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Expression, purification, crystallization and preliminary X-ray crystallographic analysis of the L,D-transpeptidase Ldt_{Mt1} from *Mycobacterium tuberculosis*

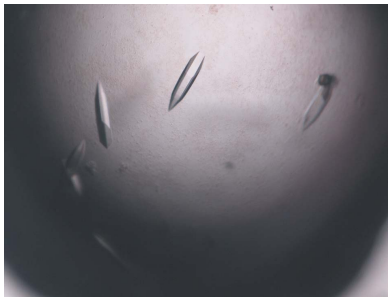
Mycobacterium tuberculosis is capable of adapting to prolonged periods of dormancy, a state which is resistant to killing by antimycobacterial agents. The L,D-transpeptidation reaction catalysed by the L,D-transpeptidase Ldt_{Mt1} is likely to play an essential role in the adaptation of *M. tuberculosis* to its dormant state. Ldt_{Mt1} has been successfully crystallized using vapour-diffusion methods. The crystals of this protein belonged to space group $P6_522$, with unit-cell parameters $a = 57.25$, $b = 57.25$, $c = 257.96$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. Diffraction data have also been collected from a selenomethionine derivative to 2.9 Å resolution. Model building using the phases derived from the multiwavelength anomalous dispersion experiment is in progress.

1. Introduction

Tuberculosis (TB), an infectious airborne disease, is a major global health problem. Each year, there are about nine million new cases of TB and close to two million deaths (Kaufmann, 2008; Kaufmann & McMichael, 2005). The aetiological agent of TB, *Mycobacterium tuberculosis*, can act both intrapulmonarily and extrapulmonarily (Kaufmann, 2008; Esposito *et al.*, 2008, 2010, 2012) and is able to survive in humans without producing apparent symptoms (Kaufmann, 2008). However, this apparent dormancy can develop into active disease even decades after initial infection, when the immune response weakens (Kaufmann, 2008; Keep *et al.*, 2006). Since about one-third of the world's population is infected with dormant *M. tuberculosis*, the risk of disease reactivation is troublesome. For this reason, the Global Plan to Stop TB 2011–2015 of the World Health Organization recognizes that effective new treatments for latent TB infection are necessary to reach full TB elimination. Over the years, it has become clear that reactivation from dormancy, growth and division of bacteria require modelling of the cell-wall peptidoglycan (PG; Mukamolova, Yanopolskaya *et al.*, 1998; Mukamolova, Kaprelyants *et al.*, 1998; Ruggiero *et al.*, 2009, 2010; Ruggiero, Marchant *et al.*, 2012), in a complex mechanism which also involves STPK kinases (Squeglia *et al.*, 2011; Ruggiero *et al.*, 2011; Dworkin & Shah, 2010; Ruggiero, De Simone *et al.*, 2012). However, the precise nature of *M. tuberculosis* cells associated with latent tuberculosis is not yet clear.

L,D-Transpeptidases are responsible for an alternate transpeptidation pathway which leads to the formation of 3,3 cross-links instead of the typical 4,3 cross-links catalysed by penicillin-binding proteins (Fig. 1; Dubée *et al.*, 2012; Gupta *et al.*, 2010). The genome of *M. tuberculosis* contains five putative L,D-transpeptidases (Ldt_{Mt1}, Ldt_{Mt2}, MT0202, MT0501 and MT1477, with sequence identities ranging from 35 to 45%), of which only Ldt_{Mt1} and Ldt_{Mt2} have actually been shown to be endowed with L,D-transpeptidase activity (Gupta *et al.*, 2010).

Cross-links generated by L,D-transpeptidation (3,3 cross-links; Fig. 1) are predominant (80%) in the peptidoglycan of *M. tuberculosis* in the stationary phase (Lavollay *et al.*, 2008). Consistently, microarray analyses have shown that the gene encoding Ldt_{Mt1} is upregulated 17-fold under nutrient starvation (Betts *et al.*, 2002). Also, analysis of the peptidoglycan structure in a stationary phase culture of a mutant lacking Ldt_{Mt2} showed that 3,3 cross-links were synthesized by Ldt_{Mt1} (Gupta *et al.*, 2010). These findings suggested



that the L,D-transpeptidation reaction catalysed by Ldt_{Mt1} is likely to play an essential role in the adaptation of *M. tuberculosis* to the stationary phase (Lavollay *et al.*, 2008; Gupta *et al.*, 2010) and identified Ldt_{Mt1} as an attractive target for the development of efficient drugs.

In this study, we have successfully expressed, purified and crystallized Ldt_{Mt1} using vapour-diffusion methods. Analysis of the PFAM database (Finn *et al.*, 2006) shows that Ldt_{Mt1} contains two domains: an N-terminal domain, the structure of which cannot unambiguously be predicted, and a C-terminal L,D-transpeptidase catalytic domain (residues 125–249, Ykud family, PF03734). The catalytic domain of Ldt_{Mt1} shares 29% sequence identity with that of the L,D-transpeptidase from *Enterococcus faecium* (Biarrotte-Sorin *et al.*, 2006). The results obtained here will allow us to gather clues on *M. tuberculosis* adaptation phenomena and on the molecular basis for the design of enzyme inhibitors with therapeutic interest.

2. Experimental methods

2.1. Cloning, expression and purification

Ldt_{Mt1} is thought to contain a signal peptide at its N-terminus (residues 1–28) as predicted by *SignalP* 3.0 (Bendtsen *et al.*, 2004). We cloned, expressed and purified Ldt_{Mt1} deprived of its signal peptide starting at residue 32. The primers Rv0116cF, 5′-TTCCATGGCGC-CACTCCAACCGATCCCA-3′, and Rv0116cR, 5′-CCCAAGCT-TACTAGCCGACCACCTCAATGGGA-3′, containing *Nco*I and *Hin*dIII restriction sites were used to amplify the Ldt_{Mt1} coding sequence starting at residue Pro32 from the H37Rv strain of *M. tuberculosis*. The PCR product (660 bp) was cloned into the pETM-11 expression vector (Novagen), giving a protein with a TEV-cleavable N-terminal poly-His tag. *Escherichia coli* BL21 (DE3) strain was co-transformed with the resulting recombinant plasmid and the pREP4 GroESL plasmid. The overnight culture was used to inoculate 1 l LB medium containing 50 µg l^{-1} kanamycin; protein induction was performed by the addition of 0.5 m*M* IPTG at 289 K when an OD_{600} value of 0.7 was reached. After approximately 16 h, the cells were harvested and the protein was isolated by sonicating cell pellets resuspended in 30 ml lysis buffer {50 m*M* Tris–HCl, 150 m*M* NaCl, 5%(*v*/*v*) glycerol, 0.01% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) pH 7.5} in the presence of a protease-inhibitor cocktail (Roche Diagnostics). The crude cell extract was cleared by centrifugation at 39 000*g* and the supernatant was loaded onto a 5 ml Ni–NTA column connected to an ÄKTA FPLC system (GE Healthcare) equilibrated with binding buffer [50 m*M* Tris–HCl, 300 m*M* NaCl, 5%(*v*/*v*) glycerol, 0.01% CHAPS, 10 m*M* imidazole pH 7.5]. A high NaCl concentration was used to reduce nonspecific binding by impurities during nickel-affinity chromatography (Kim *et al.*, 2008). After washing with ten volumes of binding buffer, a step gradient of imidazole (40–250–500 m*M*) was applied to elute the protein. The fractions containing Ldt_{Mt1} were pooled and dialysed against 2 l of 50 m*M* Tris–HCl, 200 m*M* NaCl, 5%(*v*/*v*) glycerol, 0.01% CHAPS pH 7.5 with one exchange at 277 K. After removal of the His tag using TEV protease, the cleaved protein was purified by a second Ni–NTA affinity chromatography step. A final purification step was carried out using a Superdex 200 10/30 (GE Healthcare) column [50 m*M* Tris–HCl, 200 m*M* NaCl, 5%(*v*/*v*) glycerol, 0.01% CHAPS pH 7.5]. All purification steps were carried out at 277 K. The homogeneity of the protein was tested by SDS–PAGE. Freshly concentrated protein was used for crystallization experiments.

A selenomethionine derivative of Ldt_{Mt1} ($_{SeMet}Ldt_{Mt1}$) was prepared by growing *E. coli* BL21 (DE3) cells expressing the recombinant protein in 1 l minimal medium (M9) containing 0.4%(*w*/*v*) glucose, 1 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, 50 µg l^{-1} kanamycin and 1 m*M* thiamine at 310 K. After reaching an OD_{600} of 0.7, an amino-acid mixture (50 mg l^{-1} Ile, Leu and Val and 100 mg l^{-1} Phe, Thr and Lys) was added to the culture, which was then shifted to 289 K. After equilibration, 60 mg l^{-1} seleno-L-methionine was added and induction was performed. The labelled protein was purified as described above.

2.2. Crystallization experiments

Crystallization was performed at 293 K by hanging-drop vapour-diffusion methods. Preliminary crystallization trials were carried out using a crystallization workstation (Hamilton Robotics). 192 high-throughput reagents (Hampton Research) were tested. Optimization of the crystallization conditions was performed by fine-tuning the protein and precipitant concentrations using a drop consisting of 1 µl protein solution and 1 µl precipitant solution and a reservoir volume of 400 µl.

2.3. Data collection and processing

Preliminary diffraction data at 2.9 Å were collected in-house at 100 K using a Rigaku MicroMax-007 HF generator producing Cu *Kα* radiation and equipped with a Saturn 944 CCD detector. Higher diffraction data for both native Ldt_{Mt1} and $_{SeMet}Ldt_{Mt1}$ were collected on synchrotron beamline BM14 at the ESRF, Grenoble, France at 100 K. Cryoprotection of the crystals was achieved by rapid soaking (1–2 s) in a solution of 14%(*v*/*v*) glycerol. An oscillation range of 1° and an X-ray dose corresponding to about 4 s exposure were adopted for all experiments. The data sets were scaled and merged using the *HKL*-2000 program package (Otwinowski & Minor, 1997). Multi-wavelength anomalous diffraction (MAD) was carried out using three different wavelengths determined from the selenium absorption spectrum and the native data set (Table 1).

2.4. Structure determination

The C-terminal part of Ldt_{Mt1} (residues 123–249) shares 29% sequence identity with the L,D-transpeptidase from *E. faecium*

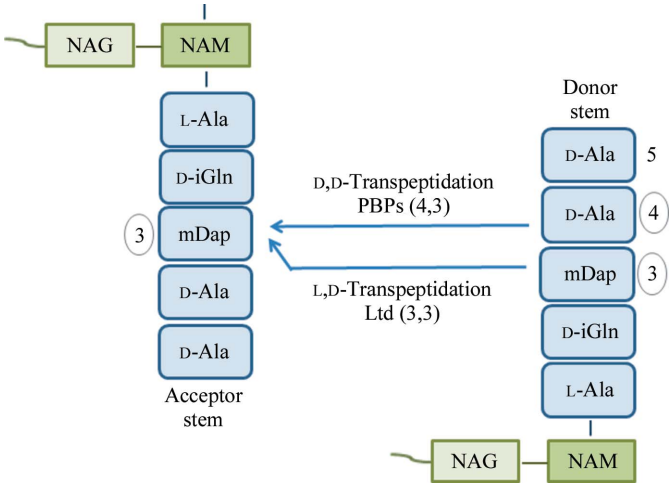


Figure 1
Schematic representation of peptidoglycan transpeptidation. L,D-Transpeptidases cleave the mDap3—D-Ala4 bond and link mDap3 of the donor to the acceptor stems (3,3 cross-link).

Table 1
Data-collection statistics.

Values in parentheses are for the highest-resolution shell.

	SeMet derivative			Native
	Peak	Inflection point	Remote	
Beamline	BM14	BM14	BM14	BM14
Space group	$P6_522$	$P6_522$	$P6_522$	$P6_522$
Unit-cell parameters (Å)				
a	56.80	56.85	56.84	57.114
b	56.80	56.85	56.84	57.114
c	256.76	257.08	257.05	256.997
Resolution range (Å)	50.00–2.95 (3.01–2.95)	50.00–2.95 (3.01–2.95)	50.00–2.90 (2.95–2.90)	50.00–1.90 (1.93–1.90)
Wavelength (Å)	0.9784	0.9786	0.9724	0.9786
Mosaicity (°)	0.9	0.8	0.8	0.5
Average multiplicity	9.0 (6.2)	6.1 (5.6)	6.7 (6.9)	9.6 (5.6)
Unique reflections	5590	5742	6076	20581
Completeness (%)	97.8 (98.5)	97.4 (98.8)	99.5 (99.5)	98.9 (96.0)
R_{merge}† (%)	7.4 (44.5)	7.0 (45.1)	4.7 (34.8)	7.6 (41.3)
Average $I/\sigma(I)$	18.1 (2.1)	23.2 (3.0)	25.6 (4.1)	29.9 (2.6)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean value of the intensity of reflection hkl.

(Biarrotte-Sorin *et al.*, 2006; PDB entry 1zat). Using this structure, we attempted molecular replacement (MR) using the program *Phaser* (McCoy *et al.*, 2007). However, all trials were unsuccessful. The structure of the enzyme was solved by MAD using data sets collected at wavelengths optimized for SeMet (Table 1) and a data set from a native crystal. Phases were derived using *SHELXD* (Sheldrick, 2008) implemented in the *Auto-Rickshaw* pipeline (Panjikar *et al.*, 2005). These phases were improved by solvent-flattening and phase-extension routines using the program *RESOLVE* (Terwilliger, 2003*b*).

3. Results and discussion

The gene of Ldt_{Mt1} consists of 660 bp encoding the protein sequence (residues 32–251) deprived of its N-terminal signal peptide. This construct has been successfully cloned, expressed, purified and crystallized using vapour-diffusion methods. The purified Ldt_{Mt1} showed a single band of approximately 24 kDa on SDS–PAGE, which is in good agreement with the molecular mass derived by mass spectrometry (23 935 Da). The initial screening using commercially available solutions revealed some initial indications of crystallization in four conditions containing polyethylene glycol (PEG) 3350. Fine-tuning of crystallization conditions using the hanging-drop method produced crystals suitable for X-ray diffraction studies (Fig. 2). The best crystals of Ldt_{Mt1} with dimensions of 0.05 × 0.05 × 0.2 mm were obtained by mixing protein solution at 5 mg ml^{-1} with 0.16 *M* ammonium citrate tribasic pH 7.0, 16%(*w*/*v*) PEG 3350 (Fig. 2).

The crystals diffracted to 1.90 Å resolution on the BM14 beamline at the ESRF (Fig. 3) and belonged to space group $P6_522$, with unit-cell parameters a = 57.25, b = 57.25, c = 257.96, α = 90, β = 90, γ = 120.00 (Table 1). Matthews coefficient calculations suggested the presence of one molecule in the asymmetric unit (V_M = 2.5 Å^3 Da^{-1}, 52% solvent content; Matthews, 1968). All attempts to solve the structure by MR using the available structure of the L,D-transpeptidase from *E. faecium* (29% sequence identity between residues 123–249; PDB entry 1zat) were unsuccessful. This suggested that the available model was not suitable for use in MR. Therefore, a SeMet derivative of the protein was prepared in order to perform MAD experiments. Crystals of $_{SeMet}Ldt_{Mt1}$ were obtained using the same

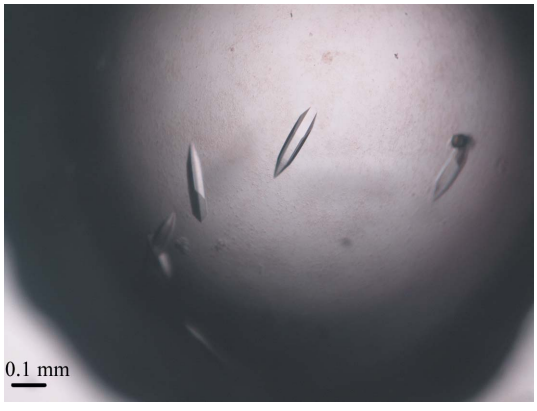


Figure 2
Image of typical Ldt_{Mt1} crystals grown by vapour diffusion using 0.16 *M* ammonium citrate tribasic pH 7.0, 16%(*w*/*v*) polyethylene glycol 3350 as precipitant.

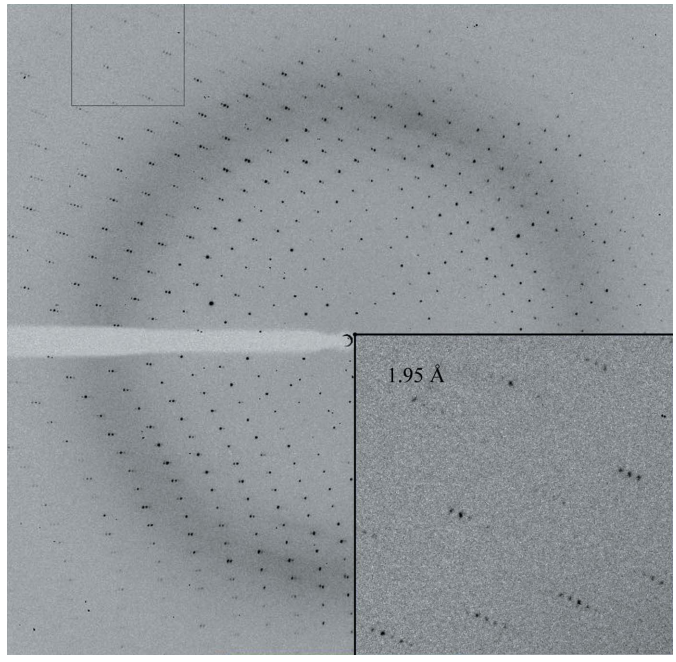


Figure 3
Diffraction pattern of a native Ldt_{Mt1} crystal.

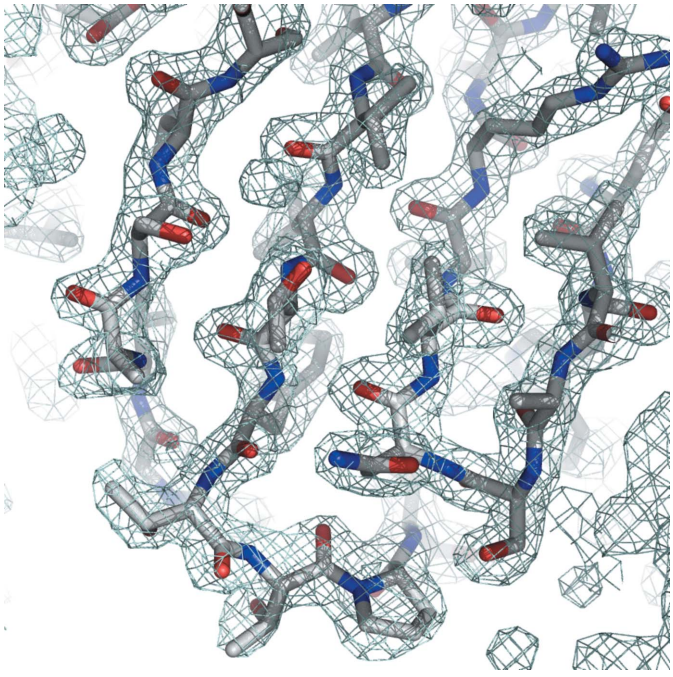


Figure 4
($2F_o - F_c$) electron-density map contoured at 2.0σ. The density clearly shows a β-sheet region of the N-terminal domain of Ldt_{Mt1}.

procedure as adopted for the native protein. The best crystals of $_{SeMet}Ldt_{Mt1}$ grew using protein solution at 3 mg ml^{-1} mixed with 0.18 *M* ammonium citrate tribasic pH 7.0, 18%(*w*/*v*) PEG 3350 and diffracted to 2.95 Å resolution on the BM14 beamline at the ESRF. For peak and inflection wavelength determination, a fluorescence scan was recorded from a single $_{SeMet}Ldt_{Mt1}$ crystal.

The *Auto-Rickshaw* pipeline was used to combine phases derived from the three wavelengths corresponding to peak, inflection and remote regions of the fluorescence scan (Panjikar *et al.*, 2005). *SHELXD* identified four selenium sites in the asymmetric unit of the protein: three in the protein sequence plus one at the N-terminus (Sheldrick, 2008). The initial set of phases was improved using the solvent-flattening and phase-extension methods implemented in the program *RESOLVE* (Terwilliger, 2003*a*,*b*). Manual model-building sessions aimed at defining the complete structure of Ldt_{Mt1} are in progress (Fig. 4). This work will produce precious information for understanding the structural features associated with Ldt_{Mt1} activity and inhibition.

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References

Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). *J. Mol. Biol.* **340**, 783–795.
Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. & Duncan, K. (2002). *Mol. Microbiol.* **43**, 717–731.
Biarrotte-Sorin, S., Hugonnet, J. E., Delfosse, V., Mainardi, J. L., Gutmann, L., Arthur, M. & Mayer, C. (2006). *J. Mol. Biol.* **359**, 533–538.
Dubée, V., Triboulet, S., Mainardi, J. L., Ethève-Quelquejeu, M., Gutmann, L., Marie, A., Dubost, L., Hugonnet, J. E. & Arthur, M. (2012). *Antimicrob. Agents Chemother.* **56**, 4189–4195.
Dworkin, J. & Shah, I. M. (2010). *Nature Rev. Microbiol.* **8**, 890–896.
Esposito, C., Cantisani, M., D'Auria, G., Falcigno, L., Pedone, E., Galdiero, S. & Berisio, R. (2012). *FEBS Lett.* **586**, 659–667.
Esposito, C., Carullo, P., Pedone, E., Graziano, G., Del Vecchio, P. & Berisio, R. (2010). *FEBS Lett.* **584**, 1091–1096.
Esposito, C., Pethoukov, M. V., Svergun, D. I., Ruggiero, A., Pedone, C., Pedone, E. & Berisio, R. (2008). *J. Bacteriol.* **190**, 4749–4753.
Finn, R. D., Mistry, J., Schuster-Böckler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., Eddy, S. R., Sonnhammer, E. L. & Bateman, A. (2006). *Nucleic Acids Res.* **34**, D247–D251.
Gupta, R., Lavollay, M., Mainardi, J. L., Arthur, M., Bishai, W. R. & Lamichhane, G. (2010). *Nature Med.* **16**, 466–469.
Kaufmann, S. H. (2008). *Nature (London)*, **453**, 295–296.
Kaufmann, S. H. & McMichael, A. J. (2005). *Nature Med.* **11**, S33–S44.
Keep, N. H., Ward, J. M., Cohen-Gonsaud, M. & Henderson, B. (2006). *Trends Microbiol.* **14**, 271–276.
Kim, Y. *et al.* (2008). *Adv. Protein Chem. Struct. Biol.* **75**, 85–105.
Lavollay, M., Arthur, M., Fourgeaud, M., Dubost, L., Marie, A., Veziris, N., Blanot, D., Gutmann, L. & Mainardi, J. L. (2008). *J. Bacteriol.* **190**, 4360–4366.
McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mukamolova, G. V., Kaprelyants, A. S., Young, D. I., Young, M. & Kell, D. B. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 8916–8921.
Mukamolova, G. V., Yanopolskaya, N. D., Kell, D. B. & Kaprelyants, A. S. (1998). *Antonie Van Leeuwenhoek*, **73**, 237–243.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Panjikar, S., Parthasarathy, V., Lamzin, V. S., Weiss, M. S. & Tucker, P. A. (2005). *Acta Cryst.* D**61**, 449–457.
Ruggiero, A., De Simone, P., Smaldone, G., Squeglia, F. & Berisio, R. (2012). *Curr. Protein Pept. Sci.* **13**, 756–766.
Ruggiero, A., Marasco, D., Squeglia, F., Soldini, S., Pedone, E., Pedone, C. & Berisio, R. (2010). *Structure*, **18**, 1184–1190.
Ruggiero, A., Marchant, J., Squeglia, F., Makarov, V., De Simone, A. & Berisio, R. (2012). *J. Biomol. Struct. Dyn.*, doi:10.1080/07391102.2012.698243s.
Ruggiero, A., Squeglia, F., Marasco, D., Marchetti, R., Molinaro, A. & Berisio, R. (2011). *Biochem. J.* **435**, 33–41.
Ruggiero, A., Tizzano, B., Pedone, E., Pedone, C., Wilmanns, M. & Berisio, R. (2009). *J. Mol. Biol.* **385**, 153–162.
Sheldrick, G. M. (2008). *Acta Cryst.* A**64**, 112–122.
Squeglia, F., Marchetti, R., Ruggiero, A., Lanzetta, R., Marasco, D., Dworkin, J., Petoukhov, M., Molinaro, A., Berisio, R. & Silipo, A. (2011). *J. Am. Chem. Soc.* **133**, 20676–20679.
Terwilliger, T. C. (2003*a*). *Acta Cryst.* D**59**, 45–49.
Terwilliger, T. C. (2003*b*). *Methods Enzymol.* **374**, 22–37.