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GlxA is a new structural member of the radical copper oxidase family and is required for glycan deposition at hyphal tips and morphogenesis of *Streptomyces lividans*

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Streptomyces lividans displays a distinct dependence on copper to fully initiate morphological development. Evidence has accumulated to implicate the participation of an extracytoplasmic cuproenzyme in morphogenesis. In the present study, we show that GlxA fulfils all criteria to be that cuproenzyme. GlxA is membrane associated and has an active site consisting of a mononuclear copper and a cross-linked Y-C cofactor. The domain organization of the tertiary structure defines GlxA as a new structural member of the mono-copper oxidase family, with copper co-ordination geometry similar to, but spectroscopically distinct from fungal galactose oxidase (Gox). EPR spectroscopy reveals that the oxidation of cupric GlxA generates a protein radical residing on the Y-C cross-link. A variety of canonical Gox substrates (including D-galactose) were tested but none were readily turned over by GlxA. A *glxA* null-mutant leads

to loss of glycan accumulation at hyphal tips and consequently a drastically changed morphology both on solid substrates and in liquid-grown environments, a scenario similarly observed in the absence of the neighbouring glycan synthase CslA (cellulase synthase-like protein). In addition the *glxA* mutant has lost the stimulation of development by copper, supporting a model whereby the enzymatic action of GlxA on the glycan is required for development and morphology. From a biotechnology perspective, the open mycelium morphology observed with the *glxA* mutant in submerged culture has implications for use as an enzyme production host.

Key words: cuproenzyme, development, glycan biotechnology, protein radical, streptomycetes, X-ray structure.

INTRODUCTION

Streptomycetes are filamentous monoderm soil bacteria that form networks of branching hyphae called mycelia. These organisms display a complex developmental life cycle on solid substrates. Following spore germination, a vegetative mycelium is established that in response to nutrient depletion and other signals initiates both secondary metabolite production and morphological differentiation [1,2]. This leads to the formation of aerial hyphae that will develop into chains of spores. The richness and varied production of secondary metabolites in streptomycetes is co-ordinated with development and these metabolites have long been a source of interest due to their pharmaceutical properties [1,2]. Streptomycetes also hold promise as a large-scale production host in biotechnology for the heterologous production of proteins and enzymes at high levels for therapeutic, scientific, diagnostic and agricultural purposes [3]. *Streptomyces lividans* is a preferred choice as an industrial host primarily due to its low level of endogenous extracellular proteolytic activity. As a production host the morphology of the mycelial growth in submerged culture is important as this can play a significant role in its production capacity [4].

Copper (Cu) is an essential redox-active metal ion in living organisms. In *S. lividans* a distinct dependence on the bioavailability of Cu in order to fully initiate morphological development has been described [5–7]. *In vitro* studies have

revealed that two extracytoplasmic Cu metallochaperones, ECuC (extracytoplasmic Cu chaperone) and Sco (synthesis of cytochrome c oxidase), facilitate a Cu trafficking pathway whereby the Sco protein can receive Cu from ECuC and deliver it to the Cu_A site of an aa₃-type cytochrome c oxidase (CcO). Mutant analysis in *S. lividans* has revealed that morphological development proceeds in the absence of ECuC ($\Delta ecuc$) and CcO (Δcco), but not in the absence of Sco (Δsco). Whereas $\Delta ecuc$ and Δsco significantly reduce CcO activity, these data indicate that morphological development is not linked to impaired CcO activity [7,8]. Notably the addition of exogenous Cu to the Δsco mutant rescues development [7]. This implies that a branched Cu trafficking pathway is prevalent under Cu homeostasis conditions in *S. lividans*, whereby Sco is most certainly required to act as a Cu chaperone for CcO and possibly for other extracytoplasmic cupro-proteins/enzymes that trigger formation of aerial hyphae [8].

Analysis of the *S. lividans* genome has revealed a number of genes that encode putative extracytoplasmic cuproenzymes [9,10]. One of these, SLI_3188, has a weak sequence homology with the secreted fungal cuproenzyme galactose oxidase (Gox), with putative ligands to the Cu ion (two tyrosine and two histidine residues) seemingly conserved (Supplementary Figure S1). Gox houses a catalytic unit, which combines two distinct redox centres; a mononuclear Cu site capable of one electron redox cycling and a Y-C cofactor, whereby a cysteine residue is cross-linked to one

Abbreviations: CcO, cytochrome c oxidase; CFW, calcofluor white; Gox, galactose oxidase; HRP, horseradish peroxidase; LMCT, ligand-to-metal charge transfer; WT, wild-type.

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of the Cu-co-ordinating tyrosine residues and can form a stable protein radical [11–16]. This redox unit enables the two-electron oxidation of a range of D-isomers of primary alcohols (including the C6-hydroxymethyl group of mono- and poly-saccharides) to aldehydes with the reduction of dioxygen to hydrogen peroxide [17]. The physiological function of Gox remains unknown, but the broad substrate specificity inherent in this cuproenzyme suggests that maintaining hydrogen peroxide production in the extracytoplasmic environment may be important for function.

In *Streptomyces coelicolor* a homologous gene to *SLI_3188*, annotated as *glxA*, is required for aerial hyphae development under conditions of osmotic stress [18]. The *glxA* gene is the distal gene in an operon with *csIA*, which encodes a family 2 glycosyltransferase that synthesizes a glycan at hyphal tips [19,20]. Orthologues of *glxA* and *csIA* are present in all streptomycetes and several other actinobacteria with some species having paralogues, e.g. *Streptomyces griseus* and *Streptomyces albus*. The genetic locus contains several other genes encoding enzymes implicated in glycan processing, including a Cu-containing lytic polysaccharide mono-oxygenase, suggesting a role of GlxA in polysaccharide modification or synthesis. In the present study, we show that GlxA is a membrane-associated cuproenzyme with a mononuclear Cu site and a Y-C redox cofactor. However, GlxA is distinct from fungal Gox through a unique tertiary structure, atypical spectroscopic properties and a lack of enzymatic activity with a range of 'classical' Gox substrates. As observed in the absence of *csIA*, we find that a *glxA* null-mutant stalls aerial hyphae development on solid media, lacks the hyphal tip glycan and has a dramatically different morphology in liquid-grown cultures. Taken together, our work implies GlxA as the likely candidate mediating Cu-dependent morphogenesis in *Streptomyces*.

EXPERIMENTAL

Mycelium washes, fractionation and GlxA detection

S. lividans 1326 was grown in TSBS (tryptic soy broth supplemented with sucrose) for 18 h at 30 °C with shaking at 200 rpm. Mycelium was harvested in 1.5 ml aliquots by centrifugation at 15 000 g for 10 min and stored at –80 °C until required. Mycelium aliquots were first resuspended in 25 mM Tris/HCl, pH 7.5, 100 mM NaCl centrifuged at 25 000 g and the supernatant was kept as the 100 mM NaCl wash. The pellet was resuspended in buffer containing 1 M NaCl, incubated for 30 min on ice and centrifuged at 25 000 g, which produced the 1 M NaCl wash. For mycelium extracts the suspended mycelium pellets were sonicated (Bioruptor, 12 cycles: 30-s on, 30-s off). The soluble (S30) and insoluble fractions (P30) were obtained by centrifugation at 30 000 g. The supernatant (S30) was subjected to a second centrifugation step at 100 000 g to remove all membrane particles and ribosomes to give the S100 and P100 fractions. The pellets (P30 and P100) were resuspended in buffer containing 1 % Triton X-100, incubated on ice for 30 min and centrifuged again at 30 000 g and 100 000 g respectively to separate Triton soluble (P30-TS, P100-TS) and insoluble (P30-TP, P100-TP) fractions. Fractions obtained were run on SDS/PAGE (10 % gel) and blotted to Hybond-P membranes for immuno-detection of GlxA and EF-Tu1. The antibodies against GlxA were a kind gift of James W. Whittaker (Oregon Health & Science University, U.S.A.). The EF-Tu1 antibodies were raised in rabbits against *Streptomyces ramocissimus* EF-Tu1 [21]. Incubation with antibodies (GlxA antibodies 10 000× diluted and EF-Tu1 antibodies 5000× diluted) was carried out for 18 h at 4 °C with gentle rocking in PBS with 5 % milk (FrisoLac Extra,

FrieslandCampina). The bound antibodies were detected with GARAP (Sigma) as the secondary antibody and NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as the substrate. Digital images were taken of the Western blots and total signal intensities were determined with ImageJ [22].

Creation of the *S. lividans* $\Delta csIA$ and $\Delta glxA$ null-mutants, complementation plasmids and analysis of growth morphology

The $\Delta glxA$ and $\Delta csIA$ mutants were prepared and isolated as previously described [7]. In the $\Delta glxA$ mutant, nucleotides +60 to +1916 relative to the start codon of *SLI_3188* were replaced by a 62 nt scar of the loxP recombination site including two XbaI sites. In the $\Delta csIA$ mutant, nts +79 to +1827 relative to the start of *SLI_3187* were replaced. The mutant is consistent with the one described by Xu et al. [20]. Plasmid pGlxA contains the *glxA* ORF (*SLI_3188*) and the promoter region (–545 to –1) upstream of *csIA* (*SLI_3187*). For growth on solid, R5 agar medium [23] was used and supplemented with Cu as required. Morphology in liquid medium was determined following 24 h growth with shaking in flasks equipped with coils containing tryptic soya broth (TSB) supplemented with 10 % sucrose and Cu as indicated. All solid and liquid-grown cultures were incubated at 30 °C. Samples from liquid cultures were analysed by light microscopy with a Zeiss Standard 25 microscope and digital pictures were taken with an AxioCam camera linked to AxioVision software. All spore stocks were obtained from cultures grown on MS (soy flour mannitol medium) agar plates [23] and stored in 20 % glycerol at –20 °C.

Analysis of glycan production

Glycan production was determined following 24 h growth at 30 °C in eight-well microscopy plates (Lab-TEK II Chambered Coverglass) containing 500 μ l of mNMMP (minimal NMMP medium with mannitol) medium [24]. Mycelium was stained by adding 50 μ l of calcofluor white (CFW) solution (Remel Bactidrop) to each well. After 5 min, but within 20 min, stained mycelium was analysed with a laser-scanning confocal microscope (Zeiss LSM5 Exciter/Axio Observer) by excitation with a 405 nm laser, a 405/488 nm beamsplitter and a bandpass emission filter of 420–480 nm. Images were adjusted for brightness and contrast using ImageJ [22].

Cloning, overexpression and purification of GlxA

The *glxA* gene with 200 flanking nt at the 5'- and 3'-ends was amplified from *S. lividans* 1326 and cloned into a pUC19 vector. This construct was used to create an N-terminal deleted *glxA* gene for overexpression in *Escherichia coli* by amplifying the *glxA* nt sequence that starts coding for amino acid 35 and restricted using the NdeI and BamHI sites of a pET28a vector (Novagen) to create an N-terminal His₆-tagged *glxA* construct. The overexpression and purification of recombinant GlxA are described in Supporting Information.

Preparation of GlxA samples and UV-visible spectroscopy

GlxA concentration was determined by UV-visible spectroscopy (Varian Cary 50 UV-visible spectrophotometer) using an absorption coefficient (ϵ) at 280 nm of 78730 M^{–1}·cm^{–1}. The various forms of GlxA were prepared through the addition of ~5-fold excess of [Fe(CN)₆]^{3–} (Sigma), [Ir(Cl)₆]^{3–} (Acros) or Na₂S₂O₄ (Sigma) followed by removal via a PD-10 column (GE Healthcare).

Table 1 X-ray data processing and refinement parameters

The GlxA crystal structure was in space group $P2_1$ with unit cell parameters 50.4, 126.6, 107.6 Å, 90, 91.1, 90°. Values in parentheses refer to the outermost resolution shell (1.80–1.77 Å).

Resolution (Å)	53.3–1.77
Unique reflections	130533 (21898)
Completeness (%)	99.7 (99.6)
Redundancy	3.6 (3.4)
R_{merge}	0.064 (0.633)
Mn(I/sd)	10.5 (2.0)
Wilson B factor (Å ²)	20.5
R_{cryst}	0.188
R_{free}	0.228
RMSD bond lengths (Å)	0.011
RMSD bond angles (°)	1.41
ESU based on ML (Å)	0.096
Ramachandran favoured (%)	95.9
PDB accession code	4unm

Crystallization and structure determination

An ARI-Gryphon 96-well crystallization robot was used to screen crystallization conditions for GlxA. A crystal hit was discovered in 0.1 M sodium acetate, pH 4.6, 15 % PEG 20000 (PEG suite, Qiagen). Scaling-up and optimization of GlxA crystals from the initial hit was carried out in 24-well VDX plates (Molecular Dimensions) using the hanging drop vapour diffusion method at 20°C. Equal volumes of GlxA solution at a concentration of 15 mg·ml⁻¹ and reservoir solution containing 0.1 M sodium acetate, pH 4.0, 20 % PEG 20000 were mixed. Crystals suitable for diffraction studies grew within 1 week. A single crystal was transferred to a cryoprotectant solution containing the respective reservoir solution and 20 % glycerol prior to flash-cooling by plunging into liquid nitrogen. Crystallographic data were measured at the beamline I03, Diamond Light Source, using an X-ray wavelength of 0.979 Å (1 Å = 0.1 nm) and a Pilatus 6 M-F detector (Dectris). Details of structure determination are given in Supporting Information. Co-ordinates and structure factors were deposited in the RCSB Protein Data Bank with accession number 4unm. A summary of data and refinement statistics and the quality indicators for the structure are given in Table 1. CAVR analyst 1.0 was used for the identification of tunnels [25]. Chain B of GlxA was analysed using the tunnel computation tool within CAVR with a starting point defined using the first co-ordination sphere Cu-binding residues (Tyr²⁸⁹, Tyr⁵⁰¹, His⁵⁰², His⁵⁸⁹). Default settings for tunnel analysis were used, including the minimum probe radius set to 0.9 Å.

EPR spectroscopy

GlxA samples (85–120 µM) for EPR were prepared in duplicate in a mixed buffer system consisting of 10 mM each of Tris, potassium acetate, MES, MOPS and 200 mM KCl with the pH adjusted to 7.0. Wilmad SQ EPR tubes (Wilmad Glass) were filled with the GlxA solutions and frozen in methanol kept on dry ice. The tubes were then transferred to liquid nitrogen. All EPR spectra were measured on a Bruker EMX EPR spectrometer (X band). A spherical high-quality Bruker resonator ER 4122 SP 9703 and an Oxford Instruments liquid helium system were used to measure the low-temperature EPR spectra. Digitizing of a published EPR spectrum was performed using Un-Scan-It, v.6, Silk Scientific.

Activity assays

A variety of putative substrates for GlxA were assayed. These included the following monosaccharides; D-galactose, D-glucose, D-sucrose, D-fructose *N*-acetyl-D-glucosamine and D-glucuronic acid; the disaccharides, D-lactose and D-cellobiose; and the aldehydes and primary alcohols glycolaldehyde, glyoxal and glycerol (all purchased from Sigma). Catalytic turnover was measured using a coupled assay, whereby the production of H₂O₂ by GlxA was detected by the presence of horseradish peroxidase (HRP; Sigma) and the subsequent oxidation of guaiacol (Sigma). Samples were prepared in 3 ml quartz cuvettes containing 0.1 M sodium phosphate, pH 7.4, 1 mM guaiacol, 1 µl of HRP (10 mg·ml⁻¹), 20–30 µM GlxA and various concentrations of the individual substrate. The oxidation of guaiacol was monitored at 470 nm using a Hewlett–Packard 8453 diode-array spectrophotometer scanning between 190 and 1100 nm and thermostatted at 25°C. Plots of turnover rate (k , s⁻¹) compared with substrate concentration were constructed, whereby k was calculated from $((\Delta A_{470}/\epsilon_{\text{gc}})/t)/[\text{GlxA}]$ where ΔA_{470} is the absorbance change at 470 nm upon guaiacol oxidation, ϵ_{gc} is the absorption coefficient of the guaiacol oxidation product taken as 5.57 mM⁻¹·cm⁻¹, t is the time in seconds and [GlxA] is the total millimolar concentration of GlxA in the assay.

RESULTS

GlxA is associated with the membrane

The distribution of GlxA in the soluble and insoluble fractions of liquid grown *S. lividans* mycelium was first determined. Upon thorough mycelium sonication followed by centrifugation steps, a small fraction of GlxA was detected in the soluble protein fraction (S100) with the majority remaining in the pellet fraction (P30 and P100; Figures 1A and 1B). In contrast, the majority of the cytoplasmic marker EF-Tu1 is detected in the soluble fraction (S100; Figures 1A and 1B). Resuspension of the insoluble pellet fraction in buffer containing 1 % Triton X-100 solubilizes a significant amount of GlxA (Figures 1A and 1B), indicating that GlxA is not covalently bound to the membrane. However, salt washes did not remove GlxA from intact mycelium (Figure 1C), but small amounts of EF-Tu1 were detected probably due to some lysis. These data show that GlxA is not a peripheral membrane protein but is firmly associated with the membrane, although not via a covalent bond. These results are in agreement with *in silico* analysis, which predicts residues 1–11 of GlxA to be an N-terminal signal peptide [26], with a weak signal peptidase cleavage site between residues 11–12, followed by a transmembrane helix (residues 12–32), which could function as the membrane anchor [27,28].

The tertiary structure of GlxA consists of three distinct domains

To elucidate the molecular features of GlxA, an N-terminal truncated expression construct ($\Delta 1$ –34) for overexpression in *E. coli* was generated, which yielded 45 mg·l⁻¹ of purified GlxA. The crystal structure of GlxA was determined to 1.77 Å resolution and contains two protein molecules in the crystallographic asymmetric unit. Molecule A (residues 38–645) exhibits significantly lower B-factors and higher quality electron density than molecule B, but contains a disordered region between residues 198 and 206 (not visible in the electron density), whereas the main chain for molecule B was complete between residues 37 and 645. The overall structure of GlxA (Figure 2) consists of three predominately β -sheet domains. Preceding the first domain

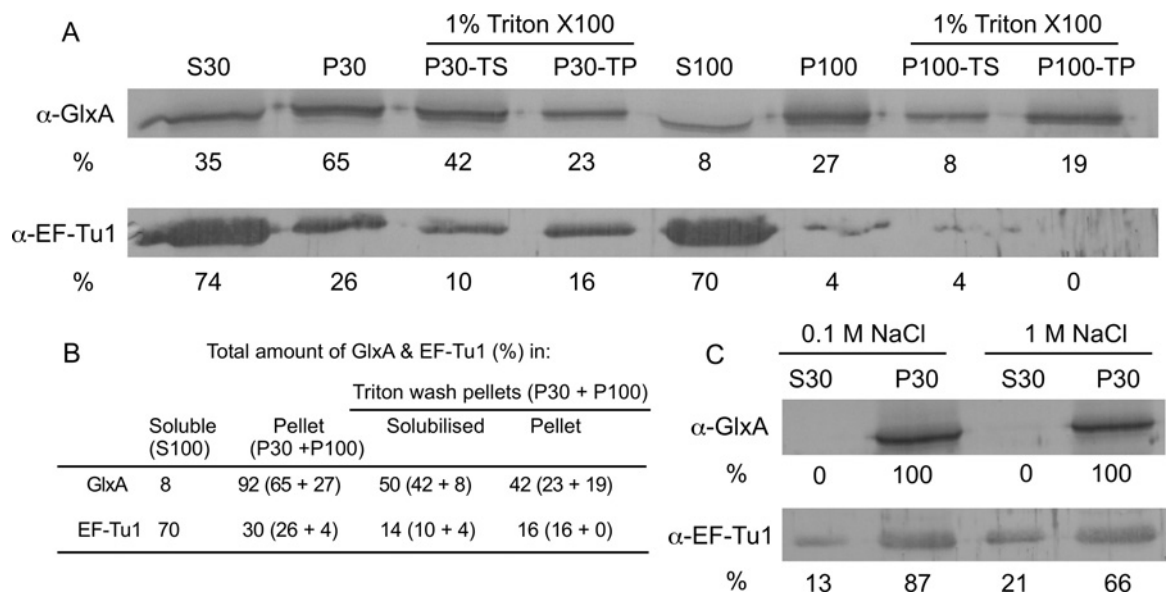


Figure 1 Location of GlxA in the mycelium determined by Western blotting

(A) Detection of GlxA and EF-Tu1 in the various mycelium fractions. Samples were prepared as described in 'Experimental'. The band intensities are expressed as percentages relative to the total amount of GlxA or EF-Tu1 in the S30 plus P30 fraction, the intensity of which was set at 100 %. (B) The Table presents the total soluble (S100), 1 % triton soluble (P30-TS + P100-TS) and insoluble fraction (P30-TP + P100-TP) of GlxA and EF-Tu1 expressed as a percentage according to (A). (C) NaCl washes of the intact mycelium. The signals detected in the S30 plus P30 samples of the 100 mM NaCl wash were set at 100 %.

are two short N-terminal α -helices (α 1 and α 2), with the α 1 helix orientated approximately perpendicular to the α 2 helix. Domain 1 of GlxA consists of seven Kelch motifs (blades) arranged in a β -propeller tertiary structure (Figure 2). Each blade of the propeller consists of four anti-parallel β -sheets (*a*, *b*, *c*, *d*) with each sheet connected through variable length loops (Figure 2). Blades 1 and 2 each possess structural inserts in the loops connecting sheet *b* to sheet *c*. In blade 1 a short platform-like α -helix (α 3) is inserted which has an overall negative charge, whereas in blade 2, sheets *b* and *c* are connected via domain 2 (Figure 2). Domain 2 consists of 10 β -sheets and 1 short α -helix (α 4). According to the CATH database [29] the domain 2-fold is classified as mainly β , with a β -barrel architecture formed by sheets 1, 3, 4, 8, 9 and 10 (Figure 2) and a representative domain topology with thrombin, subunit H. Protruding out from the core β -barrel is a long β -hairpin loop (residues 194–210) consisting of β -sheets 6 and 7 (Figure 2). In molecule A, this β -hairpin loop is disordered and not built into the model, whereas this is not the case for molecule B due to stabilizing polar interactions with molecule A residues from a symmetry related GlxA molecule in the unit cell (Supplementary Figure S2). Domain 2 is positioned in the overall tertiary structure such that the β -barrel core lies across the top of approximately half of domain 1 with the β -hairpin loop extending into the solvent (Figure 2). Further analysis of domain 2 using PDBFold identifies no structures deposited in the PDB with a high structural homology (the highest Q-score obtained was only 0.14). Thus the fold of domain 2 in GlxA is relatively novel compared with known structures. The third domain starts immediately after the seventh Kelch motif and is located at the bottom of domain 1. Domain 3 consists of eight β -sheets and one short α -helix forming a β -sandwich fold with a high structural homology to domain 3 of Gox (1.18 Å RMSD from superimposition). A loop (residues 583–590 in GlxA) connecting β -sheets 3 and 4 of domain 3 penetrates upwards into domain 1 along the 7-fold symmetry axis and provides one of the ligands (His⁵⁸⁹) to the Cu ion of

GlxA (see below). The charge distribution of GlxA (pI 8.2) is displayed through electrostatic surface representations (Figure 2) and indicates a rather disperse distribution of charge across the whole molecular surface.

Architecture and spectroscopic properties of the Cu site in GlxA

A well-defined 16 σ peak in the σ -weighted F₀-F_c difference map was present in each GlxA chain in the asymmetric unit, into which a Cu ion was modelled. The Cu is co-ordinated in a square pyramidal geometry with the OH of Tyr⁵⁰¹ acting as the axial ligand and the equatorial coordination positions occupied by the OH of Tyr²⁸⁹, the N ϵ 2 of His⁵⁰² and His⁵⁸⁹ and a H₂O molecule (Figure 3A). Bond lengths to the Cu ion are reported in Table 2 together with a comparison to those in Gox. Unbroken electron density is observed between the side chain S γ atom of Cys¹²¹ and the C ϵ 1 ring atom of Tyr²⁸⁹, providing clear evidence that a cross-linked Y-C cofactor is formed in GlxA, with a bond length of 1.9 Å (2.0 Å in chain B; Figure 3A). Adjacent to Tyr²⁸⁹ is Trp²⁸⁸, which has its side chain indole ring orientated such that the benzene ring is π - π stacking with the phenoxyl ring of Tyr²⁸⁹ with C^{Tyr}-C^{Trp} distances between ~3.5 and 4 Å (Figure 3A).

The UV-Vis absorbance spectrum of the blue-grey Cu(II)-GlxA has a broad, low intensity band in the visible region (λ_{max} ~ 577 nm) and two distinct shoulders (λ_{max} ~362 and 320 nm; Figure 3B). No change to these absorption features in the pH range 4–8 was observed, with the addition of Cu(II) ions resulting in only a small absorbance increase in λ_{max} ~ 577 nm over a 12-h period (Figure 3B). In contrast, the Cu(II)-Gox absorption spectrum has weak intensity bands at λ_{max} 441 nm attributed to phenolate (Tyr) to Cu(II) ligand-to-metal charge transfer (LMCT) and at λ_{max} 630 nm arising from mixed Cu ligand field transition and LMCT [11,14]. Addition of the reductant Na₂S₂O₄ bleaches all absorption features (Figure 3B) with removal of Na₂S₂O₄ and

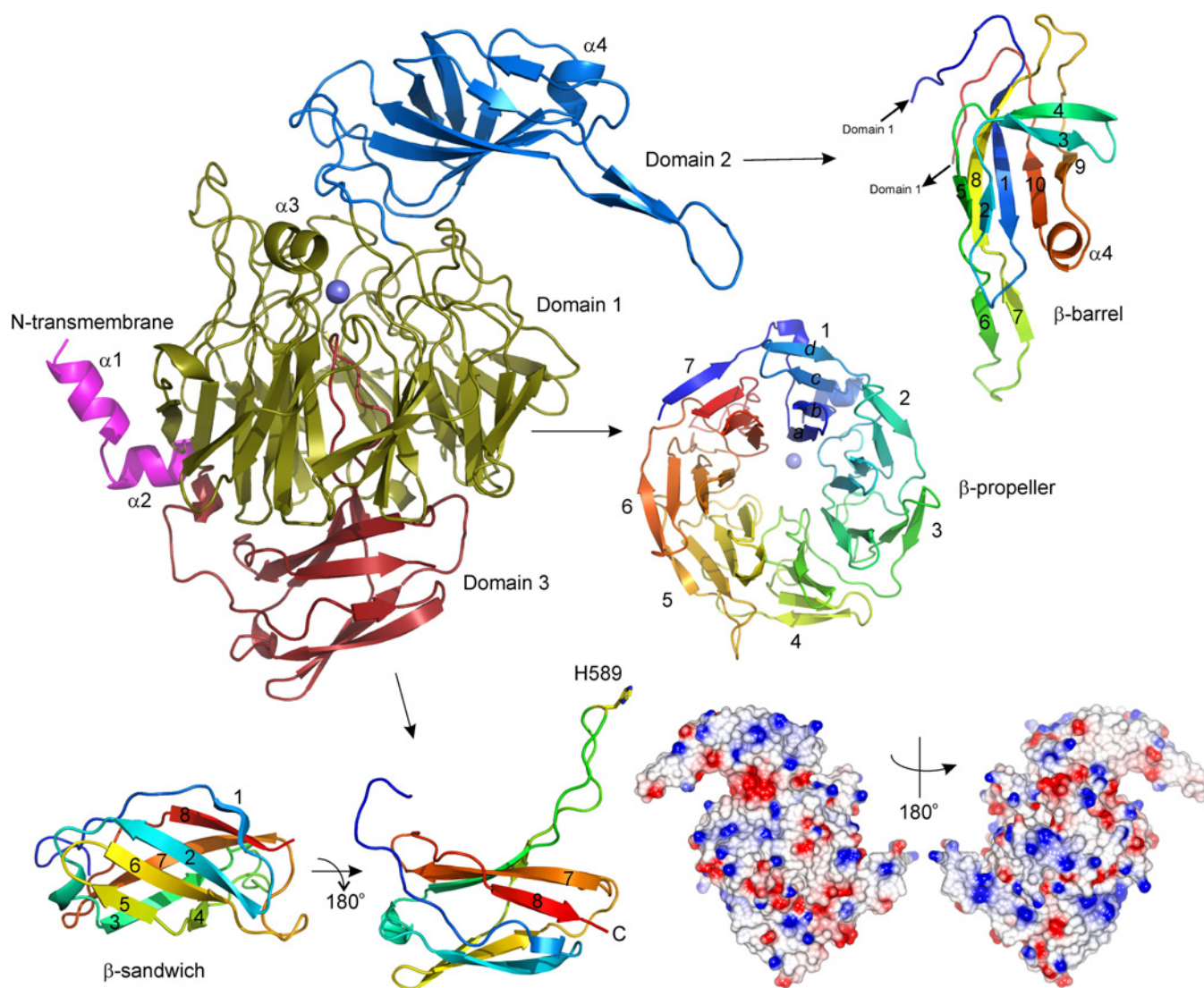


Figure 2 X-ray crystal structure of *S. lividans* GlxA

The three domains forming the tertiary structure are indicated and coloured gold (domain 1), blue (domain 2) and red (domain 3). The four short α -helices present in the structure are labelled. Each domain is shown individually and in more detail as discussed in the main text. The Cu atom is represented as a sphere and electrostatic surface representations in two GlxA orientations are shown. Images were prepared in PyMol and CCP4MG [48].

subsequent exposure to air resulting in the return of the Cu(II)-GlxA spectrum. This infers that the Cu is redox active and can cycle between the Cu(II) and Cu(I) states.

The EPR spectrum of Cu(II)-GlxA at pH 7 displays features consistent with an axial Cu(II) g-tensor, $g_{\parallel} = 2.182$ and $g_{\perp} = 2.05$ (Figure 3C). The strong line on the right from the indicated g_{\perp} region is not a principal g-factor component but an 'overshoot' line [30] occurring for specific orientations of the Cu(II) complexes, subject to particular relationships between the values of g_{\parallel} , g_{\perp} and the anisotropic components of the hyperfine interaction of the electron spin ($S = 1/2$) with the Cu nuclear spin ($I = 3/2$), A_{\parallel} and A_{\perp} . The EPR spectrum line shape is similar to that of Cu(II)-Gox [31], which also has an axial Cu(II) g-tensor [32–34]. However, the GlxA spectrum exhibits a more distinctly resolved overshoot line, likely to be a consequence of a smaller g_{\parallel} (2.182 as compared with 2.21–2.23 in Gox). The g_{\perp} component displays a hyperfine interaction of the electron spin with the Cu(II) ligands, which for

GlxA gives seven lines with relative intensities of 1:4:8:10:8:4:1 (Figure 3C, inset) compared with five lines in Gox [33,34].

Substrate access sites and binding pocket

Due to the buried nature of the Cu site, it is likely that a substrate would have to gain access via a tunnel. CAVER was used to identify tunnels in the GlxA structure [25,35], defined as void pathways leading from a cavity inside GlxA, i.e. the Cu site, that transverse to the protein surface. Three tunnels of different lengths were identified (Figure 4A). The entrance to tunnel I lies between the $\alpha 3$ helix on the loop connecting sheets *a* and *b* in blade 1 and a well-ordered loop in blade 7 of domain 1 (Figures 4A and 4B). The tunnel has an opening of $\sim 8 \times 8$ Å, is void of H₂O molecules and leads directly to the equatorially Cu co-ordinating H₂O molecule, with a distance to the surface

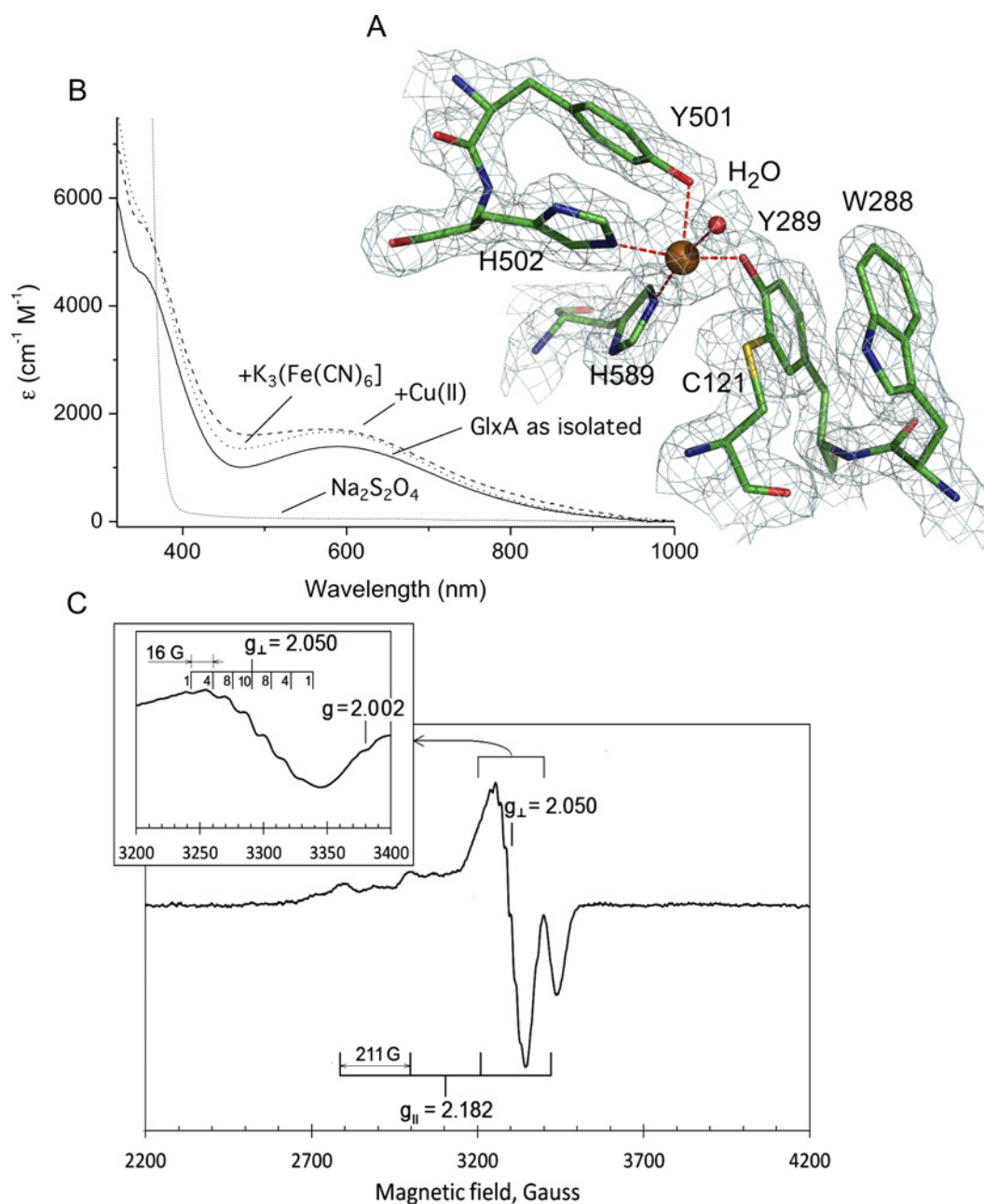


Figure 3 Structure and spectroscopy of the GlxA Cu site

(A) $2F_o - F_c$ electron-density map contoured at 2σ of the Cu site in GlxA. The Cu ion is represented as a brown sphere with the ligands to the Cu ion depicted as sticks and the equatorially coordinating H_2O molecule as a red sphere. (B) Absorbance spectra at pH 7 and 20 °C of purified GlxA (80 μM). (C) Cu(II)-GlxA EPR spectrum (85 μM) at pH 7. The $g_{||}$ component shows a hyperfine interaction of the electron spin ($S = 1/2$) with the $I = 3/2$ Cu nuclear spin, four components 1:1:1:1 separated by $A_{z^{Cu}} = 211$ G. Inset: the g_{\perp} region of the spectrum, which displays unpaired electron ($S = 1/2$) hyperfine interaction with the Cu(II) ligands, seven components of relative intensities 1:4:8:10:8:4:1 separated by 16 G. Instrumental conditions: temperature 10 K, microwave frequency $\nu_{MW} = 9.47$ GHz, microwave power $P = 3.18$ mW, modulation frequency $\nu_M = 100$ kHz, modulation amplitude $A_M = 5$ G, time constant $\tau = 82$ ms, scan rate $V = 22.6$ G s^{-1} , number of scans per spectrum NS = 1.

of ~ 10 Å. The dimensions of the surface opening are such that pyranose carbohydrates, e.g. D-glucose or D-galactose and also C2 substituted pyranose carbohydrates such as N-acetyl-glucosamine would be able to pass. The opening of tunnel II is located beneath the β -hairpin loop of domain 2 (Figure 4B), with the dimensions of the opening, $\sim 9 \times 7$ Å. A network of six H-bonded H_2O molecules are present in the tunnel that thread down to the Cu co-ordinating H_2O molecule, with a distance to the surface of ~ 25 Å. Tunnel III spans a large section of domain 1, starting from below

the equatorial histidine ligands and leading to a surface entrance formed by residues Ala¹²⁸, Gly²⁹⁸, Pro³²² and Ser⁶²². The tunnel is lined with a continuous network of well-ordered H_2O molecules (13 in total) and has a distance from the Cu ion to the surface of ~ 70 Å. A putative substrate pocket is identified above the axial Tyr⁵⁰¹ ligand and is directly accessible from either tunnel I or II (Figure 4C). The pocket is occupied by several H_2O molecules, all of which are well ordered and form an extensive H-bond network, which also includes an H-bond interaction with the Tyr⁵⁰¹ ligand

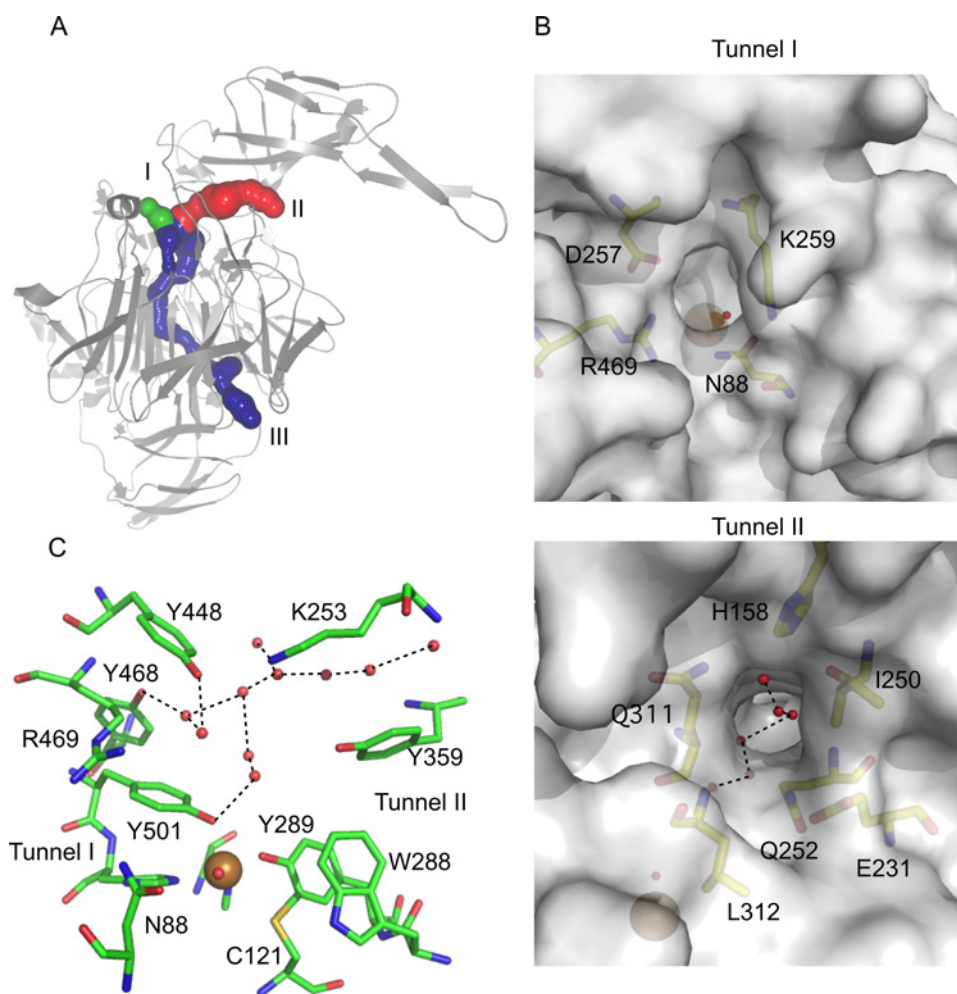


Figure 4 Substrate access channels and binding pocket

(A) Location of the three surface-to-Cu site tunnels (I, II, III) in *S. lividians* GlxA identified using the program CAVER [25]. (B) Partial transparent surface views of the openings to tunnels I and II. The amino acids forming the openings are labelled and shown as sticks, H₂O molecules are depicted as red spheres and the Cu ion as a brown sphere. In tunnel I the equatorially Cu co-ordinated H₂O molecule is visible and tunnel II illustrates the H-bonded H₂O network leading from the surface to the Cu site. (C) The putative substrate-binding pocket in GlxA.

Table 2 Bond lengths of the Cu sites (monomer A and B) in *S. lividians* GlxA and *F. graminearum* Gox (PDB 1gof) [13]

GlxA (A/B)		Gox	
Cu-Tyr ²⁸⁹ OH	1.84/1.97 Å	Cu-Tyr ²⁷² OH	1.93 Å
Cu-Tyr ⁵⁰¹ OH	2.24/2.14 Å	Cu-Tyr ⁴⁹⁵ OH	2.69 Å
Cu-His ⁵⁰² Nε2	2.13/2.18 Å	Cu-His ⁴⁹⁶ Nε2	2.11 Å
Cu-His ⁵⁸⁹ Nε2	2.18/2.23 Å	Cu-His ⁵⁸¹ Nε2	2.14 Å
Cu-H ₂ O	2.44/2.50 Å	*Cu-acetate	*2.26 Å
Tyr ²⁸⁹ -Cys ¹²¹	1.92/1.97 Å	Tyr-Cys	1.83 Å

*1gof crystallized in acetate buffer, pH 4.5, (GlxA acetate buffer, pH 4.0), with an acetate molecule found in place of the H₂O molecule in GlxA. In a Gox structure (1gof) [13] crystallized in the absence of acetate buffer at pH 7.0 a H₂O molecule is found with a bond length to the Cu ion of 2.81 Å.

(Figure 4C). The pocket is completely polar and is formed from the side chains of six amino acids (Figure 4C). Side-chain H-bonding interactions with the H₂O network in the pocket are apparent for some of the residues (Figure 4C), with Asn⁸⁸ and Arg⁴⁶⁹, part

of the entrance to tunnel I (Figure 4B), also capable of H-bond interactions.

The Y-C cross-link is redox active

The Y-C cross-link identified in the GlxA X-ray structure is redox active in Gox. Addition of the oxidants, [Fe(CN)₆]³⁻ or [Ir(Cl)₆]³⁻, to Cu(II)-GlxA does not perturb the absorbance spectrum (Figure 3B) in contrast with Gox where formation of the fully oxidized Cu(II)-Tyr-Cys• form leads to distinct spectral features. However, a change in the EPR spectrum of GlxA following addition of [Fe(CN)₆]³⁻ is observed, with a three-line free radical EPR spectrum detected (Figure 5). The spectrum is very similar to the spectrum of the radical on the Y-C cross-link reported for Gox [12], glyoxal oxidase [36] and SCO2837p from *S. coelicolor* [37] (Figure 5). This observation taken together with the result of Na₂S₂O₄ reduction indicates that the active site of GlxA can access three oxidation states [reduced (Cu(I)), semi-reduced [Cu(II)] and fully oxidized [Cu(II)-Tyr-Cys•]] as is the case for Gox and should therefore be able to catalyse the two-electron oxidation of a substrate.

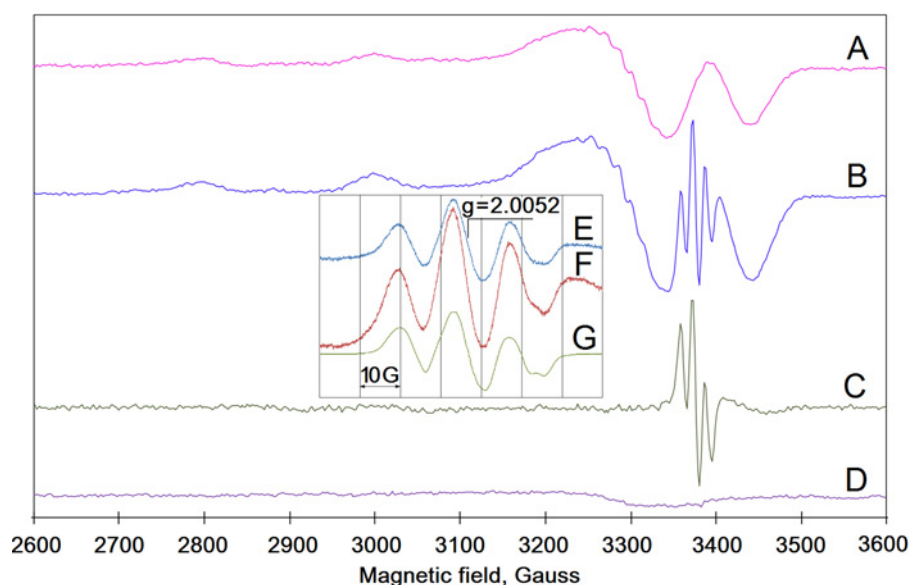


Figure 5 EPR spectra of Y-C radicals

(A) As isolated GlxA, (B) after $[\text{Fe}(\text{CN})_6]^{3-}$ treatment, (C) difference spectrum ($B - 1.46 \times A$) showing the free radical EPR signal in its pure form (the coefficient 1.46 was found empirically to minimize input of other EPR signals to the difference spectrum), (D) as in (A), but after $\text{Na}_2\text{S}_2\text{O}_4$ treatment. Inset: the same sample as the one used to detect spectrum (B), was used to measure the free radical EPR signal in greater detail using two different microwave power levels, 50 μW (E) and 3.18 mW (F). (G) The EPR spectrum of the radical in SCO2837p from *S. coelicolor* [37]. All spectra were recorded at 40 K using 120 μM GlxA at pH 7, with the instrumental conditions for (A, B and D) the same as in Figure 3(C) and for spectra (E) and (F) as follows: modulation amplitude $A_M = 3$ G, scan rate $V = 0.596$ G s^{-1} . Spectrum vii is a digitized image from [37], aligned with the GlxA spectrum on the basis of g-factors, therefore the magnetic field axis is not indicated.

Enzymatic activity of GlxA

A number of compounds were assayed for activity with GlxA using a coupled peroxidase assay (see 'Experimental'), with only four showing significant oxidase activity. Figure 6(A) reveals that aerobic oxidation rates for D-galactose, D-glucose and glycerol, follow a linear substrate relationship for the concentrations used. From the slopes of these plots, second order rate constants (k_{red}) were determined for D-galactose, $8.4 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$; for D-glucose, $8.8 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$; and for glycerol, $1.7 \times 10^{-2} \text{ M}^{-1} \cdot \text{s}^{-1}$. No activity was observed for the C2 position modified monosaccharide *N*-acetyl-D-glucosamine or for fructose, a five-membered ring monosaccharide. Likewise D-glucuronic acid gave no activity, illustrating that a C1 glycosidic (aldehyde) group is not effective as a reductant when the C6 group is modified (COOH compared with CH_2OH). Furthermore, no activity was detected with the disaccharides D-lactose and D-cellobiose. In addition to catalysing the oxidation of primary alcohols, Gox has been reported to convert aldehydes to the corresponding carboxylates [38]. However, the aldehyde, glyoxal, turned over by glyoxal oxidase, also did not give activity with GlxA. In contrast, glycolaldehyde ($\text{C}_2\text{H}_4\text{O}_2$) the smallest molecule to contain both an aldehyde and a hydroxy group gave significant activity with GlxA that followed Michaelis–Menten kinetics (Figure 6B). From these data a K_m for glycolaldehyde of 115 mM and a maximal aerobic turnover (k_{cat}) of 0.14 s^{-1} was determined. The corresponding k_{cat}/K_m value of $1.22 \text{ M}^{-1} \cdot \text{s}^{-1}$ enables a direct comparison with the two monosaccharides and glycerol, with glycolaldehyde reacting 100-times faster. A further possibility for the enzymatic role of GlxA is that the production of H_2O_2 is required for development rather than the substrate product. However, addition of sub-lethal concentrations of H_2O_2 to young cultures of both the wild-type (WT) and the ΔglxA strains did not stimulate development (Supplementary Figure S3).

GlxA is required for glycan synthesis at hyphal tips and morphogenesis

To investigate a functional role of GlxA, we created a null-mutant in *S. lividans*. Consistent with previous work in *S. coelicolor*, our *S. lividans glxA* null-mutant has a bald phenotype (i.e. no aerial hyphae formation) on solid media, as is also the case for the *csIA* null-mutant [18,20] (Figure 7A). Notably, the bald phenotype in the *glxA* and *csIA* null-mutants cannot be rescued upon addition of exogenous Cu(II) to the medium (Supplementary Figure S4) as has previously been reported for the Δsco null-mutant [7]. Furthermore, the *glxA* and *csIA* mutants have an identical morphology in liquid-grown cultures. Instead of the dense compact pellets seen in WT, the mutants grow with an open mycelium phenotype [4] (Figure 7A). The transcriptomes of the *glxA* and the *csIA* mutant as analysed by RNA-seq revealed increased expression of genes related to osmoprotection, indicating that the mutants suffer from osmotic stress (result not shown), and is in agreement with the observation of Liman et al. [18]. Excitingly, mycelial staining with CFW, which binds to β -(1–4) glycans indicated that the hyphal tips in both the *csIA* and *glxA* null mutants were no longer stained, in contrast with those of the WT strain (Figure 7B). This indicates that in addition to *CsIA*, GlxA is required for glycan synthesis or attachment to hyphal tips.

DISCUSSION

Results from the present study reveal that *S. lividans* GlxA is a membrane-associated cuproenzyme required for the production or localization of the hyphal tip glycan. The spectroscopic properties, tertiary structure and enzymatic profile are all distinctly different

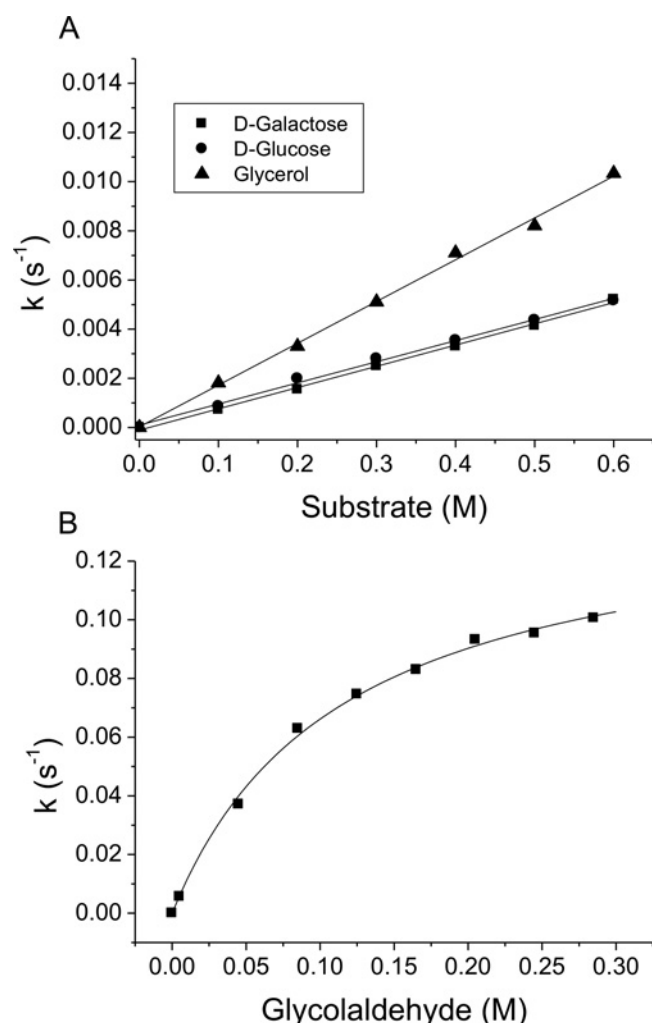


Figure 6 Enzyme activity of *S. lividans* GlxA

(A and B) Plots of turnover rates (k) for GlxA with four different substrates (25°C). In (A), the line of best fit through the data points enables a second-order rate constant ($\text{M}^{-1}\text{s}^{-1}$) to be determined. In (B) the data points have been fitted to the Michaelis–Menten equation to yield a K_m value and turnover (k_{cat}) reported in the main text.

to its fungal counterpart Gox, thus making GlxA a new structural and functional member of the mononuclear Cu oxidase family.

From a spectroscopic perspective a number of features in GlxA stand out. In the Cu(II)-GlxA EPR spectrum, the g_{\perp} component displays a hyperfine interaction of the electron spin with the Cu(II) ligands, giving rise to seven lines with relative intensities 1:4:8:10:8:4:1 yielding a 16 G splitting (Figure 3C, inset). For Cu(II)-Gox, a quintet line structure of relative intensities 1:2:3:2:1 [33,34] and a distance between the lines typical for a N-hyperfine interaction (14–18 G) [39,40] consistent with two histidine ligands is observed. The source of the additional hyperfine splitting in GlxA is presently unclear and requires further investigation, but would suggest that differences in the electronic properties of the Cu(II) site in GlxA and Gox exist. A further anomaly in the spectroscopic properties between GlxA and Gox is in relation to the spectral observation of the Y-C• radical. EPR spectroscopy clearly indicates the presence of this radical (Figure 5) following addition of an oxidant to Cu(II)-GlxA. However, no perturbation of the Cu(II)-GlxA absorbance spectrum is observed as is the case on forming Cu(II)-Y-C• in Gox. Furthermore, the Cu(II)-GlxA optical spectrum does not resemble that of Cu(II)-Gox [11]. The

co-ordination geometry of the Cu(II) site in both GlxA and Gox are identical, but as indicated by EPR could be electronically distinct with second sphere co-ordination contributing. In this respect, the most pertinent difference between GlxA and Gox is in the side chain orientation of Trp²⁸⁸ (Trp²⁹⁰ in Gox). In Gox, Trp²⁹⁰ has been proposed to provide an extended aromatic system with its indole ring stacking over the Y-C bond, stabilizing the radical (Figure 8D) [13,41]. However, in GlxA Trp²⁸⁸ no longer has its indole ring stacking with the Y-C cross-link, but instead has the benzene ring component π - π stacking with the Tyr²⁸⁹ Cu ligand (Figure 8D). In combination with a more insulated (buried) Cu site, the re-orientation of the tryptophan side chain may influence the electronic transitions of the Cu and the radical and result in the markedly different absorbance spectrum for GlxA compared with Gox.

The ability to generate three oxidation states in GlxA suggests an enzymatic function in *S. lividans*. However, none of the substrates tested exhibit activity with GlxA that is comparable to Gox (with the exception of D-glucose). This is exemplified with D-galactose, the canonical monosaccharide substrate for Gox, where under aerobic conditions a k_{cat}/K_m value of $10^{-3} \text{ M}^{-1}\text{s}^{-1}$ for GlxA is determined, which is a million times lower than for Gox [42] and is equivalent to Gox with D-glucose [43]. The most active substrate tested with GlxA, glycolaldehyde, has a K_m value (115 mM) comparable to Gox for D-galactose (70–80 mM), but a k_{cat}/K_m value >4-orders of magnitude lower. For Gox the turnover of glycolaldehyde has been reported to be 75 % of the D-galactose rate [44]. These slow turnover kinetics indicate that GlxA has very different substrate specificity compared with Gox, a likely consequence of gross structural differences.

The distinctness of the GlxA structure can be appreciated from the superposition with Gox in Figure 8(A). In Gox, the domain arrangement and surface flatness of the β -propeller domain (domain 2) contributes to a readily accessible Cu site and substrate-binding pocket (Figures 8B and 8C) [13]. This is not the case in GlxA, due to the positioning of domain 2 and the longer loop structures of the Kelch motifs. The more accessible nature of the Cu site in Gox is further apparent in the presence of an acetate ion (a buffer component of the crystallization solution) co-ordinating the Cu in the X-ray structure. Despite crystals of GlxA also being grown from acetate buffer, no evidence for an acetate ion, in or close, to the Cu site is observed, highlighting the protection afforded to the Cu site in GlxA. Furthermore, a very different structural arrangement of the substrate pocket in GlxA compared with Gox is apparent (Figure 8D). On one side of the Gox pocket, the side chains of residues Arg³³⁰, Gln⁴⁰⁶ and the N ϵ 1 atom of Trp²⁹⁰ are posed to provide H-bonds to the canonical D-galactose substrate, with the opposite side of the pocket formed by the aromatic residues, Phe¹⁹⁴ and Phe⁴⁶⁴, creating an asymmetric polar/apolar substrate pocket. For GlxA the residues in the pocket, with the exception of Trp²⁸⁸, differ and are not spatially conserved (Figure 8D). Furthermore, Trp²⁸⁸ in GlxA, has its indole ring ‘flipped’ relative to Trp²⁹⁰ in Gox, making this orientation less favourable from a distance perspective for a substrate H-bond interaction in the pocket (Figure 8D) [15]. These differences serve to illustrate that despite the Cu co-ordination being similar, the substrate pockets between Gox and GlxA contain no conserved features. Therefore substrates displaying high turnover kinetics with Gox are unlikely to be optimally accommodated or positioned in GlxA enabling similar high turnover rates to be achieved.

The observations from the molecular genetics and CFW staining (Figure 7) indicate that GlxA is directly involved in the production or localization of the hyphal tip glycan. This is consistent with GlxA localization on the hyphal tip [18].

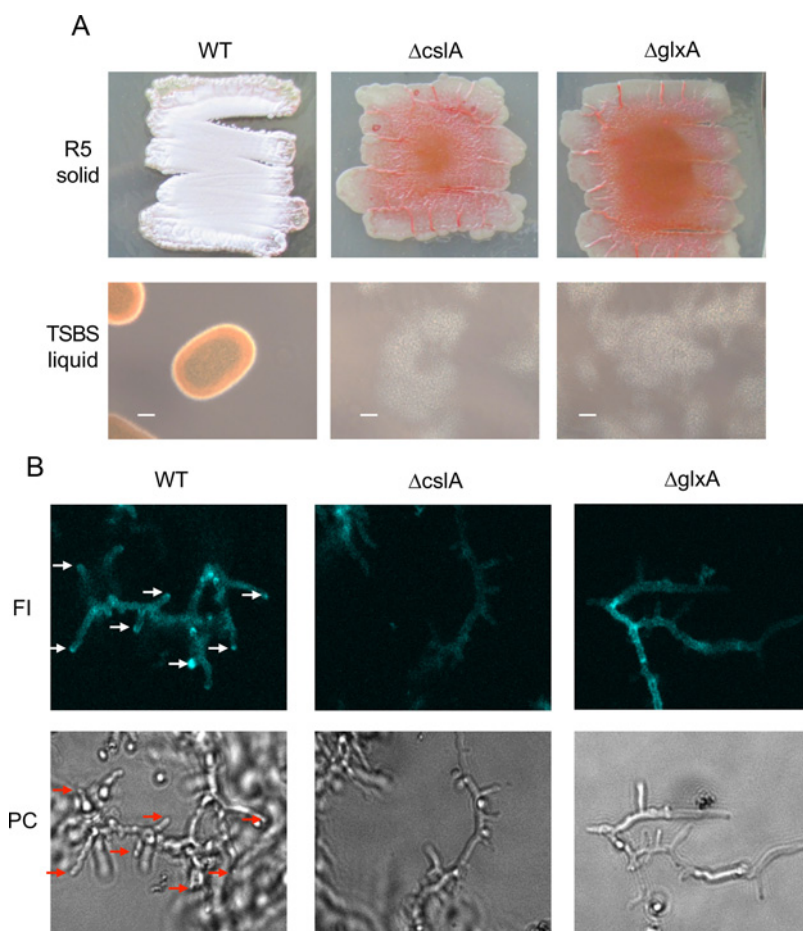


Figure 7 Deletion of *cslA* or *glxA* in *S. lividans* leads to a block in development and abolishes pellet formation and glycan deposition

(A) Growth on solid R5 medium and pellet formation and dispersed growth in TSBS 24 h liquid cultures. Scale-bar (white line) is 100 μ m. (B) CFW staining in 24 h mNMMP standing cultures shown in fluorescence image (FI) and phase contrast brightfield (PC). Arrows indicate tip staining that is present in WT cultures but not in mutants.

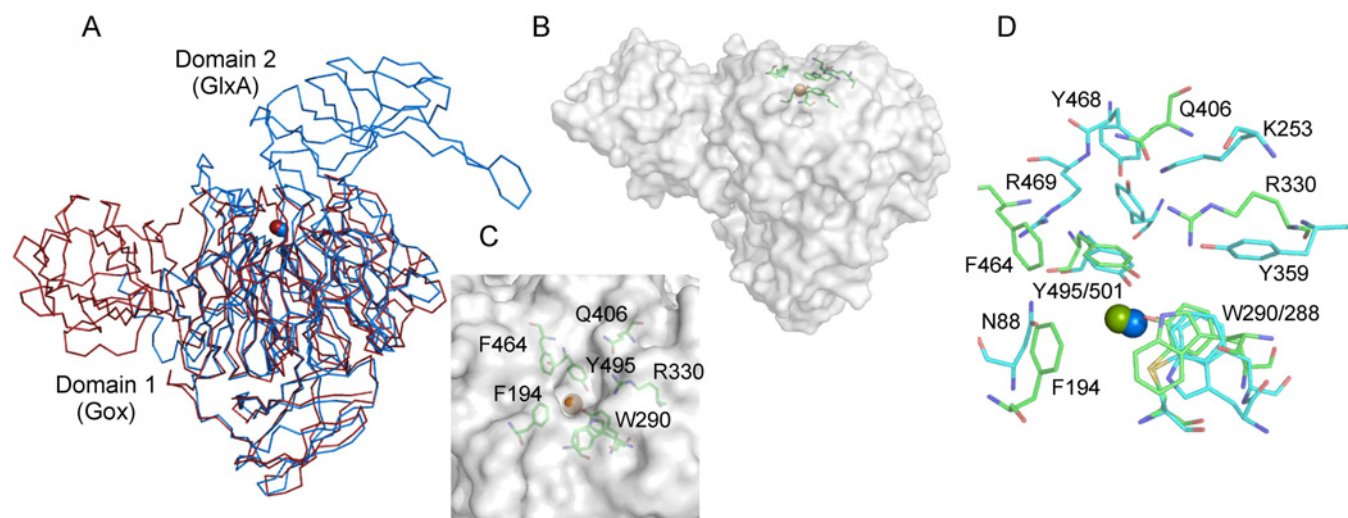


Figure 8 Structural comparison of GlxA with Gox (1gof) [13]

(A) Ribbon representation of a superposition of the α atoms of GlxA (blue) with Gox (red). The Cu ions are shown as spheres. (B) Surface representation of Gox, with location of the Cu ion (brown sphere) and residues in the substrate pocket indicated in sticks. (C) View of the substrate pocket looking down the 7-fold symmetry axis towards domain 3 of Gox. The location of the surface exposed Tyr⁴⁹⁵ Cu ligand and the stacking Trp²⁹⁰ as well as residues forming the substrate pocket are shown in sticks, with the solvent exposed Cu represented as a sphere. (D) Superposition of the substrate pockets in GlxA (blue) and Gox (green).

Synthesis of this glycan also requires CslA, which is a cellulose synthase-like enzyme encoded by the translationally-coupled gene upstream of *glxA*. This glycan accumulates at apical sites during vegetative growth and is absent in the *cslA* mutant [19,20,45] and also in the *glxA* mutant. The molecular identity of this glycan is not yet known, but we propose that GlxA acts to modify it through a two-electron oxidation process. It is tempting to speculate that the orientations of tunnels I and II, both connecting at the Cu site (Figure 4), could be a key structural feature utilized by GlxA in the oxidation of this glycan. A scenario whereby the nascent glycan produced by CslA is fed down into one of these tunnels, orientated in the substrate pocket, oxidized and then released through the other tunnel may be envisaged. From a biotechnology perspective the open mycelium structure of the *glxA* and *cslA* null-mutants in liquid-grown cultures is of interest for improvement in the utilization of *S. lividans* as an enzyme production host. It has been shown that a more fragmented and therefore also more open mycelium growth in *Streptomyces* increases enzyme production several fold [4,46] and that the absence of other glycans result in a distinct morphology in liquid cultures [47].

Finally, the inability of the $\Delta glxA$ phenotype to be rescued by Cu links GlxA to the Cu dependency of *S. lividans* (Supplementary Figure S4) and raises the question of how GlxA acquires Cu. Our previous work has inferred that the Cu chaperone Sco delivers Cu to an *aa₃*-type CcO and also to an unidentified target, which is required to trigger aerial hyphae growth [7,8]. To date no other Sco target except CcO in either eukaryote or prokaryote species has been identified. The possibility therefore arises that the second Sco target in *S. lividans* is GlxA. Further experiments aimed at exploring whether this is the case are planned.

AUTHOR CONTRIBUTION

Jonathan Worrall, Erik Vijgenboom and Dennis Claessen conceived and designed the project. Amanda Chaplin cloned, overexpressed and purified recombinant GlxA, crystallized and solved the X-ray structure and performed all spectroscopic and enzymatic activity measurements. Marloes Petrus, Giulia Mangiameli, Dennis Claessen and Erik Vijgenboom carried out the molecular genetics, GlxA location and glycan detection. Michael Hough assisted with X-ray data collection and monitored the structural work. Dimitri Svistunenko helped interpret the EPR data. Peter Nicholls advised and assisted with enzymatic assays. Jonathan Worrall, Erik Vijgenboom and Dennis Claessen wrote the manuscript with contributions from all authors.

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