
Structure of the nucleotide-binding domain of a dipeptide ABC transporter reveals a novel iron–sulfur cluster-binding domain

Li *et al.*

Synopsis

The crystal structure of DppD, the nucleotide-binding domain of a dipeptide ABC transporter from *T. tengcongensis*, bound with an iron–sulfur cluster is reported at 2.89 Å resolution.

Keywords: dipeptide permease; ABC transporters; nucleotide-binding domains; iron–sulfur clusters.

Queries and comments

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Structure of the nucleotide-binding domain of a dipeptide ABC transporter reveals a novel iron–sulfur cluster-binding domain

Dipeptide permease (Dpp), which belongs to an ABC transport system, imports peptides consisting of two or three L-amino acids from the matrix to the cytoplasm in microbes. Previous studies have indicated that haem competes with dipeptides to bind DppA *in vitro* and *in vivo* and that the Dpp system can also translocate haem. Here, the crystal structure of DppD, the nucleotide-binding domain (NBD) of the ABC-type dipeptide/oligopeptide/nickel-transport system from *Thermoanaerobacter tengcongensis*, bound with ATP, Mg²⁺ and a [4Fe–4S] iron–sulfur cluster is reported. The N-terminal domain of DppD shares a similar structural fold with the NBDs of other ABC transporters. Interestingly, the C-terminal domain of DppD contains a [4Fe–4S] cluster. The UV-visible absorbance spectrum of DppD was consistent with the presence of a [4Fe–4S] cluster. A search with DALI revealed that the [4Fe–4S] cluster-binding domain is a novel structural fold. Structural analysis and comparisons with other ABC transporters revealed that this iron–sulfur cluster may act as a mediator in substrate (dipeptide or haem) binding by electron transfer and may regulate the transport process in Dpp ABC transport systems. The crystal structure provides a basis for understanding the properties of ABC transporters and will be helpful in investigating the functions of NBDs in the regulation of ABC transporter activity.

1. Introduction

ATP-binding cassette (ABC) transporters are ubiquitous membrane proteins that use energy from ATP binding and hydrolysis to transport different substrates ranging from small ions to large macromolecules across the membrane bilayer (Dean & Allikmets, 1995). Members of the ABC transporter family are found in organisms from all kingdoms of life (Higgins, 1992). They can function as exporters or importers. Exporters are found in both eukaryotes and prokaryotes, while importers seem to exclusively be present in prokaryotic organisms. ABC transporters comprise the largest protein family in *Escherichia coli*, including 79 proteins from 69 independent functional systems representing 5% of the genome (Linton & Higgins, 1998), whereas only 50 ABC transporters are present in humans (Dean *et al.*, 2001). Members of the seven families of human ABC transporters (Dean & Annilo, 2005) participate in cholesterol and lipid transport (Voloshyna & Reiss, 2011), multidrug resistance (Moitra *et al.*, 2011), antigen presentation, mitochondrial iron homeostasis and ATP-dependent regulation of ion channels (including the cystic fibrosis transmembrane conductance regulator and the sulfonyleurea receptors; Higgins, 1992). A

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115 wide range of disorders, including cystic fibrosis (Wang, 2010),
 116 hypercholesterolaemia, atherosclerosis (Voloshyna & Reiss,
 117 2011) and diabetes (Dean *et al.*, 2001; Dean & Annilo, 2005),
 118 have been related to mutations in these proteins.

119 Typical ABC transporters have a characteristic architecture
 120 that minimally consists of four domains: two transmembrane
 121 domains (TMDs) that are embedded in the membrane bilayer
 122 and two ABC domains (or nucleotide-binding domains;
 123 NBDs) that are located in the cytoplasm (Doige & Ames,
 124 1993). In prokaryotes these domains are often independent
 125 subunits which are assembled into a membrane-bound
 126 complex, whereas in eukaryotes these domains are generally
 127 fused into a single polypeptide chain. The TMDs have low
 128 sequence similarity, but the NBDs contain a set of highly
 129 conserved motifs. NBDs contain Walker A (GxxGxGKS/T,
 130 where *x* can be various amino acids) and Walker B (hhhhDEP,
 131 where *h* is a hydrophobic residue) motifs with consensus
 132 sequences for nucleotide binding (Walker *et al.*, 1982) and the
 133 ABC 'signature' sequence LSGGQ. The high similarity among
 134 the NBDs of all ABC transporters suggest that the mechanism
 135 of energy utilization by these systems is conserved. Recent
 136 structural studies of the ABC transporter NBDs, comple-
 137 menting the genetic and biochemical data already available in
 138 the literature, provide a glimpse at the atomic level of the
 139 specific residues involved in ATP binding and hydrolysis
 140 (Hung *et al.*, 1998).

141 *E. coli* dipeptide/oligopeptide/nickel ABC transporter
 142 (DppBCDF) transports peptides consisting of two or three
 143 L-amino acids and may also transport haem (Abouhamad &
 144 Manson, 1994). Haem, a major iron source, is transported
 145 through the outer membrane of Gram-negative bacteria by
 146 specific haemoprotein receptors and through the inner
 147 membrane by haem-specific periplasmic binding protein-
 148 dependent ATP-binding cassette transporters (Ferguson &
 149 Deisenhofer, 2004; Wandersman & Delepelaire, 2004; Stojilj-
 150 kovic & Hantke, 1994). Haem can competitively bind to DppA
 151 both *in vitro* and *in vivo* as revealed by biochemical experi-
 152 ments (Létoffé *et al.*, 2006). However, whether the DppD
 153 system is a haem transporter remains unknown and requires
 154 further investigation. A dipeptide/oligopeptide/nickel ABC
 155 transporter is also found in *Thermoanaerobacter tengcon-*
 156 *gensis*, an anaerobic Gram-negative bacterium (Wang *et al.*,
 157 2004) that utilizes the physical and chemical properties of Fe-
 158 S compounds for fundamental metabolism (such as amino-
 159 acid metabolism), as might have been present in the earliest
 160 forms of life under an anaerobic environment (Sheftel *et al.*,
 161 2010). It has been reported that [4Fe-4S] clusters occur in
 162 eukaryotic ABC transporter proteins. However, to date no
 163 [4Fe-4S] clusters have been reported in prokaryotic NBDs.
 164 Here, we report the crystal structure of DppD, the nucleotide-
 165 binding domain of the ABC-type dipeptide/oligopeptide/
 166 nickel-transport system from *T. tengcongensis*. This structure
 167 is the first report of a native NBD monomer bound with ATP,
 168 Mg²⁺ and a [4Fe-4S] cluster. The crystal structure may provide
 169 a basis for understanding the properties of ABC transporters
 170 and the functions of iron-sulfur clusters in the process of
 171 transporting substrates.

172 2. Materials and methods

173 2.1. Materials

174 Enzymes for recombinant DNA technology such as *Pfu*
 175 polymerase, T4 DNA ligase, *NdeI* and *XhoI* were purchased
 176 from New England Biolabs. PCR buffer and dNTP mixture
 177 were also obtained from New England Biolabs. The DNA
 178 Quick Purify/Recover Kit and Plasmid Mini Kit were products
 179 of Omega Co. The detergents Triton X-100 and *n*-decyl-β-
 180 D-maltopyranoside (DM) were purchased from Affymetrix.
 181 Chromatographic columns, such as Ni Sepharose 6 Fast Flow
 182 and Superdex 200 HR 10/30 columns, were purchased from
 183 GE Healthcare. Crystal screening kits were purchased from
 184 Hampton Research and Molecular Dimensions.

185 2.2. Construction, expression and purification

186 The *T. tengcongensis dppD* gene, which encodes a mature
 187 protein of 326 amino acids, was PCR-amplified using the
 188 genomic DNA of *T. tengcongensis* as a template. The forward
 189 primer was 5'-CGCCATATGAGCATAATCATAAGAGTT-
 190 GAGG-3' and the reverse primer was 5'-CCGCTCGAGT-
 191 TTTAATGGTCTCCCTTCTTC-3'. The expression construct
 192 was generated using a standard PCR-based cloning strategy.
 193 In order to allow easy purification, a 6×His tag was added to
 194 its C-terminus. The expression construct was subcloned into
 195 the prokaryotic expression vector pET21b with *NdeI* and
 196 *XhoI* restriction-enzyme sites. The recombinant plasmid was
 197 transformed into *E. coli* strain C43 (DE3). Protein expression
 198 was induced by the addition of IPTG (0.5 mM final concen-
 199 tration) when the OD₆₀₀ of the culture reached approximately
 200 1.2 in LB medium. The cell culture was allowed to grow for a
 201 further 12 h at 296 K. The bacterial cells were harvested by
 202 centrifugation at 4000 rev min⁻¹ for 15 min at 277 K. The cell
 203 pellets were resuspended in ice-cold lysis buffer (50 mM Tris-
 204 HCl pH 8.0, 200 mM NaCl, 1 mM PMSF) and homogenized by
 205 sonication on ice. The cell debris was removed completely by
 206 centrifugation of the lysate at 15 000 rev min⁻¹ for 15 min at
 207 277 K. The supernatant was collected and subjected to ultra-
 208 centrifugation at 30000 rev min⁻¹ for 1 h. The membrane
 209 fraction was harvested and resuspended in buffer A (25 mM
 210 Tris-HCl pH 8.0, 20 mM imidazole, 500 mM NaCl). After the
 211 addition of Triton X-100 to a final concentration of 0.5% (v/v)
 212 and 1 mM PMSF, the sample was incubated for 5 h with slow
 213 stirring at 277 K. The membrane was removed completely by
 214 further ultracentrifugation at 30 000 rev min⁻¹ for 30 min at
 215 277 K.

216 All of the following purification steps were performed in a
 217 277 K cold room. The clear supernatant was loaded onto a Ni
 218 Sepharose 6 Fast Flow column pre-equilibrated with buffer A
 219 containing 0.1% Triton X-100. The contaminant proteins were
 220 eluted with wash buffer (buffer A containing 0.2% DM). The
 221 target protein was eluted with buffer B (25 mM Tris-HCl pH
 222 8.0, 300 mM imidazole, 500 mM NaCl, 0.2% DM). The eluate
 223 was further purified by gel filtration using a Superdex 200 HR
 224 10/30 column. The buffer for gel filtration consisted of 25 mM
 225 Tris-HCl pH 7.5, 500 mM NaCl, 2.5 mM MgCl₂, 2.5 mM ATP,
 226 5 mM DTT, 0.2% DM. The purified protein was concentrated
 227

using an Amicon Ultra centrifugal filter to a concentration of about 15 mg ml⁻¹ as measured by the Bradford protein assay (Bradford, 1976). The purified protein was analyzed by SDS-PAGE and LTQ Orbitrap mass spectrometry.

2.3. LTQ Orbitrap mass-spectrometric analysis

The gel strip containing the target protein was removed from the SDS-PAGE gel, cut into small pieces and washed with 100 µl 25 mM ammonium bicarbonate pH 8.0 containing 50% acetonitrile for 15 min twice with vortexing. Gel pieces were dehydrated with 100 µl acetonitrile and completely dried with a Speed-Vac before tryptic digestion. The volume of the dried gel was evaluated and three volumes of trypsin (Promega, Madison, Wisconsin, USA) at 12.5 ng ml⁻¹ in 25 mM NH₄HCO₃ (freshly diluted) were added. The digestion was performed at 303 K overnight. The mixture was sonicated for 10 min and centrifuged. The supernatant was removed and analyzed by high-resolution nano-LC-MS on an Orbitrap (LTQ Orbitrap Velos, Thermo Fisher Scientific).

All data files were processed using *Sequest*. Protein identification was performed by searching against bacterial genomes in the NCBI nonredundant database using the *Mascot* search engine (Matrix Science, London, England). The parameters used were monoisotopic, mass accuracy 0.1 Da, missed cleavages 1.

2.4. Crystallization, data collection and processing

The purified protein sample at a concentration of 15 mg ml⁻¹ was centrifuged at 20 000g for 45 min to clarify the solution prior to initiating the crystallization trials. Initial screening was performed at 291 K in 48-well plates by the sitting-drop vapour-diffusion method using sparse-matrix screen kits from Hampton Research (PEGRx 1 and PEGRx 2) and Molecular Dimensions (MemStart, MemSys, MemPlus and MemGold) and was followed by a round of refinement of the initial conditions with variation of the precipitants, pH, protein concentration and additives using the hanging-drop vapour-diffusion method in 24-well plates. Typically, 2 µl droplets formed by mixing 1 µl protein solution with 1 µl reservoir solution were placed on siliconized coverslips and equilibrated against 200 µl reservoir solution.

X-ray diffraction data sets were collected from a single native crystal using an ADSC Quantum 315 detector on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China). The presence of iron was investigated using anomalous scattering. The reservoir solution supplemented with 12.5% (v/v) glycerol was used as a cryoprotectant. The crystals were mounted in a suitable nylon-fibre loop, immersed in the cryoprotectant solution for 5–10 s and flash-cooled in a liquid-nitrogen stream at 100 K. The wavelength used was 1.54148 Å, the exposure time was 1 s per frame, the crystal-to-detector distance was 150 mm and the oscillation range was 1° per frame. Highly redundant data sets were collected for the detection of anomalous signal. All intensity data were indexed, integrated

and scaled using the *HKL-2000* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

2.5. Structure determination

The structure of *T. tengcongensis* DppD was determined by the single-wavelength anomalous dispersion (SAD) method using the data collected at the iron peak. In order to determine the iron positions, we collected highly redundant data (data set 2). The iron positions in the crystal were determined using the program *SHELXD* (Sheldrick, 2008). Although four iron sites were expected, only one iron site was found by *SHELXD*. The identified iron site was refined and the initial phases were generated using the SAD experimental phasing module in *Phaser* (McCoy *et al.*, 2007). The phase was then extended to data set 1 using *DM* (Winn *et al.*, 2011). Solvent flattening and histogram matching were performed using *DM*. The initial model was traced automatically using *Buccaneer* (Cowtan, 2006) and was manually rebuilt using *Coot* (Emsley & Cowtan, 2004). The final structure was refined with *PHENIX* (Adams *et al.*, 2002). The *PyMOL* molecular-graphics program (<http://www.pymol.org>) was used to illustrate the final structure and to produce the figures.

2.6. Spectroscopic analysis

2.6.1. UV-visible absorption spectroscopic analysis. UV-visible absorption spectroscopy of *T. tengcongensis* DppD between 300 and 700 nm was carried out using an Ultraspec 4300 spectrophotometer. All measurements were performed at room temperature using quartz cuvettes with 1 mm path length and a scan step of 1 nm. DppD protein (0.8 mg ml⁻¹) was solubilized in 50 mM sodium phosphate buffer pH 7.5. The UV-visible absorption spectrum of BSA at the same concentration was also recorded. A suitable baseline correction was made before recording each spectrum.

2.6.2. Far-UV circular-dichroism (CD) spectroscopic analysis. For far-UV CD spectroscopic analysis, the purified DppD protein was solubilized in 50 mM sodium phosphate buffer pH 7.5. To study the effect of ATP hydrolysis on the conformation of DppD, samples of DppD were incubated with 0.5 mM ATP and 1 mM MgCl₂ for 15 min at room temperature before recording each spectrum. To study the effect of reductants on the conformation of DppD, samples of DppD were incubated with 1 mM DTT, 0.5 mM GSH or 0.5 mM NADH for 5 min at 328 K. When recording the spectra, the actual concentration of the protein was 0.1 mg ml⁻¹. Far-UV CD spectroscopy was carried out using an Applied Photophysics Pistar-180 spectropolarimeter with a 1 mm path-length cell and a bandwidth of 3.0 nm. Spectra were recorded from 250 to 190 nm at an interval of 1 nm and were repeated three times. All resultant spectra were obtained by subtraction of the spectrum of the buffer. The results were expressed as the mean residue ellipticity [θ]_{MRW}.

3. Results and discussion

3.1. Expression and purification of the DppD protein

The construct expressing *T. tengcongensis* DppD was successfully generated using a standard PCR-based cloning strategy. The recombinant protein was expressed in *E. coli* strain C43. DppD with a C-terminal 6×His tag was purified using an Ni Sepharose 6 Fast Flow column followed by gel filtration using a Superdex 200 HR 10/30 column.

The purified DppD protein was yellow and its colour became dark yellow when the protein was concentrated, suggesting the presence of a bound ligand molecule in the structure. Previous studies have suggested that the dipeptide/oligopeptide/nickel ABC transporter (DppBCDF) can transport haem (Abouhamad & Manson, 1994); we thus propose that the ligand might be a molecule containing iron. The existence and position of iron was examined in anomalous difference Fourier maps calculated from highly redundant data sets. The anomalous difference Fourier computed with the data collected at the iron peak revealed a bound iron-sulfur cluster in the structure (Fig. 1).

3.2. LTQ Orbitrap mass-spectrometric analysis

The purified DppD protein was analyzed by LTQ Orbitrap mass spectrometry. The resulting peptide mass fingerprint (PMF) of the protein was submitted to NCBIInr. Only the ABC-type dipeptide/oligopeptide/nickel-transport system ATPase component (*T. tengcongensis* MB4) was obtained as a result of the search, with a score of 109. The results provided convincing evidence that the purified protein was the ABC-

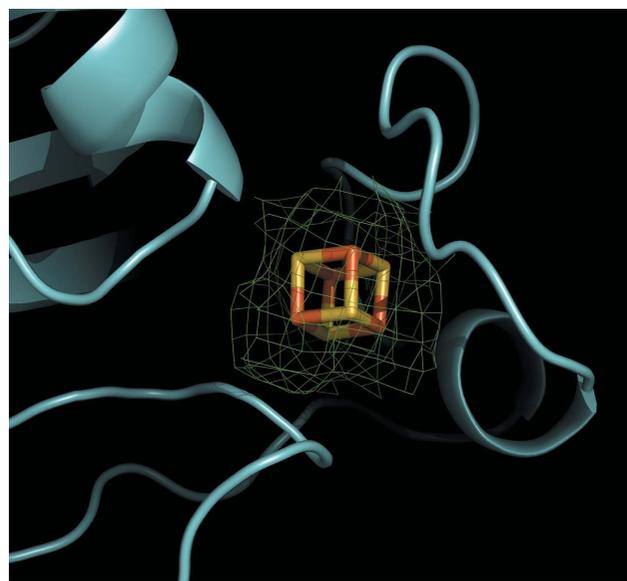


Figure 1
The difference Fourier map of *T. tengcongensis* DppD. The electron-density map for the [4Fe-4S] cluster is coloured green. The [4Fe-4S] cluster is shown as sticks and coloured red for Fe atoms and yellow for S atoms.

Table 1
Data-collection and refinement statistics.

	Data set 1†	Data set 2‡
Data-collection statistics		
Unit-cell parameters		
<i>a</i> (Å)	64.247	64.247
<i>b</i> (Å)	64.247	64.247
<i>c</i> (Å)	320.608	320.608
$\alpha = \beta = \gamma$ (°)	90	90
Space group	<i>P</i> ₄ ₃ ₂ ₂	<i>P</i> ₄ ₃ ₂ ₂
Wavelength (Å)	1.54148	1.54148
Resolution (Å)	50.00–2.89	50.00–2.89
	(2.99–2.89)	(2.99–2.89)
Total No. of reflections	59602	110339
No. of unique reflections	15715	15883
Completeness (%)	97.5 (90.3)	98.2 (96.8)
Average <i>I</i> / σ (<i>I</i>)	24.57 (2.04)	22.06 (2.23)
<i>R</i> _{merge} § (%)	8.9 (58.7)	10.2 (73.6)
Multiplicity	3.8 (3.4)	6.9 (6.6)
Wilson <i>B</i> factor (Å ²)	88.9	87.5
Refinement statistics		
No. of reflections used [σ (<i>F</i>) > 0]	14810	
<i>R</i> _{work} ¶ (%)	21.19	
<i>R</i> _{free} ¶ (%)	26.79	
R.m.s.d. from ideal bond lengths (Å)	0.015	
R.m.s.d. from ideal bond angles (°)	1.477	
No. of atoms		
Protein atoms	2425	
Ligand atoms	40	
Solvent atoms	0	
<i>B</i> value (Å ²)	88.6	
Ramachandran plot, residues in (%)		
Favoured regions	84.2	
Additional allowed regions	15.8	
Disallowed regions	0	

† For data set 1, all of the .x files were input into *SCALEPACK* for merging and scaling. ‡ For data set 2, the data were scaled separately and output as *I*⁺ and *I*⁻ for each reflection for the detection of anomalous signal. § *R*_{merge} = $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. ¶ *R*_{work} = $\sum_{hkl} |F_{obs}| - |F_{calc}| / \sum_{hkl} |F_{obs}|$. *R*_{free} was calculated in the same way as *R*_{work} but for a test set of reflections that were not included in refinement.

type dipeptide/oligopeptide/nickel-transport system ATPase component from *T. tengcongensis*.

3.3. Crystallization and structure determination

Small crystals were observed after about 2 d from one condition of the PEGRx 2 kit (Hampton Research) containing polyethylene glycol monomethyl ether 5000 as the precipitant. This condition was further optimized and harvestable crystals were obtained using the hanging-drop vapour-diffusion method by mixing 1 µl protein solution with 1 µl reservoir solution [4% (v/v) 2-propanol, 0.05 M CAPSO pH 11.0, 12.25% (w/v) polyethylene glycol monomethyl ether 5000] and equilibrating the drop against 200 µl reservoir solution. An X-ray diffraction data set was collected from a single crystal. Data-collection statistics and final refinement statistics are summarized in Table 1.

The structure was solved using an X-ray diffraction data set collected from a native crystal. The crystal belonged to space group *P*₄₃₂₂, with unit-cell parameters *a* = *b* = 64.247, *c* = 320.608 Å, and there was one molecule in the asymmetric unit. The model was further improved by cycles of manual building and refinement using *Coot* and *PHENIX*. The structure was subsequently refined to a final resolution of

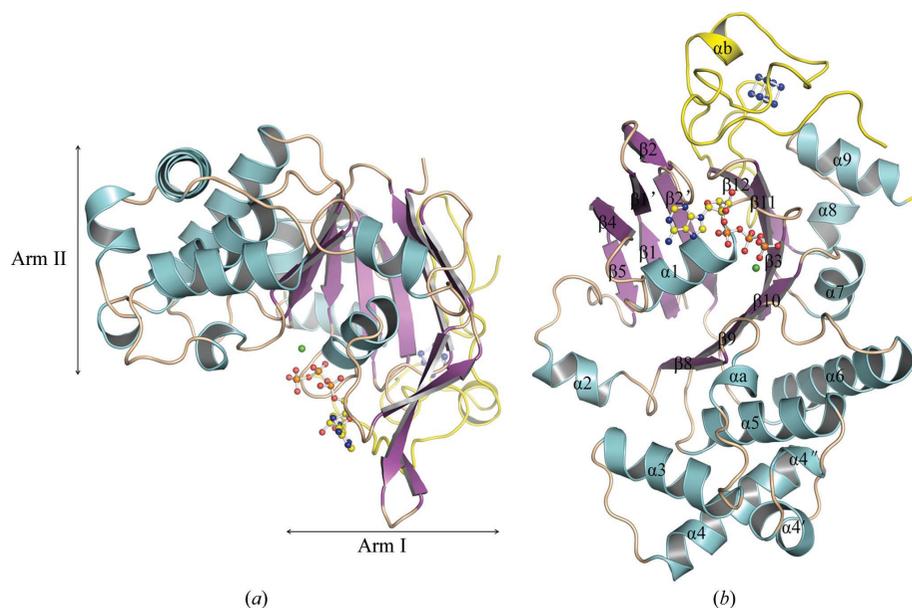


Figure 2
The three-dimensional structure of *T. tengcongensis* DppD. Cartoon representation of *T. tengcongensis* DppD in two views. ATP is coloured by element, Mg^{2+} in green and the iron-sulfur cluster in blue. The C-terminal domain is coloured yellow. (a) The L-shaped conformation of DppD with two thick arms (arm I and arm II); the top and the bottom of the protomer face towards the periplasmic and cytoplasmic sides, respectively. α -Helices (cyan) and β -strands (magenta) are differentiated by colour. (b) View from the bottom of the structure, as shown in (a), showing the ATP-binding pocket and the iron-sulfur cluster located in the C-terminal domain. The secondary-structural elements ($\alpha 1$ – $\alpha 9$, αa , αb , $\beta 1$ – $\beta 5$ and $\beta 8$ – $\beta 12$) are labelled.

2.89 Å with a working R value of 21.19% and a free R value of 26.79%. The final model of DppD consisted of residues 2–259 and 270–321. Residues 260–269 were not visible in electron-density maps. Analysis of the Ramachandran plot using PROCHECK (Laskowski *et al.*, 1993) showed that 84.2% of the modelled residues were in preferred and allowed regions.

3.4. Structure description

3.4.1. Overall structure. *T. tengcongensis* DppD is composed of two domains: the conserved nucleotide-binding domain (residues 2–259), which is homologous to other NBDs (r.m.s.d. of ~ 1.83 Å with MetN), and the C-terminal domain (residues 270–321) bound with a [4Fe–4S] cluster. Residues 260–269 are missing in the structure, probably because this segment, which links the two domains, is flexible. Although the linker is disordered, the C-terminal domain forms a stable loop region bound with a [4Fe–4S] cluster and interacts with the N-terminal domain (Fig. 2). A notable feature of the structure is that the C-terminal domain contains a [4Fe–4S] cluster with four Fe atoms covalently binding to Cys285, Cys291, Cys298 and Cys316. A DALI search (Holm & Sander, 1996) revealed no structural fold similar to this domain.

As first observed for HisP, the DppD ABC subunit adopts an L-shaped conformation with a two-arm architecture including 11 α -helices and ten β -sheets. The ATP-binding pocket is near the end of arm I, which consists of seven α -helices and four β -sheets. A six-stranded β -sheet ($\beta 3$ and $\beta 8$ – $\beta 12$) spans both arms, with a domain consisting of an α + β -

type structure ($\beta 1$, $\beta 2$, $\beta 4$, $\beta 5$, $\alpha 1$ and $\alpha 2$) on one side (within arm I) and a domain with mostly α -helices ($\alpha 3$ – $\alpha 9$, αa and αb) on the other side (arm II) of the sheet (Fig. 2). Arms I and II are connected from β -strand 4 to helix 3 via the Q-loop (residues 96–98) and are also connected by a small helix (α -helix a) and the following loop, which is different from other NBDs. The smaller arm II, also termed the signalling domain, is composed of five α -helices and contains the signature motif (residues 155–159; LSGGM), which extends into helix 5 (Holland & Blight, 1999). The whole topology is depicted in Fig. 3. The N-terminus of the structure shows the same ATPase topology and sequence motifs as previously observed in other structures of NBDs from ABC transporters.

3.4.2. ATP-binding pocket. The ATP-binding site observed in the structures of nucleotide-binding proteins such as Ras contains a phosphate-binding loop (P-loop; Saraste *et al.*, 1990). The residues involved in forming the ATP-binding site shape the active-site groove (Fig. 4a). ATP interacts with the residues of the Walker A motif, Walker B

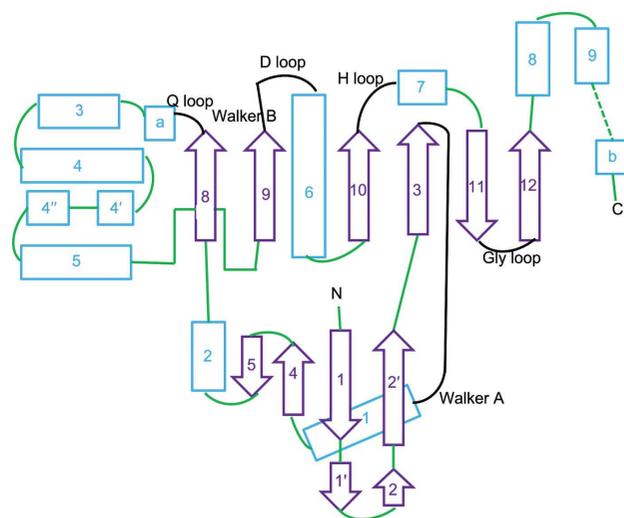


Figure 3
The topology of *T. tengcongensis* DppD. Helices are shown in cyan, β -strands in magenta and loops in green. ATP, Mg^{2+} and the iron-sulfur cluster are omitted for clarity. The conserved motifs are shown in black and labelled. The missing region between $\alpha 9$ and αb (residues 260–269) is represented as a dotted line.

motif and Q-loop. Both the Walker A (residues 41–49) and the Walker B (residues 175–180) motifs are located within arm I (Fig. 3). The P-loop (residues 43–49) surrounds the β -phosphate of ATP and forms extensive hydrogen bonds between its

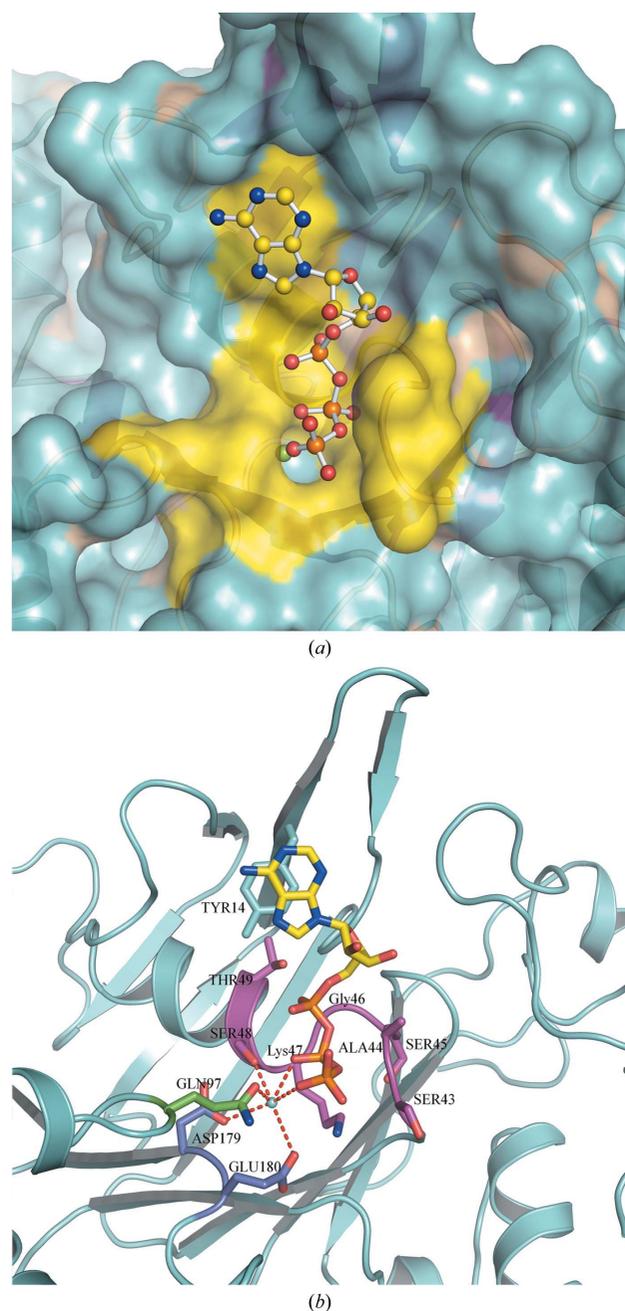


Figure 4
The arrangement of ATP, Mg²⁺ and key amino-acid residues in the active sites present in *T. tengcongensis* DppD. (a) Cartoon representation and surface of *T. tengcongensis* DppD. The residues coloured yellow interact with ATP and Mg²⁺ and form the binding pocket. (b) ATP interacts with key amino acids of DppD. The Walker A motif (residues 43–49) is shown in magenta, the Walker B motif (residues 179–180) in blue and the Q-loop (residues 97) in green. The residues that form polar interactions with Mg²⁺ are shown.

main chain and the β -phosphate. Tyr14 provides additional stabilization by hydrophobic interactions between its aromatic ring and the adenine base of ATP. Given the existence of magnesium in the crystallization conditions, it is highly probable that the ions observed in the active site of the structure are magnesium ions. The polar contacts of magnesium with other residues revealed that Asp179 is probably the residue that interacts with the divalent magnesium cation in the process of ATP hydrolysis when substrate is transported (Fig. 4b). The interaction of magnesium with the γ -phosphate of ATP can induce ATP hydrolysis when the substrate is transported. In this process, Gln97 or Glu180 could become activated and interact with the γ -phosphate of ATP by forming hydrogen bonds to water (Hung *et al.*, 1998). All of the residues forming hydrogen bonds or stacking with ATP are shown in Fig. 4(b).

3.4.3. C-terminal domain bound with an iron–sulfur cluster. In contrast to the structures of other NBDs such as MetN, the C-terminal domain of *T. tengcongensis* DppD shows a different structural fold (Fig. 5b): a V-shaped structure with a [4Fe–4S] cluster embedded in the turn region (Fig. 6a). The [4Fe–4S] cluster is coordinated in a tetrahedral geometry by Cys285, Cys291 and Cys316 from the loops between α 9 and the C-terminus and by Cys298 from α -helix b (Fig. 6b). The residues surrounding the cluster are mostly aromatic amino acids. His248, His288 and His317 make hydrophobic interactions with the [4Fe–4S] cluster. The three histidines may act as ligands for iron when translocating the substrates, as in the [FeFe]-hydrogenase (Peters *et al.*, 1998). Tyr293 interacts with Cys291 and may facilitate substrate binding; thus, it is possibly involved in the substrate-transport process. In order to examine the possible variations in the NBDs of ABC proteins, the sequences and structures of known NBDs were aligned (Fig. 5). It was observed that the residues in the NBDs have very low sequence conservation, and no conserved cysteine residues which can bind to the iron–sulfur cluster were found. *T. tengcongensis* DppD forms a different fold from those of the available NBD structures. The C-terminal [4Fe–4S] cluster could stabilize the structure and we propose that it may function in the regulation of transport of dipeptides or haem.

3.5. Spectroscopic analysis

3.5.1. UV–visible absorbance spectroscopic analysis. In order to verify the existence of the modelled [4Fe–4S] in the crystal structure, the purified DppD protein was subjected to UV–visible absorbance spectroscopic analysis. The absorption spectrum showed a broad peak at \sim 400–405 nm (Fig. 7) characteristic of [4Fe–4S] cluster proteins (Alam *et al.*, 2007), whereas the absorption spectrum of BSA showed no peak at \sim 400–405 nm. Thus, it was concluded that the freshly purified DppD protein was coordinated with a [4Fe–4S] cluster.

3.5.2. Far-UV CD spectroscopic analysis. To test the conformational changes of the DppD protein upon ATP binding, we recorded far-UV CD spectra in the wavelength range 240–190 nm. Only by incubating the protein with ATP and Mg²⁺ simultaneously could we detect the shift of the

characteristic negative peaks for α -helices (208 and 222 nm; Fig. 8a), indicating that the hydrolysis of ATP could induce conformational changes in DppD.

Far-UV CD spectra were also collected to study the functional characteristics of the [4Fe-4S] cluster with different reductants. Incubation of DppD protein with DTT or GSH had little effect on its conformation, whereas the spectrum of the protein changed significantly on incubation with NADH (Fig. 8b), indicating that NADH may transfer electrons to the iron-sulfur cluster and cause conformational changes. We propose that the [4Fe-4S] cluster may participate in substrate (dipeptide or haem) binding and activation of the protein by electron transfer when transporting the substrate. However, the biological functions of the [4Fe-4S] cluster and the

substrate-binding process still require further detailed investigation.

3.6. Comparisons with other NBD structures

Structural analysis of DppD by *BLAST* and *DALI* searches of the Protein Data Bank suggests that the molecule shares the common overall architecture of ABC-transporter NBDs. From sequence and structure alignments (Fig. 5), the N-terminal domain shares conserved motifs with the NBDs of other ABC transporters, but the C-terminal domain shows a notably different fold from those of other NBDs.

The transport of dipeptides or haem requires energy which may become available owing to binding and hydrolysis of ATP

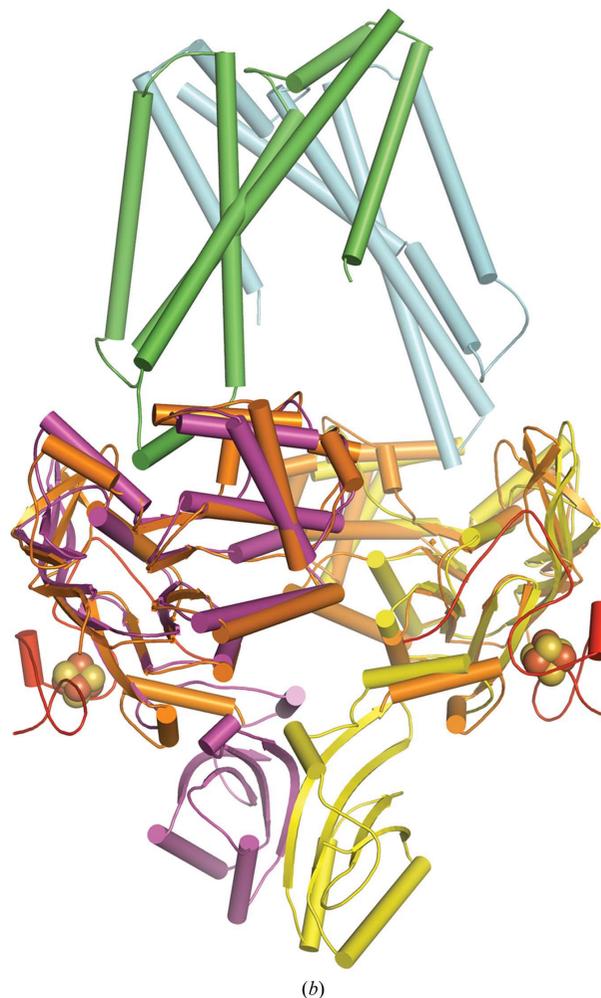
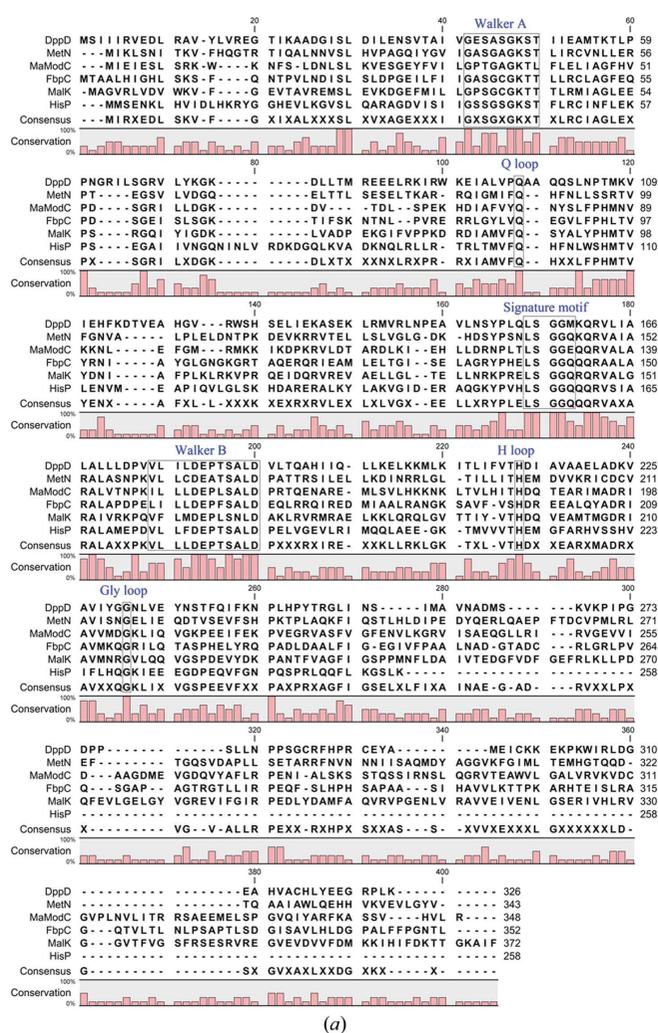


Figure 5

Sequence and structural comparisons of *T. tengcongensis* DppD with other NBDs. (a) Sequence alignment of six available NBDs. The consensus sequence is indicated, the residue conservation is shown as a bar plot and the residues framed in black are conserved motifs. The names of the conserved motifs of the NBDs are indicated above the alignment. The alignment was computed by *CLC Sequence Viewer* 6.0.2 using the following sequences retrieved from NCBI and structural templates from the PDB: MetN (NP_414741.1; PDB entry 3dhw; Kadaba *et al.*, 2008), MaModC (NP_615255.1; 3d31; Gerber *et al.*, 2008), FbpC (ZP_04720438.1; 3fvq; Newstead *et al.*, 2009), MalK (AF121946_1; 1g29; Diederichs *et al.*, 2000) and HisP (NP_461293.1; 1b0u; Hung *et al.*, 1998). (b) Superposition of *T. tengcongensis* DppD onto the MetN structure. The helices and strands of DppD are coloured orange. MetM is coloured green and cyan; MetN is coloured magenta and yellow. The [4Fe-4S] cluster is shown in sphere representation. The r.m.s.d. of DppD from MetN is ~ 1.83 Å.

by the NBD. All NBDs consist of two constituent domains: an N-terminal domain (also named the RecA-like domain; Ye *et al.*, 2004), which is a catalytic core domain with conserved motifs (Walker A motif, Walker B motif, Q-loop and H-motif), and an α -helical domain. The conformations of the two

domains are sensitive to the nucleotide-binding state (Karpowich *et al.*, 2001). It has been proposed that a subregion of the helical domain is related to the function and specific contacts of TMDs and NBDs (Schmitt *et al.*, 2003). In the maltose transporter, it was found that the conserved EAA motif of TMDs interacts with residues in the helical domain (Oldham *et al.*, 2007; Khare *et al.*, 2009). On binding and hydrolysis of ATP, the helical subdomain rotates toward the RecA-like subdomain, moving the signature motif into a favourable position to interact with ATP (Orelle *et al.*, 2010). The far-UV CD spectrum with ATP and Mg^{2+} showed a conformational change of DppD, which was likely to be the result of hydrolysis of ATP. The presence of Mg^{2+} may induce ATP hydrolysis to power the translocation.

A remarkable difference of *T. tengcongensis* DppD from other NBDs is its unique V-shaped C-terminal domain bound with a [4Fe-4S] cluster (Fig. 6a). The [4Fe-4S] cluster can covalently bind to the cysteine residues in the C-terminus, which may stabilize the structure of the C-terminal loops. Recent studies of both the methionine-uptake ABC transporter (Gerber *et al.*, 2008) and the molybdate/tungstate ABC transporter (Kadaba *et al.*, 2008) have established that substrates can bind to the extra domains at the C-terminus as allosteric regulators. A superposition of the structure of *T. tengcongensis* DppD with the Met ABC transporter MetN (Fig. 5b) revealed a root-mean-square deviation (r.m.s.d.) between equivalent C^α atoms in DppD and MetN of 1.83 Å. Interestingly, both of them have additional C-terminal unique domains, while the relative position of the C-terminal domain with respect to the N-terminal domain in the two proteins is different. As noted in *Bacillus subtilis* GPAT, the iron-sulfur cluster is not related to allosteric regulation and does not play a direct role in the enzymatic reaction. It appears to be

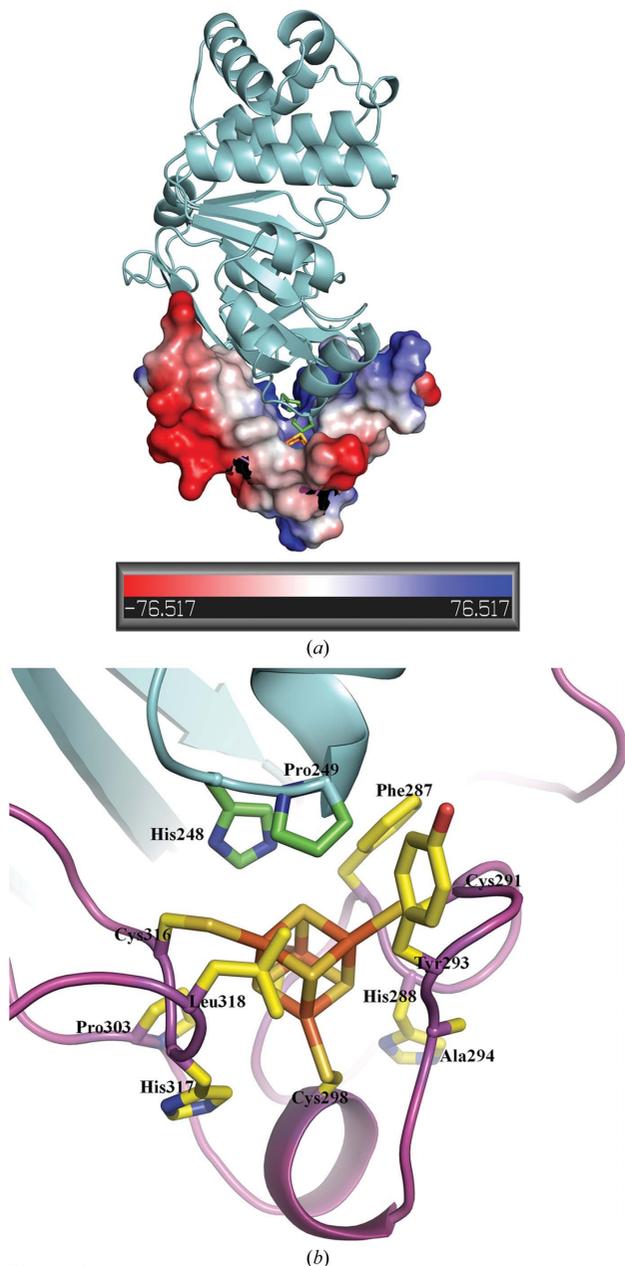


Figure 6
Interaction of the [4Fe-4S] cluster with residues in the C-terminal domain of *T. tengcongensis* DppD. Fe-S centres are shown as sticks; Fe atoms are coloured red and S atoms yellow. (a) The surface electrostatic potential of the V-shaped C-terminal domain. The N-terminal [4Fe-4S] cluster is in the turn of the V-shaped C-terminal domain. (b) Residues that interact with the [4Fe-4S] cluster in the C-terminal domain. The residues that are likely to be involved in interaction with the [4Fe-4S] cluster are shown in sticks and coloured yellow (from the C-terminal domain) or green (from the N-terminal domain).

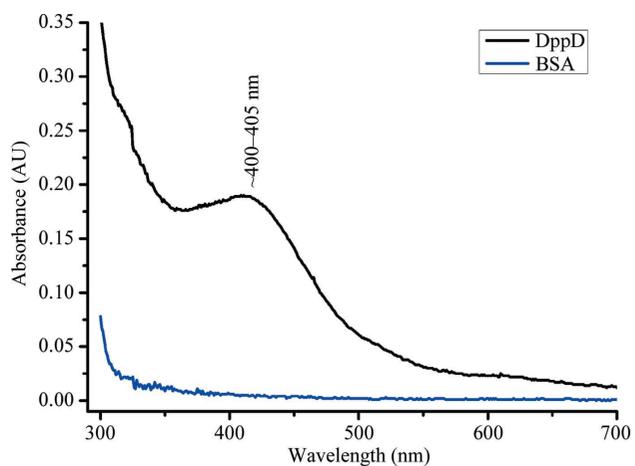


Figure 7
UV-visible absorbance spectroscopic analysis of *T. tengcongensis* DppD. The UV-visible absorption spectrum of freshly purified *T. tengcongensis* DppD shows the presence of a [4Fe-4S] cluster (black line). Purified *T. tengcongensis* DppD (0.8 mg ml⁻¹) and BSA (0.8 mg ml⁻¹) were solubilized in 50 mM sodium phosphate buffer pH 7.5. The black line represents the absorption spectrum of DppD. The broad peak (~400–405 nm) indicates the absorption spectrum of the [4Fe-4S] cluster protein. BSA has no absorption peak at this wavelength.

required for both amino-terminal processing and structural stability of the protein (Makaroff *et al.*, 1986; Grandoni *et al.*, 1989). It has also been reported that the ABC-type dipeptide/oligopeptide/nickel-transport system is actually the haem transporter in *E. coli* (Létoffé *et al.*, 2006). From the far-UV CD spectrum of DppD, we could conclude that the [4Fe-4S] cluster may participate in electron transfer. We propose that the [4Fe-4S] cluster can stabilize the protein by binding the substrate, thus stabilizing the transition state of the enzymatic reaction as in aconitase (Jarrett, 2005) in order to regulate substrate transport; thus, the C-terminal domain of DppD does not act as an allosteric regulator. The iron-sulfur cluster probably participates in substrate (dipeptide or haem) binding and activation of the protein by electron transfer during the process of transport. The cysteines, histidines and Tyr293 play important roles in this process. Iron-sulfur clusters are mostly found in eukaryotes and have been shown to play important

roles in enzyme catalysis, electron transfer and regulation (Alam *et al.*, 2007). However, how the substrate is transported and the molecular mechanism of the competitive binding of haem and dipeptides require further investigation. Further research on the biological functions of the iron-sulfur cluster in this protein is in progress.

4. Conclusions

We expressed DppD, the nucleotide-binding domain of an ABC transporter, from *T. tengcongensis* in *E. coli* strain C43 and determined its crystal structure bound with ATP, Mg²⁺ and a [4Fe-4S] cluster at 2.89 Å resolution. Structural analysis and comparisons with other ABC transporters revealed that the C-terminus of *T. tengcongensis* DppD forms a novel structural fold and is bound with a [4Fe-4S] cluster. The iron-sulfur cluster may participate in substrate binding to regulate and stabilize the protein in the ABC transport system. The three-dimensional structure of *T. tengcongensis* DppD will help us to further understand the properties of ABC transporters, the functions of NBDs and the regulation of the iron-sulfur cluster. The biological relevance of this iron-sulfur cluster in transporting substrates requires further investigation.

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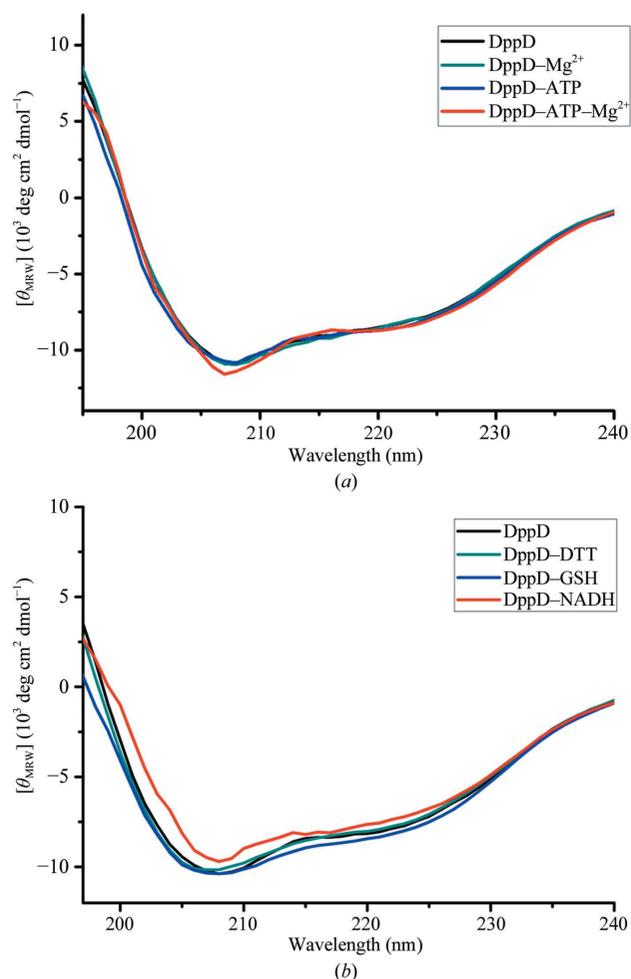


Figure 8
Far-UV circular-dichroism (CD) spectra of *T. tengcongensis* DppD with ATP, Mg²⁺ and various reductants. Purified *T. tengcongensis* DppD (0.1 mg ml⁻¹) was solubilized in 50 mM sodium phosphate buffer pH 7.5. (a) Far-UV CD spectra of DppD with Mg²⁺ (dark cyan), ATP (blue) and ATP-Mg²⁺ (red). (b) Far-UV CD spectra of DppD with DTT (dark cyan), GSH (blue) and NADH (red).

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