Structure of a fungal form of aspartate-semialdehyde dehydrogenase from *Aspergillus fumigatus*

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Aspartate-semialdehyde dehydrogenase (ASADH) functions at a critical junction in the aspartate biosynthetic pathway and represents a validated target for antimicrobial drug design. This enzyme catalyzes the NADPH-dependent reductive dephosphorylation of β -aspartyl phosphate to produce the key intermediate aspartate semialdehyde. The absence of this entire pathway in humans and other mammals will allow the selective targeting of pathogenic microorganisms for antimicrobial development. Here, the X-ray structure of a new form of ASADH from the pathogenic fungal species *Aspergillus fumigatus* has been determined. The overall structure of this enzyme is similar to those of its bacterial orthologs, but there are some critical differences both in biological assembly and in secondary-structural features that can potentially be exploited for the development of species-selective drugs with selective toxicity against infectious fungal organisms.

1. Introduction

Aspartate-semialdehyde dehydrogenase (ASADH; EC 1.2.1.11) catalyzes the reductive dephosphorylation of the substrate β -aspartyl phosphate into aspartate semialdehyde, a key intermediate in the aspartate biosynthetic pathway (Viola *et al.*, 2011). This pathway is responsible for the biosynthesis of both essential amino acids and essential cellular metabolites in most microorganisms. Inhibition of any of the key enzymes involved in this pathway has been shown to be lethal to the microorganism (Kobayashi *et al.*, 2003), making the enzymes present in this pathway attractive targets for antimicrobial drug design (Viola, 2001). Both superficial and invasive fungal infections are among the leading causes of human mortality, especially in immunocompromised patients (Brown *et al.*, 2012). *Aspergillus*, *Candida* and *Cryptococcus* are the major fungal pathogens responsible for human infections (Miao *et al.*, 2016). Significant information has been generated about the structures and the structure-guided inhibitors of a range of bacterial ASADHs (Blanco *et al.*, 2003; Faehnle *et al.*, 2006; Viola *et al.*, 2008; Thangavelu *et al.*, 2015), whereas information regarding the structure and inhibition of the fungal enzyme counterparts are relatively sparse. This is likely to be owing to the significant overlap of metabolic pathways between humans and fungi, so that the identification of specific antifungal drugs has been more limited compared with antibacterial drug development.

Aspergillus fumigatus is a ubiquitous, saprophytic fungus found both in plants and as a human pathogen responsible for causing the destructive lung disease chronic pulmonary aspergillosis (Brown *et al.*, 2012). In addition, this organism is

responsible for causing a wide range of other human ailments depending upon the immune system of the host. Mortality rates from *Aspergillus* infections range from 40% to as high as 90% in high-risk populations, and also depend upon the site of infection, the treatment regimen and the immune system of the infected host (Lin *et al.*, 2001).

2. Materials and methods

2.1. Enzyme production

The AFUA_3G06830 gene (UniProt accession ID Q4WWR8) encoding the full-length *AfASADH* enzyme from *A. fumigatus* was codon-optimized for *Escherichia coli* expression and synthesized by Invitrogen (Life Technologies). The synthesized gene was ligated between the NcoI and XhoI restriction sites of the pET-28a+ plasmid (Novagen, USA) to produce an expression construct with a C-terminal hexahistidine tag (Supplementary Table S1). The integrity of the ligated plasmid was confirmed by restriction digestion and by DNA sequencing. Additional cloning details are summarized in the Supporting Information. The recombinant enzyme was expressed in *E. coli* BL21 (DE3) cells during the growth cycle (A_{600} between 0.6 and 0.8) at 300 K by induction with 100 μ M IPTG added to LB medium containing 30 μ g ml⁻¹ kanamycin. The temperature was reduced to 289 K and the cells were grown for an additional 16 h; they were then harvested by centrifugation at 17 000g for 15 min and stored at 193 K.

The frozen cell pellet was suspended in 50 mM potassium phosphate pH 7.2, 200 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol (buffer A) and homogenized using a hand homogenizer. Lysozyme (10 μ M, Sigma) and DNase I (100 μ M, Sigma) were added to the lysis buffer and incubated on ice for 1 h. Cells were lysed by ultrasonication for a total of 8 min at 30 s intervals with a Fisher model 550 sonic dismembrator. Insoluble protein and other cellular debris was removed by centrifugation at 12 000g for 40 min and the supernatant was filtered through a 28 mm 0.45 μ m membrane syringe filter. The clarified soluble lysate was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated with buffer A. The column containing the bound protein was washed with ten column volumes of buffer A and the target enzyme was eluted using a 20–400 mM imidazole gradient. The protein was further purified using an anion-exchange Source 30Q column (GE Healthcare) with a 50–600 mM NaCl gradient. Protein purity was analyzed by SDS-PAGE, and the active and pure fractions showing a single band on Coomassie-stained polyacrylamide gel were pooled and dialyzed overnight against a buffer consisting of 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.0, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT. Protein concentration was determined from the absorbance at 280 nm using a Nanodrop 2000 spectrophotometer, and the protein was subsequently concentrated to 40–50 mg ml⁻¹ by ultracentrifugation using a 10 kDa molecular-weight cutoff spin concentrator (Millipore). The enzyme was stored at 253 K until use.

2.2. Protein characterization

A standard in-gel digestion protocol (Shevchenko *et al.*, 2007) was followed for identification of the protein sample. The expected molecular-weight band was excised from SDS gel, destained, reduced, alkylated and proteolytically cleaved with TPCK-treated trypsin (Invitrogen). The masses of the trypsin-cleaved peptides were identified by MALDI-TOF mass spectrometry using a Bruker ultrafleXtreme MALDI-TOF/TOF spectrometer. A Mascot (Perkins *et al.*, 1999) database search was performed to confirm the enzyme identity from the sequences of the cleaved peptides. The enzyme identity was confirmed by MALDI-MS with 59% sequence coverage (Supplementary Fig. S1) and an anti-His Western blot protocol.

2.3. Activity assay and sequence alignment

The enzymatic activity assay of *AfASADH* was performed in similar manner as described previously (Dahal & Viola, 2015). The protein sequences of each ASADH were obtained from the UniProt database (<http://www.uniprot.org>), and sequence alignment and secondary-structure alignment were performed using *Clustal Omega* (Sievers *et al.*, 2011) and *ESPrpt* 3.0 (Robert & Gouet, 2014), respectively.

2.4. Crystallization

Preliminary screening for crystallization conditions for *AfASADH* (14 mg ml⁻¹) was carried out in 48-well VDX plates (Hampton Research) using the Index screen (Hampton Research), as well as in-house customized crystallization kits. The hanging drops in the initial crystallization screening consisted of 1 μ l protein solution plus 1 μ l reservoir solution, with each reservoir solution consisting of 200 μ l of the unique reagents from the Index screen. Crystals were obtained after 48 h from condition 3 of Index, which consisted of 2 M ammonium sulfate, 100 mM bis-tris pH 6.5. This initial hit was further optimized by varying the concentration of ammonium sulfate (from 1.5 to 2.5 M), the pH (Tris-HCl pH 5.5, HEPES pH 7.5, Tris-HCl pH 8.5) and buffer concentration (50–100 mM). Well formed, hexagonal crystals (~40 μ m; Supplementary Fig. S2) grew from 1.9 M ammonium sulfate, 100 mM bis-tris pH 6.5 at 293 K. The crystals were either soaked or co-crystallized with 5 mM NADP (cofactor) and with several moderate inhibitors that were identified from fragment library screening.

2.5. Data collection and processing

The crystals of *AfASADH* (apo form and ligand-soaked form) were cryoprotected with reservoir solution containing 20% (v/v) ethylene glycol. The soaking experiments performed in this cryosolution contained 20 mM final concentrations of the cofactor and each inhibitor, with soaking for 1 min followed by flash-cooling in liquid nitrogen. Several crystals diffracted to between 2.8 and 3.2 Å resolution on our in-house Rigaku FR-E diffractometer equipped with an R-Axis IV image-plate detector. Six complete data sets for apo-form,

Table 1

Data collection, processing and refinement.

Values in parentheses are for the outer shell.

Data collection and processing	
Diffraction source	19-BM, APS
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	MAR 335 CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	0.5
Total rotation range (°)	110
Exposure time per image (s)	1
Space group	$P3_121$
a, b, c (Å)	94.50, 94.50, 202.14
α, β, γ (°)	90, 90, 120
Mosaicity (°)	0.19
Resolution range (Å)	75.86–2.39
Total No. of reflections	264962
No. of unique reflections	42496
Completeness (%)	100 (99.9)
Multiplicity	6.2 (5.0)
$\langle I/\sigma(I) \rangle$	9.2 (3.2)
R_{merge}	0.060 (0.224)
Overall B factor from Wilson plot (Å ²)	34.1
Refinement statistics	
Completeness (%)	100.0
No. of reflections, working set	40287 (2906)
No. of reflections, test set	2141 (184)
Final R_{cryst}	0.201 (0.240)
Final R_{free}	0.250 (0.284)
No. of non-H atoms	
Total	5598
Protein	5466
Ligand	25
Solvent	107
R.m.s. deviations	
Bonds (Å)	0.018
Angles (°)	1.92
Average B factors (Å ²)	
Overall	39.0
Protein	39.4
Ligand	47.6
Solvent	35.3
Ramachandran plot	
Most favored (%)	95.0
Additionally allowed (%)	3.9
Outliers (%)	1.1

cofactor-soaked and four moderate inhibitor-soaked crystals were subsequently collected on the SBC 19-BM beamline at the Advanced Photon Source (APS), Argonne National Laboratory using a MAR 335 detector. Each data set consisting of 220 frames was collected with a crystal-to-detector distance of 250 mm, an oscillation width of 0.5° and an exposure time of 1 s per frame (Table 1). The diffraction data were integrated and scaled using *iMosflm* (Battye *et al.*, 2011) and *AIMLESS* (Evans & Murshudov, 2013) from the CCP4 suite (Winn *et al.*, 2011).

2.6. Structure solution and refinement

The apo form of the *AfASADH* structure was solved by molecular replacement using *Phaser* with our published structure of *Candida albicans* ASADH (*CaASADH*; PDB entry 3hsk; Arachea *et al.*, 2010) as the search model. The search model gave a solution with a TFZ score of 17.1 using a monomer of *CaASADH*, an enzyme that shares 55%

sequence similarity with *AfASADH*. The initial R_{work} and R_{free} were 0.34 and 0.39, respectively, after 25 cycles of rigid-body refinement to a medium resolution of 3.5 Å, followed by restrained-body refinement at 2.38 Å using *REFMAC5* (Murshudov *et al.*, 2011). The model was rebuilt using *Buccaneer*, which automatically built most of the residues. The final model was built using numerous cycles of restrained refinement in *REFMAC* and manual rebuilding in *Coot*. The final R_{work} and R_{free} were 0.20 and 0.25, respectively, and the stereochemical quality was checked using *PROCHECK* (Laskowski *et al.*, 1993). *PyMOL* (DeLano, 2002) was used to visualize the final structure and *PDBEPIA* (Krissinel & Henrick, 2007) was used for surface calculations. The final model was deposited in the Protein Data Bank (<http://www.rcsb.org>; Berman *et al.*, 2000) as PDB entry 5jw6. The phasing and final refinement statistics are shown in Table 1.

3. Results and discussion

3.1. Overall structure

The crystal structure of *AfASADH* was determined at 2.38 Å resolution by molecular replacement using *CaASADH* (Arachea *et al.*, 2010) as the search model. The entire sequence (363 amino acids, full length) was fitted into electron density, except for amino acids 1–3 and 189–190 from each monomer, which lacked interpretable electron density. *AfASADH* displays a crystallographic dimer (Fig. 1) in its crystalline state similar to *CaASADH* (Arachea *et al.*, 2010). However, this enzyme is functionally active as a tetramer in solution, consistent with the other fungal ASADHs (Dahal & Viola, 2015; Li *et al.*, 2016). Each asymmetric unit contains two monomers, and a comparison of the monomer structures yields an r.m.s.d. value of 0.254 Å, indicating that the monomers are nearly identical. Similar to other dehydrogenases, *AfASADH* belongs to the Rossmann-fold superfamily of pyridine-linked dehydrogenases, and shares similar overall

Figure 1

Overall structure of aspartate-semialdehyde dehydrogenase from *A. fumigatus*. The monomers of this crystallographic dimer are shown in green and blue, with the bound sulfate molecules (numbered 1–5) in yellow and pink. The structure of *AfASADH* is superimposed on the *CaASADH* structure (in gray). The overall structure is similar, with only minor differences in the orientation of some secondary-structural elements.

structural features with other ASADHs. Each monomer is comprised of an N-terminal coenzyme-binding domain (residues 1–152 and 346–359) and a C-terminal catalytic and dimerization domain (residues 153–345).

3.2. Absence of cofactor and inhibitor binding

Despite crystallization in the presence of excess concentrations of the cofactor NADP and four different moderate inhibitors, no electron density was found corresponding to either NADP or the inhibitors in any of these solved structures. Instead, five sulfate molecules were found bound near the substrate-binding site and also near the cofactor-binding sites (Fig. 1). Because the crystallization solution contains nearly 2 M ammonium sulfate, this high concentration of sulfate anion is likely to be competing with and preventing either the cofactor or the inhibitors from binding at their respective sites. Attempts to lower this sulfate competition by decreasing the sulfate concentration yielded only clear crystallization drops at sulfate concentrations lower than 1.8 M. Switching to ammonium salts with different anions such as phosphate, chloride or fumarate did not support crystal growth, suggesting that the presence of sulfate helps to stabilize this crystal form. Microseeding of apo *Af*ASADH crystals into precipitation solutions containing different molecular-weight PEGs instead of ammonium sulfate also did not produce any crystal growth.

3.3. Secondary-structural arrangement

The N-terminal domain of *Af*ASADH starts with a β -strand followed by an α -helix and two short β -strands (β_2 and β_3) completing the β - α - β motif of the Rossmann fold (β_1 - α_1 - β_2 - β_3 ; amino acids 7–39) that supports the NADPH-binding site

in this dehydrogenase family (Fig. 2). This highly conserved motif is connected to a long surface loop (amino acids 40–81) consisting of two 3_{10} -helices (η_1 and η_2) and two short α -helices (α_2 and α_3) that are primarily involved in cofactor binding. This surface loop is connected to a second β - α - β motif (β_4 - α_4 - β_5) region (amino acids 82–108) which is also highly conserved in the ASADH family. The third, less conserved β - α - β motif is replaced in *Af*ASADH by a long α -helix (α_5) and a short β -strand (β_6). This region is connected by a long conserved helix (α_6 ; amino acids 157–170), followed by the first β -strand (β_7) of the dimerization domain. The helical subdomain, consisting of a helix–turn–helix, that is found in many bacterial ASADHs (Faehnle *et al.*, 2006) is absent in *Af*ASADH (Fig. 3) and is replaced by two residues (amino acids 189–190) with no significant electron density, consistent with the other fungal ASADH structures. The catalytic and dimerization domains of *Af*ASADH are more similar to the other fungal ASADHs than to the bacterial ASADHs, with mixed groups of parallel β -strands assembled with flanking α -helices on both sides. There is a slight variation in the length of each α -helix and β -strand in each secondary structure compared with other fungal ASADHs, but the overall structural arrangement is similar among all fungal ASADHs.

3.4. Comparison with related structures

Only three prior structures have been determined of ASADHs from pathogenic fungal species: *Cryptococcus neoformans* (*Cn*ASADH), *Candida albicans* (*Ca*ASADH) and *Trichophyton rubrum* (*Tr*ASADH). Two of these fungal enzyme forms, *Ca*ASADH and *Tr*ASADH, have been solved with the cofactor NADP bound. In an attempt to determine the structures of different inhibitors bound to *Af*ASADH, we have collected and processed several data sets from crystals that have been either soaked or co-crystallized with the cofactor NADP and with several moderate inhibitors. Unfortunately, all of these data sets showed electron density corresponding to sulfate ions bound at both the active site and the cofactor-binding site, but no electron density for any of these ligands. Superposition of the *Af*ASADH dimer with the dimer of *Cn*ASADH shows no significant difference in the overall structure, with only slight differences in the orientation and length of some α -helices and β -sheets (Fig. 1). The segment containing amino acids 189–190 with missing electron density was also missing in the other fungal ASADH structures, with the exception of a modelled loop in *Tr*ASADH, suggesting that this loop is highly mobile and quite flexible. The conformation of this loop plays an important role in NADP binding. The formation of a dimer of dimers in these fungal ASADHs helps to stabilize this loop in a productive conformation to assist in binding the cofactor (Li *et al.*, 2016). The r.m.s.d. value between the aligned *Af*ASADH structure and the other fungal ASADH structures was found to be 1.79 Å for *Cn*ASADH and 1.64 Å for *Ca*ASADH, based on 716 aligned C α atoms. However, this difference is only 0.62 Å for the alignment with *Tr*ASADH, showing that the

Figure 2
*Af*ASADH monomer showing the secondary-structural arrangement. The N-terminal domain β -sheet regions are shown in purple and the α -helices are shown in cyan. For the C-terminal domain the β -sheet regions are shown in yellow and the α -helices are shown in red, with numbering in increasing order starting from the N-terminus.

Figure 3

Multiple sequence alignment of ASADH enzymes from the fungi *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Trichophyton rubrum*, the bacteria *Streptococcus pneumoniae* and *Vibrio cholerae* and the archaeon *Methanocaldococcus jannaschii*. Fully conserved residues are shown with a red background, conserved residues are shown with red letters and active-site residues are marked with red and blue stars. The helical subdomain of the bacterial ASADHs is outlined with a blue rectangle.

*Af*ASADH structure is more closely related to this enzyme form.

While the sequence similarity between *Af*ASADH and the other fungal ASADHs is about 55%, and decreases to about 20% for the bacterial ASADHs, the critical catalytic residues are conserved throughout the entire ASADH family (Fig. 3). Despite this conservation, the kinetic parameters are found to vary significantly among the members of the bacterial and fungal ASADHs. The k_{cat} and K_{m} values for the native substrate ASA were found to be 6.1 s^{-1} and 0.29 mM , respectively, for *Af*ASADH. This k_{cat} value is 50-fold higher than that of *Ca*ASADH, but is similar to the values measured for *Cn*ASADH (10.2 s^{-1} ; Dahal & Viola, 2015) and for *Tr*ASADH (16.5 s^{-1} ; Li *et al.*, 2016). In contrast to these similarities to the other fungal ASADHs, the k_{cat} for the bacterial ASADHs varies from 610 s^{-1} for the *E. coli* enzyme, 100-fold higher than for *Af*ASADH, to as low as 58 s^{-1} for one of the ASADH forms from *Vibrio cholerae* (Moore *et al.*, 2002). These large differences in catalytic efficiency are likely to be a consequence of the changes in quaternary structure between the fungal and bacterial enzyme forms, and also differences in the nature of the subunit–subunit interactions. The different environmental and evolutionary requirements for the survival of this specific pathogen are also likely to play a role in these kinetic differences.

3.5. Active-site comparison

The active sites of ASADHs are highly conserved, with an identical constellation of functionally essential amino acids found among the different members of this enzyme family. Superposition of the active site of *Af*ASADH with that of *Cn*ASADH shows that these conserved catalytic residues are each located in the same general positions, but with some changes in the intra-residue distances (Fig. 4). The S atom of the active-site cysteine nucleophile (Cys154) is found in a single conformation, as opposed to the multiple conformations

seen in *Ca*ASADH (Arachea *et al.*, 2010). This cysteine is positioned at the end of a conserved helix in *Af*ASADH, while in the active site of *Tr*ASADH the cysteine is located in a flexible loop before the helix. The distance between the S atom of Cys154 and the N^{δ} atom of His251 (acid/base catalyst) is only $3.6 \text{ \AA}$ in *Af*ASADH, compared with $7.0 \text{ \AA}$ in the *Cn*ASADH structure (Fig. 4). Also, the distance between this S atom and the O^{ϵ} atom of Glu208 (a substrate-binding residue) is $4.0 \text{ \AA}$ in *Af*ASADH and $7.7 \text{ \AA}$ in the *Cn*ASADH structure. The distances between each of the other fully conserved residues responsible for substrate binding (Lys211, Arg114 and Arg244) are also several angstroms closer in *Af*ASADH compared with the *Cn*ASADH structure. These closer distances between the catalytic residues show that the active site in *Af*ASADH is pre-positioned to make more favorable interactions upon the initial encounter with the substrate at the active site. An active-site isoleucine (Ile339) in *Cn*ASADH is replaced by a serine in *Af*ASADH, but this polar residue does not make any new hydrogen-bonding interactions that would contribute to the more compact active-site structure in this apoenzyme.

Several amino-acid residues surrounding the active site are altered among different fungal ASADHs. Ala155 in *Af*ASADH is replaced by Ser157 in *Cn*ASADH and *Ca*ASADH, Ile344 in *Af*ASADH is replaced by a leucine in *Cn*ASADH and *Tr*ASADH. Similarly, Thr342 is replaced by Val343, Leu212 by Ile214 and His340 by Asp341 in *Cn*ASADH. These minor changes in the residues around the active site may contribute to the small differences in catalytic properties among these fungal ASADHs. There are also some minor differences in active-site structures between *Af*ASADH and both *Ca*ASADH and *Tr*ASADH, but the overall

Figure 4
Superpositioning of the residues in the active sites of *Af*ASADH (pink) and *Cn*ASADH (green). The critical catalytic residues are further apart in the *Cn*ASADH structure, while they are several angstroms closer in the *Af*ASADH structure.

Figure 5
Representative electron density and modelling of amino-acid side chains and sulfate ion bound near the active site.

organization of each active site is found to be quite similar among these fungal ASADHs.

3.6. Binding of an exogenous ligand

The apo form of *Af*ASADH was crystallized in the presence of 1.9 *M* ammonium sulfate as the precipitant. After final refinement of the structure, the difference Fourier maps at the 3 σ level showed some additional unassigned density near the active site of each monomer as well as in the cofactor-binding site of chain *A*. Initial speculation that the unassigned density corresponds to sulfate molecules proved to be accurate, with an excellent fit into the electron density. These anions are most likely to be found at these sites because of the high concentration of sulfate in the crystallization precipitant. The sulfate ion fits nicely in the electron density, with an average *B* factor of 26.5 Å² at the active site of each monomer and an

Table 2

Buried surface area and energetics of ASADH subunit dissociation.

Fungal ASADH	PDB code	Buried surface area (Å ²)	Free energy of dissociation (ΔG_{diss}) (kcal mol ⁻¹)	Salt bridges	Hydrogen bonds	Nonbonding contacts
<i>Af</i> ASADH	5jw6	12660	52.7	6	31	275
<i>Tr</i> ASADH	4zhs	12360	17.9	6	21	212
<i>Cn</i> ASADH	5cef	9940	13.4	6	28	202
<i>Ca</i> ASADH	3hsk	11940	14.8	0	31	261

occupancy of one (Fig. 5). Each bound anion makes several hydrogen-bonding interactions with active-site residues and with residues in the cofactor-binding site. The O₃ atom of sulfate 1 in chain *A* and sulfate 2 in chain *B* each make hydrogen-bonding interactions with the S^γ atom and N atom of the active-site cysteine (Cys154) in each monomer, with

Figure 6
LIGPLOT diagrams of sulfate molecules bound at the substrate-binding site (*a*), cofactor-binding sites (*b*, *c*) and on the surface (*d*) of *Af*ASADH. Each sulfate molecule has both hydrogen-bonding and hydrophobic interactions with active-site residues in each monomer. Direct hydrogen bonds are shown as green dotted lines with their appropriate distances, and hydrophobic interactions are displayed as labelled arcs with each sulfate molecule labelled in increasing order.

distances of 3.25 and 2.88 Å, respectively (Fig. 6a). The other O atoms of this bound sulfate (O₂ and O₄) make hydrogen-bonding interactions with additional residues around the active site, including Asn153, Arg114 and Lys211. The sulfate molecule bound near the cofactor-binding site (sulfate 3) hydrogen-bonds to residues Thr15, Ala39, Ser40 and Ser43 (Fig. 6b), while sulfate 4 makes similar interactions with Ser17, Ala186 and Ala187 (Fig. 6c). Another sulfate molecule is found bound on the surface, interacting with Ser61 and Arg63 (Fig. 6d). All of these sulfate molecules are bound to both subunits, except for sulfate 2 which is bound only to chain B. The *B* factors for the sulfates bound at the active site were found to be 25.81 and 27.3 Å² for chains A and B, respectively, and the *B* factor for the other sulfate molecules are 52.8 and 64.5 Å². The surface-bound sulfate also has a higher *B* factor of 67.4 Å², which is not unusual for non-optimized ligand binding through only two hydrogen bonds at a surface site. Superposition of the bacterial *Streptococcus pneumoniae* ASADH (*Sp*ASADH) structure with this *Af*ASADH structure shows that the acetate moiety from the crystallization buffer that is bound near the active site of this bacterial ASADH (Pavlovsky *et al.*, 2014) is in the same location and makes similar interactions to those of the sulfate bound at the active site of *Af*ASADH. This sulfate molecule bound to *Af*ASADH (Fig. 5a) superimposes perfectly with the sulfate bound at the active site in the *Tr*ASADH structure (Li *et al.*, 2016).

3.7. Differences in oligomeric arrangement

There are two monomers in the asymmetric unit in this crystal form of *Af*ASADH, similar to what was observed in *Ca*ASADH. By contrast, in the other fungal enzymes *Cn*ASADH has four monomers and *Tr*ASADH has six monomers in the asymmetric unit in their corresponding crystal forms. However, despite these crystallographic differences the oligomeric state of *Af*ASADH has been found to be a tetramer in solution, as confirmed by native gel electrophoresis (Li *et al.*, 2016). The change in oligomeric structure is required to produce an active enzyme in these fungal species; mutations that cause dissociation into dimers results in a loss of activity (Li *et al.*, 2016). Crystal-packing interactions were analysed using *PDBEPIA* (Krissinel & Henrick, 2007), the results of which also concluded that *Af*ASADH is likely to organize into a dimer of dimers. The larger buried surface area and higher free energy of dissociation each show the strength of subunit interaction in this *Af*ASADH structure. For the subunit interaction surface in *Af*ASADH, 35 residues from monomer A interact directly with 33 residues in monomer B, forming 31 hydrogen-bonding interactions, along with a total of 275 nonbonded contacts. There are also six pairs of salt bridges, with the participation of Glu177–Arg334, Asp199–Arg309 and Glu209–Arg321 from chain A to chain B and the corresponding residues from chain B to chain A within each dimer. The higher free energy of subunit dissociation in *Af*ASADH results from a larger buried surface area and a greater number of productive contacts across the subunit

interface. This fungal form of ASADH has the largest number of hydrogen-bonding and nonbonding contacts among the structurally characterized fungal enzyme family (Table 2). The *C. albicans* enzyme has nearly the same number of such interactions, but the absence of any electrostatic bonds between the subunits leads to a lower energy of dissociation. This ensemble of interactions across the dimer interface contributes to the stabilization of the *Af*ASADH structure.

4. Conclusions

We present the first structure of ASADH from the pathogenic fungus *A. fumigatus*, along with a structural and functional comparison between fungal and bacterial ASADH orthologs. The structure of *Af*ASADH was found to be more similar to those of the other fungal orthologs, but with some critical differences in the orientation of active-site functional groups and in the subunit interface region. Structural analysis suggests that the differences observed in biochemical activity among the ASADH enzyme family can be correlated with changes in oligomeric assembly, differences in the strength of subunit interactions and variations in the interatomic distances between the catalytic residues in the active sites of different fungal ASADHs. This expanded structural information on fungal ASADHs will be useful in structure-guided drug design against human fungal pathogens.

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References

- Arachea, B. T., Liu, X., Pavlovsky, A. G. & Viola, R. E. (2010). *Acta Cryst.* **D66**, 205–212.
- Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* **D67**, 271–281.
- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
- Blanco, J., Moore, R. A. & Viola, R. E. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 12613–12617.
- Brown, G. D., Denning, D. W., Gow, N. A. R., Levitz, S. M., Netea, M. G. & White, T. C. (2012). *Sci. Transl. Med.* **4**, 165rv13.
- Dahal, G. & Viola, R. E. (2015). *Acta Cryst.* **F71**, 1365–1371.
- DeLano, W. L. (2002). *PyMOL*. <http://www.pymol.org>.
- Evans, P. R. & Murshudov, G. N. (2013). *Acta Cryst.* **D69**, 1204–1214.
- Faehnle, C. R., Le Coq, J., Liu, X. & Viola, R. E. (2006). *J. Biol. Chem.* **281**, 31031–31040.
- Kobayashi, K. *et al.* (2003). *Proc. Natl Acad. Sci. USA*, **100**, 4678–4683.
- Krissinel, E. & Henrick, K. (2007). *J. Mol. Biol.* **372**, 774–797.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
- Li, Q., Mu, Z., Zhao, R., Dahal, G., Viola, R. E., Liu, T., Jin, Q. & Cui, S. (2016). *Sci. Rep.* **6**, 21067.
- Lin, S.-J., Schranz, J. & Teutsch, S. M. (2001). *Clin. Infect. Dis.* **32**, 358–366.

- Miao, Y., Tenor, J. L., Toffaletti, D. L., Washington, E. J., Liu, J., Shadrick, W. R., Schumacher, M. A., Lee, R. E., Perfect, J. R. & Brennan, R. G. (2016). *Proc. Natl Acad. Sci. USA*, **113**, 7148–7153.
- Moore, R. A., Bocik, W. E. & Viola, R. E. (2002). *Protein Expr. Purif.* **25**, 189–194.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Pavlovsky, A. G., Thangavelu, B., Bhansali, P. & Viola, R. E. (2014). *Acta Cryst.* **D70**, 3244–3252.
- Perkins, D. N., Pappin, D. J. C., Creasy, D. M. & Cottrell, J. S. (1999). *Electrophoresis*, **20**, 3551–3567.
- Robert, X. & Gouet, P. (2014). *Nucleic Acids Res.* **42**, W320–W324.
- Shevchenko, A., Tomas, H., Havliš, J., Olsen, J. V. & Mann, M. (2007). *Nature Protoc.* **1**, 2856–2860.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D. & Higgins, D. G. (2011). *Mol. Syst. Biol.* **7**, 539.
- Thangavelu, B., Bhansali, P. & Viola, R. E. (2015). *Bioorg. Med. Chem.* **23**, 6622–6631.
- Viola, R. E. (2001). *Acc. Chem. Res.* **34**, 339–349.
- Viola, R. E., Faehnle, C. R., Blanco, J., Moore, R. A., Liu, X., Arachea, B. T. & Pavlovsky, A. G. (2011). *J. Amino Acids*, **2011**, 1–11.
- Viola, R. E., Liu, X., Ohren, J. F. & Faehnle, C. R. (2008). *Acta Cryst.* **D64**, 321–330.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.