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Crystallization and preliminary X-ray analysis of aspartate transcarbamoylase from the parasitic protist *Trypanosoma cruzi*

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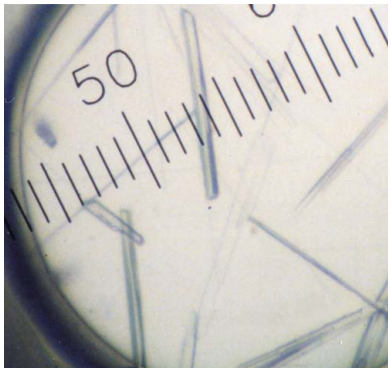


Aspartate transcarbamoylase (ATCase), the second enzyme of the *de novo* pyrimidine-biosynthetic pathway, catalyzes the production of carbamoyl aspartate from carbamoyl phosphate and L-aspartate. In contrast to *Escherichia coli* ATCase and eukaryotic CAD multifunctional fusion enzymes, *Trypanosoma cruzi* ATCase lacks regulatory subunits and is not part of the multifunctional fusion enzyme. Recombinant *T. cruzi* ATCase expressed in *E. coli* was purified and crystallized in a ligand-free form and in a complex with carbamoyl phosphate at 277 K by the sitting-drop vapour-diffusion technique using polyethylene glycol 3350 as a precipitant. Ligand-free crystals (space group $P1$, unit-cell parameters a = 78.42, b = 79.28, c = 92.02 Å, α = 69.56, β = 82.90, γ = 63.25°) diffracted X-rays to 2.8 Å resolution, while those cocrystallized with carbamoyl phosphate (space group $P2_1$, unit-cell parameters a = 88.41, b = 158.38, c = 89.00 Å, β = 119.66°) diffracted to 1.6 Å resolution. The presence of two homotrimers in the asymmetric unit (38 kDa × 6) gives V_M values of 2.3 and 2.5 Å^3 Da^{-1} for the $P1$ and $P2_1$ crystal forms, respectively.

1. Introduction

Chagas disease is a serious tropical disease that is endemic in Central and South America, affecting approximately 16–18 million people in these areas. The causative agent is a flagellate parasitic protist, *Trypanosoma cruzi*, which is transmitted by blood-feeding reduviid bugs. Manifestations of Chagas disease include severe cardiomyopathy, digestive injuries and neural disorders resulting from gradual tissue destruction caused by the parasite. Because nifurtimox and benznidazole, which are the currently used drugs for the treatment of Chagas disease, are toxic and ineffective in the chronic phase, the development of new chemotherapeutic drugs is urgently required (Urbina, 2002).

Pyrimidine biosynthesis is indispensable to all organisms and is achieved *via* the *de novo* and/or salvage pathways. *T. cruzi* possesses both pathways and their balance varies at different developmental stages of the parasite. Since the amastigote stage essentially relies on the *de novo* pathway (Gutteridge & Gaborak, 1979), in which uridine 5′-monophosphate is produced through a series of six enzymatic reactions, the enzymes of the *de novo* pathway therefore provide a greater potential as the primary targets of chemotherapy (Urbina & Docampo, 2003).

Aspartate transcarbamoylase (ATCase; EC 2.1.3.2), the second enzyme of the *de novo* pyrimidine-biosynthetic pathway, catalyzes the production of carbamoyl aspartate from carbamoyl phosphate (CP) and L-aspartate. *Escherichia coli* ATCase is a well known allosteric enzyme and is comprised of catalytic and regulatory subunits, the latter of which carries the binding site of the feedback inhibitor cytidine 5′-triphosphate (CTP; Gerhart & Pardee, 1964; Gerhart & Schachman, 1965; Shepherdson & Pardee, 1960). X-ray structural analysis of the *E. coli* enzyme demonstrated that the enzyme is composed of two trimers of the catalytic subunit (c) and three dimers of the regulatory subunit (r) to form a $(c_3)_2(r_2)_3$ quaternary structure (Krause *et al.*, 1985; Wiley *et al.*, 1972; Wiley & Lipscomb, 1968). ATCases from different strains of *Yersinia enterocolitica* and *Y. enterocolitica*-like organisms also exhibit the $(c_3)_2(r_2)_3$ structure but are sensitive to feedback inhibition by different

pyrimidine nucleotides (Foltermann *et al.*, 1981). *Bacillus subtilis* ATCase lacks the regulatory subunits (Stevens *et al.*, 1991) and in the hyperthermophile *Aquifex aeolicus* six ATCase chains noncovalently associate with six molecules of dihydroorotase (DHO), the third enzyme of the pyrimidine-biosynthetic pathway, to form a dodecamer (Zhang *et al.*, 2009).

On the other hand, eukaryotic ATCases from animals, fungi and Amoebozoa fuse with carbamoyl-phosphate synthetase II (CPS II; the first enzyme of the *de novo* pathway) and DHO to form a multifunctional fusion protein called CAD (Coleman *et al.*, 1977; Freund & Jarry, 1987; Simmer *et al.*, 1989; Souciet *et al.*, 1987), whose feedback inhibitor (CTP) binding site is located in the CPS II domain (Liu *et al.*, 1994). In contrast, *T. cruzi* ATCase (TcATCase), together with the ATCases from plants and other protists, is not part of the CAD multifunctional enzyme and is virtually insensitive to feedback inhibition by pyrimidine nucleotides (Aoki & Oya, 1987) since the enzyme lacks the regulatory subunit (El-Sayed *et al.*, 2005; Gao *et al.*, 1999). In addition, *N*-(phosphonoacetyl)-L-aspartate (PALA), a specific inhibitor of bacterial and mammalian ATCases (Aoki, 1994), only weakly inhibits TcATCase. Thus, structure determination of TcATCase, as well as of the other *de novo* pyrimidine-biosynthetic enzymes of *T. cruzi*, is considered to be crucial for the rational design of chemotherapeutic agents against Chagas disease.

Currently, the crystal structures of bacterial ATCases from *Escherichia coli* (Honzatko *et al.*, 1982), *Bacillus subtilis* (Stevens *et al.*, 1991), *Pyrococcus abyssi* (Van Boxstael *et al.*, 2003), *Sulfolobus acidocaldarius*, *Moritella profunda* (De Vos *et al.*, 2004, 2007) and *Methanococcus jannaschii* (Vitali & Colaneri, 2008), and of the *A. aeolicus* ATCase–DHO complex (Zhang *et al.*, 2009) have been reported. In the present study, we report the expression, purification, crystallization and preliminary X-ray analysis of TcATCase. This is the first crystallization report of an eukaryotic ATCase.

2. Materials and methods

2.1. Preparation of *T. cruzi* ATCase

The *T. cruzi* ATCase genes were previously cloned by screening the total DNA library of *T. cruzi* Tulahuen strain (Nara *et al.*, 2003). *T. cruzi* Tulahuen possesses three copies of the ATCase gene (*tcact1*, *tcact2* and *tcact3*, with GenBank accession Nos. AB074138, AB074139 and AB074140, respectively) and *tcact2* was selected for expression. The open reading frame of *tcact2* was amplified by PCR using 5′-CGGGATCCATGTTGGAACTGCCGCCAG-3′ and 5′-CGGG-ATCCTCACGCCAAAACGCTCCAC-3′ as the forward and reverse primers, respectively, and then subcloned into the bacterial expression vector pET14b (Novagen, EMD Biosciences Inc., Madison, Wisconsin, USA). The recombinant plasmid was introduced into *E. coli* BL21 (DE3) pLysS (Novagen). The transformant was grown in 1000 ml Luria–Bertani medium containing 50 µg ml^{-1} carbenicillin at 310 K until the absorbance at 600 nm (A_{600}) reached about 0.6. Expression of the recombinant His$_6$-tagged TcATCase was induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside at 310 K for 2 h. The cells were harvested by centrifugation at 5000*g* for 10 min and suspended in 20 ml lysis buffer (20 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl, 40 m*M* imidazole). After disruption of the cells by sonication, the lysate was centrifuged at 15 000*g* at 277 K for 20 min. The supernatant containing the His$_6$-tagged TcATCase was filtrated with a 0.22 µm pore-size filter and loaded onto a His-Trap FF column (1 ml bed volume; GE Healthcare) pre-equilibrated with lysis buffer. The column was then washed with 20 ml lysis buffer and the bound His$_6$-tagged TcATCase was eluted from the column with lysis buffer containing 500 m*M* imidazole. The fractions containing TcATCase were pooled and the buffer was exchanged to 20 m*M* Tris–HCl pH 7.4 using a PD-10 desalting column (GE Healthcare); they were then concentrated to 10 mg ml^{-1} with a centrifugal concentrator tube (Amicon Ultra-4 Ultracel-10K).

The ATCase activity was assayed by monitoring the production of carbamoyl aspartate from CP and L-aspartate by Ceriotti's colorimetric method (Prescott & Jones, 1969) with minor modifications. Briefly, 0.5 ml of a reaction mixture containing 200 m*M* Tris–HCl pH 7.9, 30 m*M* L-aspartate and purified ATCase was pre-incubated in a 1.5 ml quartz cuvette at 310 K for 5 min and the enzymatic reaction was then started by adding CP to a final concentration of 1.3 m*M*. CP was dissolved in ice-cold distilled water just before measurement. After standing for 15 min at 310 K, the reaction was stopped and 0.5 ml of a 1:1 mixture of 0.5% antipyrine in 50% sulfonic acid and 0.8% diacetylmonoxime in 5% acetic acid was added. Colorimetric development of the diazine produced from the carbamoyl aspartate and diacetylmonoxime was performed at 333 K for 2 h in the dark and the A_{466} was measured. The concentration of the carbamoyl aspartate produced was estimated from the A_{466} values of standard solutions containing carbamoyl aspartate at known concentrations. The typical specific activity of the purified TcATCase was 9 µmol min^{-1} mg^{-1} and the K_m values for CP and L-aspartate were estimated to be 0.03 and 29.4 m*M*, respectively.

TcATCase was purified to apparent homogeneity as shown by SDS–PAGE (Fig. 1), with a yield of about 2 mg from a 1000 ml culture. Gel-filtration chromatography with TSK-gel G3000SWXL (7.8 × 300 mm, Tosoh) and dynamic light-scattering analysis using DynaPro Titan (Wyatt Technology) both indicated that the purified enzyme existed as a homotrimer in solution.

2.2. Crystallization and X-ray data collection

All crystallization experiments were performed by the sitting-drop vapour-diffusion technique in 96-well Corning CrystalEX micro-

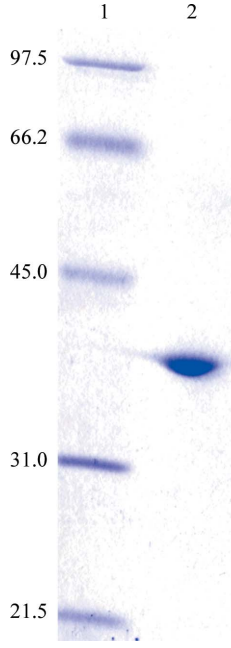


Figure 1
12.5% SDS–PAGE gel stained with Coomassie Brilliant Blue R-250 showing the apparent homogeneity of the purified TcATCase. Lane 1, molecular-weight markers (kDa); lane 2, TcATCase purified using a His-Trap FF column.

Table 1
Statistics of data collection and processing.

Values in parentheses are for the highest resolution shell.

	Ligand-free TcATCase	TcATCase–CP complex
Wavelength (Å)	0.900 (SPring-8 BL44XU)	1.000 (SPring-8 BL41XU)
Space group	*P*1	$P2_1$
Unit-cell parameters (Å, °)	*a* = 78.42, *b* = 79.28, *c* = 92.02, α = 69.56, β = 82.90, γ = 63.25	*a* = 88.41, *b* = 158.38, *c* = 89.00, β = 119.66
Solvent content† (%)	48	51
Frame number	222	180
Resolution range (Å)	50.0–2.8 (2.90–2.80)	50.0–1.60 (1.66–1.60)
No. of reflections	103124	946629
Unique reflections	44850	277190
Mosaicity	0.90	0.21
Redundancy	2.3 (2.3)	3.4 (3.4)
Completeness (%)	97.7 (98.4)	95.3 (91.4)
R_{merge}‡ (%)	7.4 (38.9)	6.5 (39.9)
Mean $I/\sigma(I)$	15.8 (4.4)	11.7 (3.9)

† Assuming the presence of six molecules in the asymmetric unit. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of reflection *hkl*.

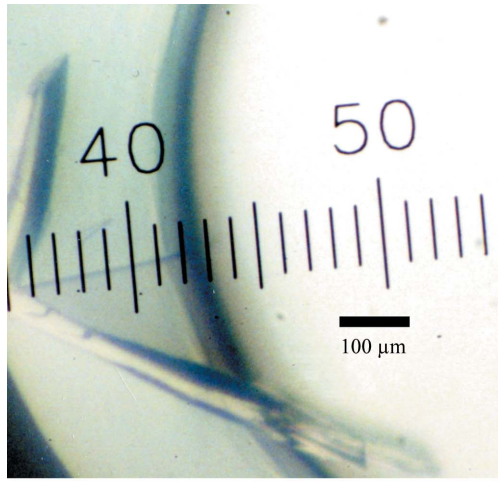
plates with a conical flat bottom (Hampton Research). In the initial screening for crystallization conditions, a 0.5 µl droplet containing around 10 mg ml^{-1} TcATCase dissolved in 20 m*M* Tris–HCl pH 7.4 was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 100 µl reservoir solution at 277 and 293 K. Commercially available screening kits purchased from Hampton Research (Crystal Screen, Crystal Screen 2, Crystal Screen Lite and SaltRx) and Emerald BioStructures (Wizard I, Wizard II, Cryo I and Cryo II) were used as reservoir solutions. Of the 434 conditions screened, condition No. 10 from Crystal Screen Lite [15%(*w*/*v*) PEG 4000, 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate] gave tiny plate-shaped crystals at 277 K. The condition was then optimized using 154 conditions by varying the PEG concentration (4–16%), the buffer pH (3.6–5.6) and the temperature (277 and 293 K) using PEG 3350, which is a monodisperse and high-purity polyethylene glycol obtained from Hampton Research, as a precipitant. For the best condition found, the effects of 72 additives on crystallization were examined using Additive Screen kits (Hampton Research) according to the manufacturer's instruction. Cobalt chloride and glycerol improved the size of the crystals; moreover, the addition of both additives gave thicker crystals. Currently, crystals larger than 0.2 × 0.1 × 0.01 mm can be grown at 277 K from reservoir solution containing 8–10%(*w*/*v*) PEG 3350, 0.1 *M* acetate buffer pH 4.6, 0.2 *M* ammonium acetate, 0.01 *M* cobalt chloride and 3% glycerol. For cocrystallization with CP, a freshly prepared 100 m*M* CP solution was added to the TcATCase solution to give a final concentration of 5 m*M* and crystallization was conducted as described above. Crystals of similar shape and size were obtained.

X-ray diffraction experiments were performed on the BL44XU beamline (λ = 0.900 Å; equipped with a Bruker DIP-6040 detector system) and the BL41XU beamline (λ = 1.000 Å; equipped with a Rayonix CCD MX225HE detector) at SPring-8 (Harima, Japan) and on the BL17A beamline (λ = 1.000 Å; equipped with an ADSC Quantum 270 detector) at Photon Factory (Tsukuba, Japan). A crystal mounted in a nylon loop was transferred and soaked briefly in reservoir solution supplemented with 20%(*w*/*v*) glycerol and then flash-cooled by rapidly submerging it in liquid nitrogen. Diffraction data were collected under cryocooled conditions at 100 K. Images were recorded with an oscillation angle of 1°, an exposure time of 1 s per frame and a crystal-to-detector distance of 150 mm and were processed with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

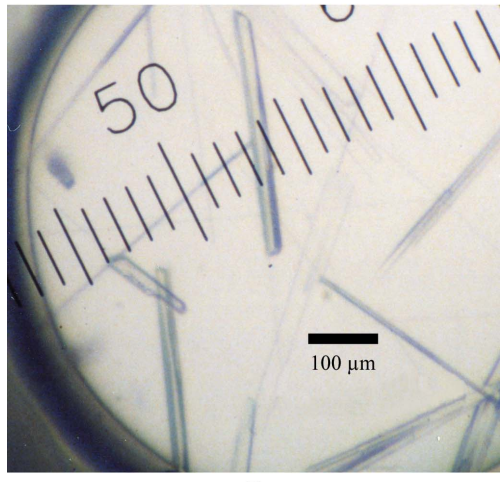
3. Results and discussion

His_6-tagged TcATCase (38 kDa) could be purified to homogeneity by one-step purification using His-Trap FF column chromatography (Fig. 1). The molecular weight of the purified enzyme estimated by gel-filtration chromatography (134 kDa) and dynamic light scattering (102 kDa; R_h = 4.3 nm, polydispersity = 13.9%, mass = 100%) indicated that the enzyme probably exists as a homotrimer in solution.

Crystals of ligand-free TcATCase were obtained at 277 K from reservoir solution containing 8–10%(*w*/*v*) PEG 3350, 0.1 *M* acetate buffer pH 4.6, 0.2 *M* ammonium acetate, 0.01 *M* cobalt chloride and 3% glycerol and reached maximum dimensions within two weeks (Fig. 2*a*). TcATCase complexed with CP was also crystallized by the cocrystallization method under the same conditions within 2 d (Fig. 2*b*). Analyses of the symmetry and systematic absences in the recorded diffraction patterns indicated that the crystals of ligand-free TcATCase belonged to the triclinic space group *P*1, with unit-cell parameters *a* = 78.42, *b* = 79.28, *c* = 92.02 Å, α = 69.56, β = 82.90, γ = 63.25°, whereas those of TcATCase complexed with CP belonged to the monoclinic space group $P2_1$, with unit-cell parameters *a* = 88.41, *b* = 158.38, *c* = 89.00 Å, β = 119.66°. Assuming the presence of two His_6-tagged TcATCase trimers (6 × 38 kDa) in the asymmetric unit, the V_M values are 2.3 and 2.5 Å^3 Da^{-1} for the triclinic and monoclinic



(*a*)



(*b*)

Figure 2
Crystals of (*a*) ligand-free TcATCase and (*b*) TcATCase complexed with carbamoyl phosphate obtained by the sitting-drop vapour-diffusion method using PEG 3350 as a precipitant.

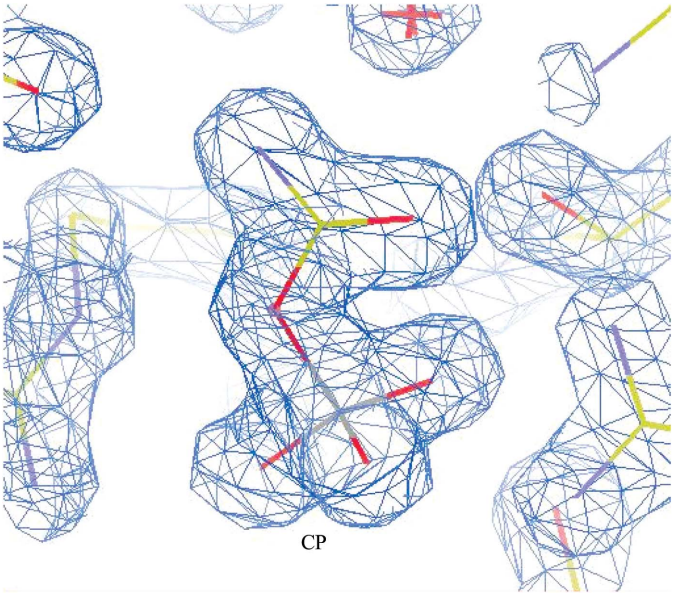


Figure 3
The $2F_o - F_c$ electron-density map around the bound CP in the TcATCase–CP complex structure contoured at 2σ. The structure is currently refined to $R = 0.151$ (1.6 Å resolution).

crystal forms, respectively; these values are within the range commonly observed for protein crystals (Matthews, 1968). A data set to 2.8 Å resolution (44 850 unique reflections) was obtained for ligand-free TcATCase after merging 103 124 reflections recorded on 222 images, while 277 190 unique reflections to 1.6 Å resolution were produced from 946 629 measured reflections on 180 images for TcATCase complexed with CP. Statistics of data collection and processing are shown in Table 1.

Attempts to solve the structures of both crystal forms by the molecular-replacement method with the *MOLREP* program (Vagin & Teplyakov, 1997) as implemented within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) were carried out using the homotrimeric structure of the catalytic subunit of *P. abyssi* ATCase (PDB code 1ml4; 40.1% amino-acid sequence identity with TcATCase), which showed a higher identity to TcATCase than to the ATCases from *E. coli* (PDB code 2atc; 36.5% identity), *B. subtilis* (PDB code 2at2; 32.5% identity), *S. acidocaldarius* (PDB code 2be9; 36.8% identity), *Moritella profunda* (PDB code 2be7; 37.7% identity), *Methanococcus jannaschii* (PDB code 2rgw; 38.6% identity) and *A. aeolicus* (PDB code 3d6n; 21.2% identity). A promising solution with two homotrimers per asymmetric unit was obtained for both the ligand-free TcATCase (correlation coefficient and *R* factor of 0.551 and 49.0%, respectively) and the TcATCase–CP complex (correlation coefficient and *R* factor of 0.615 and 50.9%, respectively). The models were subsequently subjected to rigid-body refinement and gave *R* factors of 44.8% and 44.4% for ligand-free TcATCase and the TcATCase–CP complex, respectively. Refinement of the structures is currently in the final stages. Clear electron densities for the entire protein part and the bound CP were observed for the TcATCase–CP complex (Fig. 3), but the loop of the CP-binding site (Cys85–Thr95) was disordered in the ligand-free TcATCase. The suppression of the flexibility of the loop by the bound CP may lead to the different crystal form and the enhanced X-ray diffraction of the crystals of the TcATCase–CP complex. We are now trying to prepare crystals of TcATCase complexed with potential inhibitors found in the Chemical Library of the Chemical Biology Research Initiative, University of Tokyo by *in silico* screening. Since the enzymes of the *de novo* pyrimidine-biosynthetic pathway have great potential as primary targets of chemotherapy (Urbina & Docampo, 2003), the detailed structures of TcATCase complexed with these compounds will help in structure-based drug design aimed at Chagas disease.

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References

Aoki, T. (1994). *Jpn. J. Parasitol.* **43**, 1–10.
Aoki, T. & Oya, H. (1987). *Comput. Biochem. Physiol. B*, **87**, 143–150.
Coleman, P. F., Suttle, D. P. & Stark, G. R. (1977). *J. Biol. Chem.* **252**, 6379–6385.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
De Vos, D., Van Petegem, F., Remaut, H., Legrain, C., Glansdorff, N. & Van Beeumen, J. J. (2004). *J. Mol. Biol.* **339**, 887–900.
De Vos, D., Xu, Y., Hulpiau, P., Vergauwen, B. & Van Beeumen, J. J. (2007). *J. Mol. Biol.* **365**, 379–395.
El-Sayed, N. M. *et al.* (2005). *Science*, **309**, 409–415.
Foltermann, K. F., Wild, J. R., Zink, D. L. & O'Donovan, G. A. (1981). *Curr. Microbiol.* **6**, 43–47.
Freund, J. N. & Jarry, B. P. (1987). *J. Mol. Biol.* **193**, 1–13.
Gao, G., Nara, T., Nakajima-Shimada, J. & Aoki, T. (1999). *J. Mol. Biol.* **285**, 149–161.
Gerhart, J. C. & Pardee, A. B. (1964). *Fed. Proc.* **23**, 727–735.
Gerhart, J. C. & Schachman, H. K. (1965). *Biochemistry*, **4**, 1054–1062.
Gutteridge, W. E. & Gaborak, M. (1979). *Int. J. Biochem.* **10**, 415–422.
Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Ewards, B. F., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C. & Lipscomb, W. N. (1982). *J. Mol. Biol.* **160**, 219–263.
Krause, K. L., Volz, K. W. & Lipscomb, W. N. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 1643–1647.
Liu, X., Guy, H. I. & Evans, D. R. (1994). *J. Biol. Chem.* **269**, 27747–27755.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nara, T., Hirayama-Noguchi, Y., Gao, G., Murai, E., Annoura, T. & Aoki, T. (2003). *Int. J. Parasitol.* **33**, 845–852.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Prescott, L. M. & Jones, M. E. (1969). *Anal. Biochem.* **32**, 408–419.
Shepherdson, M. & Pardee, A. B. (1960). *J. Biol. Chem.* **235**, 3233–3237.
Simmer, J. P., Kelly, R. E., Scully, J. L., Grayson, D. R., Rinker, A. G. Jr, Bergh, S. T. & Evans, D. R. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 4382–4386.
Souciet, J. L., Potier, S., Hubert, J. C. & Lacroute, F. (1987). *Mol. Gen. Genet.* **207**, 314–319.
Stevens, R. C., Reinisch, K. M. & Lipscomb, W. N. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 6087–6091.
Urbina, J. A. (2002). *Curr. Pharm. Des.* **8**, 287–295.
Urbina, J. A. & Docampo, R. (2003). *Trends Parasitol.* **19**, 495–501.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
Van Boxstael, S., Cunin, R., Khan, S. & Maes, D. (2003). *J. Mol. Biol.* **326**, 203–216.
Vitali, J. & Colaneri, M. J. (2008). *Acta Cryst.* F**64**, 776–780.
Wiley, D. C., Evans, D. R., Warren, S. G., McMurray, C. H., Edwards, B. F., Franks, W. A. & Lipscomb, W. N. (1972). *Cold Spring Harb. Symp Quant. Biol.* **36**, 285–290.
Wiley, D. C. & Lipscomb, W. N. (1968). *Nature (London)*, **218**, 1119–1121.
Zhang, P., Martin, P. D., Purcarea, C., Vaishnav, A., Brunzelle, J. S., Fernando, R., Guy-Evans, H. I., Evans, D. R. & Edwards, B. F. (2009). *Biochemistry*, **48**, 766–778.