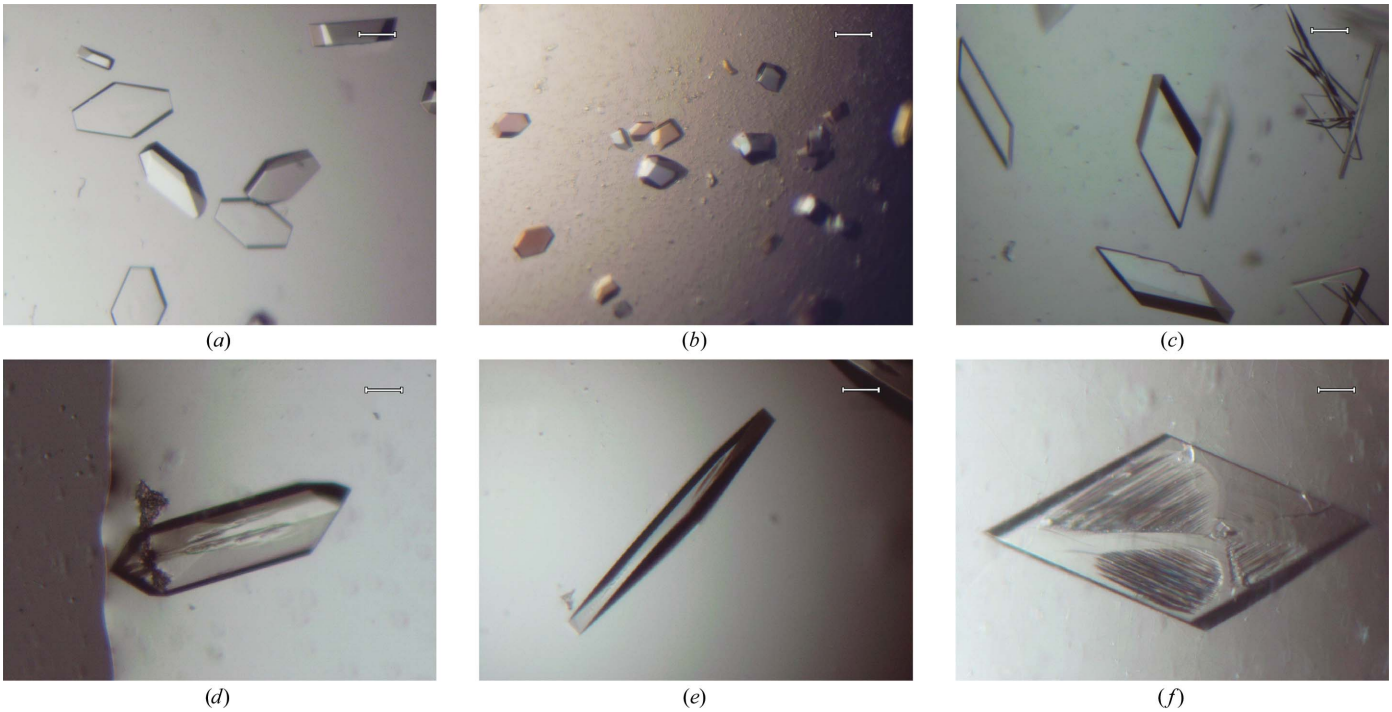

Figure 2
Crystals of apo forms of sGDH variants A (*a*), B (*b*) and G (*c*) and homologues from *E. coli* (*d*) and *S. coelicolor* (*e*, *f*). (*e*) and (*f*) show two different views of a *S. coelicolor* crystal. The scale bar represents 60 µm.

variants tried so far (Fig. 2). An additional advantage of the micro-seeding method in CHES- or HEPES-containing solutions is that no buffer exchanges are required to facilitate soaking with PQQ, which has made it more straightforward to obtain crystals of PQQ-bound enzyme forms.

Using this method, it has also been possible to reproducibly obtain crystals with a typical maximal size of 0.1 mm and 0.3 mm for the *E. coli* and *S. coelicolor* sGDH homologues, respectively (Fig. 2). In most cases, crystals were clearly visible after 3 h and were fully grown after a couple of days. *S. coelicolor* control drops did not produce crystals, whereas a small number of *E. coli* sGDH crystals grew in some control drops after several weeks.

3.2. Preliminary X-ray diffraction analysis

Data sets were collected from crystals of apo forms of each of the three sGDH variants A, B and G. The mutants crystallize in a centered orthorhombic space group rather than the monoclinic space group of wild-type crystals (Oubrie, Rozeboom, Kalk, Olsthoon *et al.*, 1999). The asymmetric unit of the $C222_1$ space group contains two sGDH monomers (Oubrie, Rozeboom & Dijkstra, 1999). Data-collection statistics are summarized in Table 1.

A data set was collected at 2.0 Å resolution from a single crystal of the *E. coli* sGDH homologue. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 59.22, b = 73.80, c = 75.80 Å. The asymmetric unit is estimated to contain one molecule of the *E. coli* protein, with a solvent content of 41.8%. Data-collection statistics are shown in Table 2. A data set was collected at 1.8 Å resolution from a single crystal of the apo form of the *S. coelicolor* sGDH homologue. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 48.94, b = 40.67, c = 86.06 Å, β = 93.41°. The asymmetric unit is estimated to contain one molecule of the *S. coelicolor* protein, with a solvent content of 47.3%. Data-collection statistics are shown in Table 2.

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