High-resolution crystal structure of *Streptococcus agalactiae* glyceraldehyde-3-phosphate dehydrogenase

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Received 17 October 2017

Accepted 5 March 2018

Edited by M. G. Joyce, Walter Reed Army Institute of Research, USA

Keywords: glyceraldehyde-3-phosphate dehydrogenase; asymmetric tetramer; NAD; phosphate-binding sites; *Streptococcus agalactiae*; group B streptococcus.

PDB reference: glyceraldehyde-3-phosphate dehydrogenase, 5y37

Supporting information: this article has supporting information at journals.iucr.org/f

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional enzyme that plays critical roles in bacterial pathogenesis in some pathogenic bacteria. In this study, the crystal structure of group B streptococcus GAPDH was determined at 1.36 Å resolution. The structure contained an asymmetric mixed holo tetramer, with two NAD ligands bound to two protomers. Further structural analysis identified interesting phosphate ion-binding sites, which shed light on its catalytic mechanism.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ~37 kDa) is the sixth enzyme in the glycolytic pathway, in which it converts D-glyceraldehyde 3-phosphate (D-G3P) to 1,3-bisphosphoglycerate and consumes inorganic phosphate using NAD as a coenzyme. The reaction consists of two steps. The first step catalyzed by GAPDH from *Bacillus stearothermophilus* (Didierjean *et al.*, 2003) is an oxidoreduction reaction, which leads to the formation of a thioacyl intermediate and NADH. The catalytic cysteine (Cys149) makes a nucleophilic attack on the aldehydic group of D-G3P, followed by a hydride transfer, assisted by His176, from the thiohemiacetal intermediate to NAD. The second step is a phosphorylation reaction, which consists of a nucleophilic attack of inorganic phosphate on the thioacyl intermediate, leading to formation of the product 1,3-bisphosphoglycerate (Segal & Boyer, 1953; Trentham, 1971; Moras *et al.*, 1975; Michels *et al.*, 1996). According to most studies (Moras *et al.*, 1975; Skarżński *et al.*, 1987; Moniot *et al.*, 2008; Chaikuad *et al.*, 2011; Danshina *et al.*, 2016), it can be concluded that there are three anion-binding sites in GAPDHs from various species, designated P_s, classical P_i and new P_i, for the binding of the C3 phosphate group of D-G3P or the inorganic phosphate ion. The P_s site has been found to occupy an identical location in all solved crystal structures of GAPDHs. The classical P_i site was found, for example, in the holoenzyme from *B. stearothermophilus* (Skarżński *et al.*, 1987), while the new P_i site was observed, for example, in the holostructure from *Leishmania mexicana* (Kim *et al.*, 1995), approximately 2.9 Å away from the classical P_i site. However, the positions of the P_s site and the P_i site in *Streptococcus agalactiae* (also known as group B streptococcus; GBS) GAPDH still remain unclear because of the lack of a high-resolution structure.

GBS is a Gram-positive coccus surrounded by a bacterial capsule composed of polysaccharides, which tend to form chains. There are ten serotypes in the GBS family defined by

the immunological reactivities of their polysaccharide capsules (Rýc *et al.*, 1988; Ferrieri & Flores, 1997; Ferrieri *et al.*, 2004). As a commensal bacterium that forms part of the human microbiota colonizing the gastrointestinal and genitourinary tracts, GBS is generally harmless to humans. However, under certain circumstances, such as pregnancy, it may become a fatal pathogen, causing serious illness in the mother and neonatal pneumonia, septicaemia and meningitis with significant mortality rates in premature infants (Phares *et al.*, 2008). Maternal vaccination has been proposed to prevent invasive GBS not only in neonates but also in mothers (Doran & Nizet, 2004; Johri *et al.*, 2006; Larsson *et al.*, 2006; Pannaraj *et al.*, 2008). An ideal universal human GBS vaccine should be directed against a structurally conserved antigen which is essential for GBS virulence and/or growth. In bacteria, GAPDH was the first identified 'moonlighting enzyme': a protein that performs more than one function (Jeffery, 2003). Despite lacking identifiable secretion signals, GAPDH has been detected on the surface of several prokaryotic and eukaryotic organisms, where it exhibits nonglycolytic functions, including adhesion, facilitating colonization and invasion of host tissues, suggesting a role in pathogenesis (Pancholi & Chhatwal, 2003; Egea *et al.*, 2007). Therefore, GAPDH from GBS constitutes an attractive target for the development of a human vaccine (Madureira *et al.*, 2011). Madureira and coworkers found that GAPDH from GBS is an extracellular virulence factor which could induce the early production of the immunosuppressive cytokine IL-10 by the host upon bacterial infection (Madureira *et al.*, 2011). They also showed that neonatal mice from mothers immunized with recombinant GAPDH were protected against infection with GBS strains by the production of neutralizing antibodies in the mothers which are sufficient to decrease IL-10 production and induce neutrophil recruitment into infected tissues in newborn mice (Madureira *et al.*, 2011). Alves and coworkers demonstrated that the GAPDH-based vaccine was able to protect susceptible and diabetic adult mice against GBS infection (Alves *et al.*, 2015). Overall, GAPDH is an important enzyme for GBS pathogenesis.

Here, we report a high-resolution (1.36 Å) crystal structure of GAPDH from GBS.

2. Materials and methods

2.1. Cloning, expression and purification

S. agalactiae 2603V/R glyceraldehyde-3-phosphate dehydrogenase (GBS GAPDH; NCBI accession No. NP_688758.1) was cloned into a pET-30a-derived vector with a cleavable N-terminal maltose-binding protein (MBP) tag. A linker containing a cleavage site for TEV protease was present between GAPDH and MBP. Transformed *Escherichia coli* BL21 (DE3) CodonPlus RIPL cells (Stratagene, Santa Clara, California, USA) were grown at 37°C and induced at 18°C with 0.2 mM IPTG for 4 h. The cells were lysed by sonication in buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl) plus 5 mM imidazole and protease inhibitors (Roche Applied

Table 1

Summary of X-ray diffraction data-collection and structure-refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	<i>P</i> 2 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	144.30, 156.74, 67.82
α , β , γ (°)	90, 90, 90
Wavelength (Å)	1.000
Wilson <i>B</i> factor (Å ²)	14.28
Resolution (Å)	50.00–1.36 (1.44–1.36)
No. of reflections (total/unique)	2227960/326253
Completeness (%)	99.3 (96.2)
Average multiplicity	6.83 (6.10)
$\langle I/\sigma(I) \rangle$	15.87 (2.14)
<i>R</i> _{meas} [†] (%)	7.6 (105.0)
CC _{1/2} (%)	99.9 (68.3)
Refinement	
Resolution (Å)	50–1.36
No. of atoms	
Protein	10217
Ligand	143
Solvent	1623
<i>B</i> factor (Å ²)	
Protein atoms	18.8
Ligand atoms	24.12
Solvent	29.99
R.m.s.d., bond lengths (Å)	0.006
R.m.s.d., bond angles (°)	0.87
<i>R</i> _{work} [‡] (%)	16.64
<i>R</i> _{free} [§] (%)	18.50
Ramachandran plot¶	
Favoured (%)	96.6
Disallowed (%)	0
PDB code	5y37

[†] $R_{\text{meas}} = \sum_{hkl} \{ [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl) \}$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the *i*th and the mean measurements of the intensity of reflection *hkl*, respectively. [‡] $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. No $\langle I/\sigma(I) \rangle$ cutoff was applied. [§] *R*_{free} is the *R* value obtained for a test set of reflections consisting of a randomly selected 10% of the data set that was excluded from refinement. ¶ Values from the MolProbity server (<http://molprobity.biochem.duke.edu/>).

Science, Indianapolis, Indiana, USA). The soluble protein was purified from the clarified lysate using a HisTrap HP IMAC column (GE Healthcare Biosciences, Piscataway, New Jersey, USA). After cleavage using TEV protease, the tag-free GAPDH was further purified using an IMAC column and size-exclusion chromatography.

2.2. Crystallization

Purified GBS GAPDH protein was concentrated to ~20 mg ml⁻¹ using Amicon centrifugal concentrators (Millipore, Billerica, Massachusetts, USA) before setting up crystallization using the hanging-drop vapour-diffusion method. Multiple commercially available crystal screens were tested. Crystals were obtained in a condition composed of 2 *M* ammonium sulfate, 0.1 *M* Tris-HCl pH 8.5.

Single blade-like crystals grew to dimensions of 0.40 × 0.30 × 0.05 mm after one to two weeks and were picked up in nylon loops and transferred to an optimized cryoprotectant solution consisting of 2 *M* ammonium sulfate, 1 *M* lithium sulfate, 5% glycerol, 0.1 *M* Tris-HCl pH 8.5. After exchange into cryoprotectant solution, single crystals were flash-cooled in liquid nitrogen for X-ray diffraction data collection.

2.3. X-ray diffraction, structure determination and refinement

X-ray diffraction data were collected on the GM/CA-CAT beamline 23-ID-D at the Advanced Photon Source (APS), Argonne National Laboratory. A data set of 180 images was collected at 12.662 keV with 1° rotation and 1 s exposure time. Data were processed with the *HKL*-2000 suite (Otwinowski & Minor, 1997) and *XDS* (Kabsch, 2010). The structure was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) in the *CCP4* suite (Potterton *et al.*, 2018) using the published structure of GAPDH as a search model (PDB entry 4qx6; Ayres *et al.*, 2014). The final model was completed by alternating manual model building in *Coot* (Adams *et al.*,

2010) and refinement using *phenix.refine* in *PHENIX*. The crystal structures were validated by *MolProbity* (Chen *et al.*, 2010) and RCSB *ADIT* validation (Yang *et al.*, 2004). Most of the values in Table 1 were generated using the 'Table 1' utility in the *PHENIX* package. Molecular graphics and structural alignments were prepared with *PyMOL* (<http://www.pymol.org>).

3. Results and discussion

3.1. Crystallization, data collection and structure determination

GBS GAPDH readily formed sizable crystals in conditions containing ammonium sulfate (Fig. 1*a*). The best GBS

Figure 1
Structure determination of GBS GAPDH. (*a*) Single crystals obtained in 2 M ammonium sulfate, 0.1 M Tris–HCl pH 8.5. (*b*) Cross-eyed stereoview of the NAD ligand in chain A. The $2F_o - F_c$ electron-density map of NAD is shown at 2σ . (*c*) Overall structure of the tetramer. Subunits A (magenta), B (green), C (sky blue) and D (yellow) of the mixed holo complex (PDB entry 5y37) are shown in cartoon representation. NAD and sulfates are shown as sticks and spheres, respectively, and are coloured by element (C, green; N, blue; O, red; P, orange; S, dark yellow).

GAPDH crystals diffracted to 1.36 Å resolution and belonged to space group $P2_12_12$, with unit-cell parameters $a = 144.3$, $b = 156.7$, $c = 67.8$ Å (see also Table 1). Data analysis by *phenix.xtriage* showed that on assuming a solvent content of 50% there are 1403 residues in the asymmetric unit, suggesting the presence of four molecules of GAPDH in each asymmetric unit. The molecular-replacement search identified four molecules and the structural model was completed with an R_{work} of 16.64% and an R_{free} of 18.50%. The $2F_o - F_c$ electron-density

map of one bound NAD ligand is shown at 2σ to show the quality of the electron-density map (Fig. 1*b*)

3.2. Overall structure

There are four copies of the GAPDH molecule in one asymmetric unit that form a tetramer or two dimers, namely the *A–B* dimer and the *C–D* dimer (Fig. 1*c*). Subunit *A* of the *A–B* dimer and subunit *C* of the *C–D* dimer bind molecules of

Figure 2

The interaction interfaces of subunits *A* and *D* as well as the NAD and sulfates in GBS GAPDH. (*a*) A cartoon drawing of subunit *A* of GBS GAPDH. (*b*) Close-up view of the active site in subunit *A*. (*c*) A cartoon drawing of subunit *D* of GBS GAPDH. (*d*) Close-up view of the active site in subunit *D*. Subunits *A* and *D* are shown in magenta and yellow, respectively. NAD and sulfates are shown as sticks and coloured by element (C, green; N, blue; O, red; P, orange; S, dark yellow). Residues within 3.5 Å of the NAD and sulfates are shown as sticks and coloured by element (C, magenta in subunit *A*, yellow in subunit *D*; N, blue; O, red). Hydrogen bonds are shown as dashed blue lines.

the coenzyme NAD to form the holo state, whereas subunits *B* and *D* remain in the unliganded apo state. Subunits *A* and *B* have a major interacting interface previously named the P

interface (Roitel *et al.*, 2003). This complex can be referred to as a mixed holoenzyme (Schormann *et al.*, 2016). Eight sulfate ions bound to the tetramer are dispersed unevenly over the four subunit molecules. Subunits *A* and *C* both contain one sulfate ion each, whereas subunits *B* and *D* both contain three sulfate ions each.

3.3. Mixed holo tetramer

We obtained GBS GAPDH crystals without using any other additives apart from the crystallization solution. It is interesting that the structures obtained from both this study and previous studies of GBS GAPDH (Ayres *et al.*, 2014; Schormann *et al.*, 2016) are in a mixed holo form rather than the holo form. In our structure, subunit *A* contains a NAD coenzyme molecule and a sulfate ion (labelled SUL) in the holo state (Fig. 2*a*). As expected, the NAD-binding site located in the N-terminal domain is highly similar to those in other GAPDHs and the residues bound to NAD coincide with those in reported structures of GBS GAPDH (Fig. 2*b*). The O atom of the nicotinamide ribose of NAD is hydrogen-bonded to the hydroxyl group in the side chain of Thr121. The NAD pyrophosphate moiety binds to the main chain of the protein with hydrogen bonds from the backbone amino groups of Arg12 and Ile13. The two hydroxyl groups of the adenosine

Figure 3
Conformational changes upon NAD binding. (*a*) Superimposition of subunits *A* and *D*. (*b*) A close-up of the view in (*a*) showing the difference in the NAD-binding pocket. Subunit *A* (magenta) and subunit *D* (yellow) are shown as tubes. Residues within 3.5 Å of NAD in subunit *A* are coloured cyan. NAD and the labelled residues in (*b*) are shown as sticks and coloured by element (C, magenta in NAD, cyan in labelled residues in subunit *A* and yellow in labelled residues in subunit *D*; N, blue; O, red; P, orange).

Figure 4
Comparison of GAPDH tetramers. Structural superposition of GAPDH tetramers from this study with those in PDB entry 5jye. The same chain names as in PDB entry 5jye were used. Subunits *A* (magenta), *B* (green), *C* (sky blue) and *D* (yellow) of the tetramer reported in this study (PDB entry 5y37) and in PDB entry 5jye (grey) are shown in cartoon representation. NAD molecules are shown as sticks in two colours (red for PDB entry 5y37, blue for PDB entry 5jye).

ribose of the NAD are hydrogen-bonded to Asp34, and Arg78 forms a hydrogen bond to the amide group of the adenine base. The sulfate ion in subunit *A* shown in Fig. 2(*b*) was fitted to a tetrahedral density that was clearly not part of the polypeptide chain in the electron-density map of GBS GAPDH, and so are those in the remaining subunits. A phosphate or a sulfate ion can be built into the tetrahedral density. Given the high concentration of sulfate in the crystallization condition, the tetrahedral densities were interpreted as sulfate ions in our model. The sulfate ion in subunit *A* forms hydrogen bonds to the side chains of Thr182, Arg199 and Arg235. It is also hydrogen-bonded to the 2'-hydroxyl of the nicotinamide ribose of the NAD.

The apo subunit *D* of the crystal structure contains three sulfate ions labelled SUL1, SUL2 and SUL3 (Fig. 2*c*). SUL1 binds to Ser151, Thr212 and Gly213 by hydrogen bonds. The location of SUL2 is similar to the position of the sulfate ion in subunit *A*, and the residues to which SUL2 binds by hydrogen bonds are identical to those in subunit *A*, including Thr182, Arg199 and Arg235. In addition, SUL3 is hydrogen-bonded to the backbone amino group of Arg12 (Fig. 2*d*).

The conformations of subunits *A* and *C* are almost the same as those of subunits *B* and *D*, respectively. However, some noticeable differences were observed between the holo subunit and the apo subunit, and the r.m.s.d. value for superposition of all atoms in subunits *A* and *D* is 0.611 Å. We

Figure 5

Superimposition of GBS GAPDH and other GAPDH structures. (*a*) A close-up view of a superimposition of subunit *A* and holo BsGAPDH (PDB entry 1gd1). (*b*) A close-up view of a superimposition of subunit *A* and holo LmGAPDH (PDB entry 1a7k). (*c*) A close-up view of a superimposition of subunit *D* and holo BsGAPDH. (*d*) A close-up view of a superimposition of subunit *D* and holo LmGAPDH. Subunits *A* and *D*, holo BsGAPDH and holo LmGAPDH are shown in magenta, yellow, green and sky blue, respectively. NADs, sulfates and phosphates are shown in stick representation and are coloured by element (C, magenta in subunit *A*, green in holo BsGAPDH and sky blue in holo LmGAPDH; N, blue; O, red; P, orange; S, dark yellow).

found that NAD binding to the N-terminal domain induced some noticeable conformational changes, especially within the interaction interface (Fig. 3*a*). The NAD-binding pocket, in which Arg78 and Gly98 in subunit *A* are 3.0 and 2.1 Å away from the equivalent residues in subunit *D*, respectively, performs a marked contraction into the location of NAD in comparison with subunit *D* (Fig. 3*b*).

3.4. Identification of pre-existing asymmetric tetramers

Structural comparison with a published structure of mixed holo GBS GAPDH (PDB entry 5jye; Schormann *et al.*, 2016), which also had two NAD molecules in its tetrameric structure, showed that the two NAD-bound molecules are different. In our structure, NAD is present in the *A* and *C* subunits, whereas it is present in the *A* and *D* subunits in PDB entry 5jye if the same chain-naming system is used (Fig. 4). The asymmetric dimers *A*–*B* of both structures can be aligned nearly perfectly, while the asymmetric dimers *C*–*D* are in very different conformations because of the different NAD-binding states of the molecules. The molecular interfaces within both tetramers are essentially the same. Overall, the two tetramers are in different conformations owing to the different ligand-binding states of the four promoters. It is likely that both conformations exist in solution, but only one of them was crystallized under the specific conditions. The pre-existing asymmetric tetramers in solution suggest that the two asymmetric P-type dimers in a tetramer act independently in solution. The mixed holo configuration may be a resting state for this enzyme.

3.5. Identification of phosphate-binding sites

The chemical natures of the substrates D-G3P and inorganic phosphate and the product 1,3-bisphosphoglycerate imply that the anion-binding sites located by sulfate ions in our structure could naturally be occupied by phosphate ions. To clarify the roles of the sulfate ion-binding sites in our structure, a superimposition of our model and other GAPDH structures was carried out. Upon the superposition of subunit *A* of GBS GAPDH onto holo BsGAPDH (GAPDH from *B. stearothermophilus*; PDB entry 1gd1; Skarżński *et al.*, 1987) and holo LmGAPDH (GAPDH from *L. mexicana*; PDB entry 1a7k; Kim & Hol, 1998), the P_s site was determined in GBS GAPDH, in which the only sulfate in subunit *A* is located. The same site is occupied by a sulfate (BsSUL2) in BsGAPDH or a phosphate (LmP2) in LmGAPDH (Figs. 5*a* and 5*b*). Meanwhile, when subunit *D* of GBS GAPDH was superimposed onto holo BsGAPDH and holo LmGAPDH, we found that a sulfate ion (SUL1) in subunit *D* binds at the position of the classical P_i site but not the new P_i site. There is still a short distance of 0.7 Å between SUL1 and the classical P_i site. Therefore, the P_i anion-binding site in GBS GAPDH was also determined to be a classical P_i site (Figs. 5*c* and 5*d*). It is also noteworthy that SUL2 of subunit *D* was found to not exactly occupy the P_s site, being at a distance of 1.0 Å in the opposite direction from the NAD (Figs. 5*c* and 5*d*), which may be caused by the absence of NAD in the apo subunit *D*. As

observed in subunit *A*, the 2'-hydroxyl of the nicotinamide ribose of the NAD forms a hydrogen bond to the sulfate in the P_s site (Fig. 2*b*). On account of the interaction between the NAD and the sulfate, the P_s site is closer to the NAD than the position of SUL2 in subunit *D*. In addition, the location originally occupied by the phosphate of the adenosine is occupied by SUL3 in subunit *D* (Figs. 5*c* and 5*d*). There is a possibility that excess sulfate ions from the crystallization solution might reduce the occupancy of NAD in the structure.

Figure 6
Superimposition of GBS GAPDH and a ternary complex of GBS GAPDH (PDB entry 5jya). (*a*) A close-up view of a superimposition of subunit *D* and the ternary complex. (*b*) A close-up view of the strand-loop-helix portion spanning residues Ser211–Lys216 (STGA_{AK}). Subunit *D*, the ternary complex and the strand-loop-helix portion of subunit *D* are shown in yellow, cyan and hot pink, respectively. NAD, sulfates, D-G3P and Ser152 and His179 bound to D-G3P as well as Thr212 and Gly213 in the strand-loop-helix portion are shown in stick representation and coloured by element (C, cyan in the ternary complex and hot pink in subunit *D*; N, blue; O, red; P, orange; S, dark yellow). Hydrogen bonds are shown as dashed blue lines.

3.6. Catalytic mechanism

Interestingly, Schormann and coworkers showed that the C3 phosphate of the substrate binds at the new P_i site in a ternary complex of GBS GAPDH by co-crystallization of an active-site mutant C152S enzyme with NAD and the substrate D-G3P (Schormann *et al.*, 2016). However, according to the identification of the P_i anion-binding site in GBS GAPDH reported here and the catalytic mechanism proposed in previous studies (Trentham, 1971; Harrigan & Trentham, 1974; Moras *et al.*, 1975; Skarżński *et al.*, 1987; Moniot *et al.*, 2008; Mukherjee *et al.*, 2010), the C3 phosphate of the substrate D-G3P should have bound at the classical P_i site. The superimposition of our model and the previously determined structure of the ternary complex (PDB entry 5jya; Schormann *et al.*, 2016) sharing the same amino-acid sequence showed that SUL1 of subunit D and the C3 phosphate of the D-G3P are located in the classical P_i site and the new P_i site, respectively (Fig. 6a). There is also a distance of 2.4 Å between the two sites. At the same time, the conformation of the strand-loop-helix portion spanning residues Ser211–Lys216 (STGAACK) in the catalytic domain of our model is different from the corresponding region in the ternary complex of GBS GAPDH (Fig. 6b). The maximum distance between the two loops is about 2.9 Å. The conformational changes may be interpreted in the same way as the NAD binding. The binding of the substrate D-G3P to Ser152 and His179 in the catalytic domain of the C152S mutant may lead to movement of the strand-loop-helix portion spanning residues 211–216 from the current location in our model to the position in the ternary complex, resulting in the concomitant repositioning of the P_i anion-binding site from the classical P_i site to the new P_i site. By contrast, in the absence of a substrate, the strand-loop-helix portion spanning residues Ser224–Ala229 (STTGAA) of holo LmGAPDH (Kim *et al.*, 1995) also adopts the same conformation as the aforementioned ternary complex, and there is an inorganic phosphate ion bound in the new P_i site (Fig. 5d). Owing to the lack of the structures of the hemithioacetal and thioacyl intermediates in the presence of NAD, the conformation changes involved in the whole reaction and the catalytic mechanism of GBS GAPDH remain unclear.

In conclusion, our study presents a high-resolution mixed holo structure of GBS GAPDH. Surprisingly, it is in a different ligand-binding state compared with the previously reported structure with PDB code 5jye. Two putative phosphate-binding sites were determined through the identification of two bound sulfate ions, and this contributes to the understanding of the catalytic reaction mechanism.

Acknowledgements

We would like to thank the staff of GM/CA-CAT at the Advanced Photon Source for their assistance during data collection.

Funding information

TJ is supported by the Fundamental Research Funds for the Central Universities, the 100 Talents Program of CAS and the

National Science Foundation of China (Grant No. U1732109).

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