Structural characterization of angiotensin I-converting enzyme in complex with a selenium analogue of captopril

Mohd Akif¹, Geoffrey Masuyer¹, Sylva L. U. Schwager², Bhaskar J. Bhuyan³, Govindasamy Mugesh³, R. Elwyn Isaac⁴, Edward D. Sturrock² and K. Ravi Acharya¹

¹ Department of Biology and Biochemistry, University of Bath, UK
² Division of Medical Biochemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory, South Africa
³ Department of Inorganic and Physical Chemistry, Indian Institute of Science, Bangalore, India
⁴ Institute of Integrative and Comparative Biology, University of Leeds, UK

Keywords
angiotensin I-converting enzyme (ACE); cardiovascular disease; inhibitor design; metalloprotease; selenium

Correspondence
K. R. Acharya, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK
Fax: +44 1225 386779
Tel: +44 1225 388238
E-mail: bsskra@bath.ac.uk

Human somatic angiotensin I-converting enzyme (ACE), a zinc-dependent dipeptidyl carboxypeptidase, is central to the regulation of the renin–angiotensin aldosterone system. It is a well-known target for combating hypertension and related cardiovascular diseases. In a recent study by Bhuyan and Mugesh [Org. Biomol. Chem. (2011) 9, 1356–1365], it was shown that the selenium analogues of captopril (a well-known clinical inhibitor of ACE) not only inhibit ACE, but also protect against peroxynitrite-mediated nitration of peptides and proteins. Here, we report the crystal structures of human testis ACE (tACE) and a homologue of ACE, known as AnCE, from Drosophila melanogaster in complex with the most promising selenium analogue of captopril (SeCap) determined at 2.4 and 2.35 Å resolution, respectively. The inhibitor binds at the active site of tACE and AnCE in an analogous fashion to that observed for captopril and provide the first examples of a protein–selenolate interaction. These new structures of tACE–SeCap and AnCE–SeCap inhibitor complexes presented here provide important information for further exploration of zinc coordinating selenium-based ACE inhibitor pharmacophores with significant antioxidant activity.

Database
Structural data for the two SeCap complexes with ACE and AnCE have been deposited with the RCSB Protein Data Bank under the codes 2YDM and 3ZQZ, respectively.

Introduction

Human angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase that plays a critical role in blood pressure regulation [1–7] by catalysing the proteolysis of angiotensin I to the vasopressor angiotensin II [8–10]. There are two isoforms of human ACE: in somatic tissues, it exists as a glycoprotein composed of a mature single polypeptide chain of 1277 amino acids with two active centres, one in each of the N- and C-domains [11]. Testis ACE (tACE) is identical to the C-terminal half of somatic ACE, except for a unique 36-residue sequence at its N-terminus [12]. Both domains are heavily glycosylated (the N-domain has 10 and the C-domain has 7 N-linked glycosylation sites), cleave angiotensin I, are dependent on chloride ion activation and share ~ 55% amino acid sequence identity.

Abbreviations
ACE, angiotensin I-converting enzyme; SeCap, selenium analogue of captopril; tACE, testis angiotensin I-converting enzyme.
ACE inhibitors are widely used in clinical practice for the treatment of hypertension, heart failure, myocardial infarction and diabetic nephropathy. In addition, a number of studies have suggested that hypertension and oxidative stress are interdependent [13,14]. Therefore, ACE inhibitors having antioxidant properties are considered beneficial for the treatment of hypertension. Because selenium compounds are known to exhibit better antioxidant behaviour than their sulfur analogues [15,16], we have recently reported the synthesis, characterization and antioxidant activity of a number of selenium analogues of the clinically used ACE inhibitor captopril [17,18]. It was shown that selenium analogues of captopril (SeCap) not only inhibit ACE activity, but also can effectively scavenge peroxynitrite, a strong oxidant found in vivo [19].

The two ACE homologues, AnCE and ACER, from a nonvertebrate, Drosophila melanogaster, have been studied in detail. AnCE is a single-domain protein, reported to have biochemical resemblance to C-domain ACE [20]. In addition the 3D structure(s) of native AnCE and AnCE in complex with ACE inhibitors has firmly established the high degree of conservation in the active site of ACE [21,22].

Here, we report for the first time, structural details on the binding of one of the potent SeCaps (Fig. 1) to tACE and AnCE, as elucidated by X-ray crystallography at 2.4 and 2.35 Å resolution, respectively. Using these structures, we have been able to make a direct comparison of the previously determined structures of native tACE [23] and AnCE [22] with their respective complex with captopril [22,24]. These structures are useful in understanding a selenolate ligand’s coordination of zinc and its binding mode at the active site of ACE and its homologue AnCE.

Results and Discussion

**tACE–SeCap complex**

The final structure contains a zinc ion, one SeCap inhibitor molecule, N-glycosylated carbohydrates at two potential binding sites (Asn72, Asn109) and 54 water molecules (Table 1). No noticeable conformational change in the protein is observed upon inhibitor binding. The topological arrangement of the final structure (Fig. 2A) is consistent with previously determined structures of tACE [23], as well as tACE in complex with captopril [24]. One bound SeCap molecule was unambiguously fitted in the catalytic site of tACE (Fig. 3A) with the aid of a clearly observed electron-density map.

The inhibitor SeCap molecule makes a direct interaction with the catalytic Zn$^{2+}$ ion (distance 2.5 Å, Table 2) deep inside the active site channel (Fig. 2A), similar to the zinc coordination in tACE–captopril [24]. This interaction results in the formation of a zinc–selenolate complex. The inhibitor is anchored through the central carbonyl group and the proline carboxylate group. The proline residue of SeCap interacts with the S$_2$¢ subsite of the active site via two strong hydrogen bonds from two histidines (His513, 3.1 Å; His353, 2.6 Å). One oxygen atom of the proline carboxylate group is held by interactions with Tyr520 (2.7 Å), Gln281 (2.7 Å) and Lys511 (3.0 Å) (Fig. 3A, Table 2). In addition, it is held by seven hydrogen bonds including two mediated through water molecules, as calculated by HBPLUS [25] (Table 2). Thus, the interactions of SeCap with tACE residues are almost identical to those observed with captopril [24] (Fig. 3B), which is not too surprising considering the similarity between the two chemical structures (Fig. 1).

**AnCE–SeCap complex**

The final structure contains a zinc ion, one inhibitor molecule, N-glycosylated carbohydrates at three potential binding sites (Asn53, Asn196, Asn311) and 201 water molecules (Table 1). No noticeable conformational change is observed upon inhibitor binding. The topological arrangement of the final structure (Fig. 2B) is consistent with previously determined structures of AnCE and AnCE in complex with captopril [21,22]. One bound SeCap molecule was unambiguously fitted in the catalytic site of AnCE (Fig. 4A) with the aid of a clearly observed electron-density map.

The inhibitor SeCap molecule makes a direct interaction with the catalytic Zn$^{2+}$ ion, displacing the water that is bound in the native enzyme (distance 2.7 Å, Table 3) (Fig. 4A), similar to the zinc coordination in AnCE–captopril [21,22]. This interaction results in the formation of a zinc–selenolate complex. The carboxy-end of the proline moiety and the central carbonyl interact with the extended S$_1$/'S$_2$'–binding site.

Fig. 1. Chemical structures of captopril and SeCap.
locking the inhibitor in position. The proline residue of SeCap makes important contacts with the S2’ subsite via two strong hydrogen bonds from two histidines (His497, 3.1 Å; His337, 2.5 Å). One oxygen atom of the proline carboxylate group interacts with Tyr504 (2.5 Å), Gln265 (2.9 Å) and Lys495 (2.9 Å) through

Table 1. X-ray diffraction data collection and refinement statistics. Values in parentheses are for last resolution shell. SeCap, selenium analogue of captopril; tACE, testis angiotensin I converting enzyme.

|                         | tACE–SeCap inhibitor complex | AnCE–SeCap inhibitor complex |
|-------------------------|------------------------------|------------------------------|
| Resolution (Å)          | 2.4                          | 2.35                         |
| Space group             | P2₁2₁2₁ (one molecule/asymmetric unit) | R₃ (one molecule/asymmetric unit) |
| Cell dimension (Å, deg) | a = 56.1, b = 84.5, c = 132.3 α = β = γ = 90 | a = 173.8, b = 173.8, c = 100.8 α = β = γ = 120 |
| Total no. of observations | 117329                      | 179815                      |
| No. of unique reflections | 23627                        | 44663                        |
| Completeness (%)      | 97.4 (83.6)                  | 94.7 (72.1)                  |
| I/σ(I)                 | 12.3 (2.2)                   | 11.5 (1.6)                   |
| R_{symm}               | 0.09 (0.62)                  | 0.075 (0.53)                 |
| R_{cryst}/R_{free}     | 0.21/0.26                    | 0.20/0.24                    |
| No. of protein atoms   | 4687                         | 4866                         |
| No. of solvent atoms   | 54                           | 201                          |
| No. of inhibitor atoms | 14                           | 14                           |
| Deviation from ideality |                             |                              |
| Bond lengths (Å)       | 0.01                         | 0.01                         |
| Bond angles (deg)      | 1.42                         | 0.88                         |
| B-factor analysis      |                              |                              |
| Protein all atoms      | 32.6                         | 35.3                         |
| Protein main chain     | 32.2                         | 35.2                         |
| Protein side chain     | 33.0                         | 35.3                         |
| Solvent atoms          | 32.3                         | 35.4                         |
| Inhibitor atoms        | 46.9                         | 51.0                         |
| Zn^{2⁺}/Cl⁻ ions (tACE); Zn^{2⁺} ion (AnCE) | 35.7/44.0                  | 33.9                         |
| Glycosylated sugars    | 52.7                         | 54.0                         |

a R_{symm} = \sum \frac{I(h)}{<I(h)>}/\sqrt{\sum I(h)}, where I(h) is the rth measurement and <I(h)> is the weighted mean of all the measurements of I(h).
b R_{cryst} = \sum F_o - F_c, where F_o and F_c are observed and calculated structure factor amplitudes of reflection h, respectively. c R_{free} is equal to R_{cryst} for a randomly selected 5% subset of reflections.

Fig. 2. (A) Structure of tACE (cyan) with inhibitor SeCap bound at the active site cavity (shown in spheres). The zinc ion (green sphere) bound in the active site and N-glycosylated sugars (brown sticks) at potential sites, Asn72, Asn109 are shown. Protein termini are labelled. (B) Structure of AnCE (blue) with inhibitor SeCap bound at the active site cavity (shown in spheres). The zinc ion (green sphere) bound in the active site and N-glycosylated sugars (brown sticks) at potential sites, Asn53, Asn196, Asn311 are shown.
hydrogen and ionic bonds (Fig. 4A, Table 3). The central carbonyl of SeCap is held by His337 and His497. In addition, it is held by seven hydrogen bonds, including two hydrogen bonds mediated through water molecules, as calculated by HBPLUS [25] (Table 3). Thus, the interactions of SeCap with AnCE residues are almost identical to those observed with captopril [21,22] (Fig. 4B).

Conclusions

There has been considerable interest in applications of selenolate compounds because of their broad therapeutic spectrum and low toxicity. The organoselenium compound ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] an anti-inflammatory and general antioxidant, is also a potent inhibitor of extracellular nucleoside diphosphokinase [26]. Furthermore, Achillion Pharmaceuticals (New Haven, CT, USA) has recently developed a selenophene inhibitor of bacterial topoisomerases that shows promise as an antibiotic [27]. However, selenolates have not been exploited as metalloprotease inhibitors despite their antioxidant- and peroxynitrite-scavenging activities. This study has, for the first time, provided molecular details on the binding and coordination of a selenium analogue of the potent ACE inhibitor captopril with ACE and its homologue AnCE. Captopril is one of the smallest ACE inhibitors and with both ACE and AnCE, key interactions of the selenium analogue’s central carbonyl and proline carboxylate anchor the inhibitor in the cavernous
S$_1'S'_2'$-binding site of the enzyme. The envelope of space surrounding the P$_2'$ proline of SeCap is further illustrated by the accommodation of silylated captopril analogues in the active site of ACE [28]. Selenolates are stronger zinc-binding groups than thiols, yet surprisingly the IC$_{50}$ value of SeCap was greater than that of captopril [19]. This anomaly could be due to the cooperativity of the two-domain somatic ACE and the modest N-domain selectivity of captopril. Structures of SeCap complex with the N-domain and somatic ACE will undoubtedly shed further light on the binding of this new zinc-binding group of compounds with ACE and aid in the design of further selenium-based ACE inhibitors.

**Experimental procedures**

**tACE–SeCap complex**

A variant of tACE (tACE36-p13, underglycosylated protein) was purified to homogeneity from Chinese hamster ovary cells [29]. The inhibitor SeCap (Fig. 1, IC$_{50}$ value of 36.4 ± 1.5 nM) was synthesized as reported recently [19]. A stock solution (5 mM) of SeCap was prepared by dissolving the inhibitor in deoxygenated water containing 10 mM dithiothreitol. The crystals of the tACE complex with SeCap were grown at 16 °C using the hanging drop vapour diffusion method. tACE protein (11.5 mg mL$^{-1}$ in 50 mM Hepes, pH 7.5) was preincubated with SeCap (1 mM) on ice for 3 h before crystallization. Preincubated sample (2 μL) was mixed with the reservoir solution consisting of 13.5% poly(ethylene glycol) 4000, 50 mM sodium acetate, pH 4.7 and 10 μM ZnSO$_4$, and suspended above the well. Diffraction quality of cocrystals appeared after ~10 days.

**Fig. 4.** (A) A stereo representation of AnCE active site with bound inhibitor. The inhibitor molecule is shown in a stick model (brown) with the electron density map contoured at 1σ level. The zinc ion is shown as a green sphere and water molecules in light blue colour. Interacting residues are labeled and atoms are coloured as follows: red for oxygen, blue for nitrogen and purple for selenium. Hydrogen bonds are shown as dotted lines. (B) Comparison of SeCap (this study, left) and captopril (yellow sticks) binding [22] to AnCE (right).

**Table 3.** Hydrogen bond contacts of AnCE with the selenium analogue of captopril (SeCap) inhibitor.

| Atom       | Inhibitor atom | Distance (Å) |
|------------|----------------|--------------|
| AnCE residue |                |              |
| Gln265 NE2 | O2             | 2.9          |
| His337 NE2 | O1             | 2.5          |
| Lys495 NZ  | O2             | 2.9          |
| His497 NE2 | O1             | 3.1          |
| Tyr504 OH  | O1             | 2.5          |
| Zinc ion    | Zn             | 2.7          |
| Water molecule | O3           | 2.9          |
|             | O3             | 3.5          |
X-ray diffraction data for the tACE–SeCap complex were collected on the PX station I02 at Diamond Light Source (Didcot, UK). A total of 150 images were collected using a Quantum-4 CCD detector (ADSC Systems, Poway, CA, USA). No cryoprotectant was used to keep the crystal at constant temperature (100 K) under the liquid nitrogen jet during data collection. Raw data images were indexed and scaled with XDS [30] and the CCP4 program SCALA [31]. Initial phasing for structure solution was obtained using the molecular replacement routines of the program PHASER [32]. The atomic coordinates of native tACE [23] (PDB code 1O8A) were used as a search model. The resultant model was refined using REFMACS [33] and adjustment of the model was carried out using COOT [34]. Water molecules were added at positions where $F_o - F_c$ electron-density peaks exceeded 3$\sigma$ and potential H-bonds could be made. Based on electron-density interpretation, the inhibitor and sugar moieties were added in the complex structure and further refinement was carried out. The coordinate and parameter files for SeCap were generated using SKETCHER [31]. Validation was conducted with the aid of MOLPROBITY [35]. Figures were drawn with PYMOL. Hydrogen bonds were verified with the program HBPLUS [25]. The detailed refinement statistics for the complex structure are given in Table 1.

**AnCE–SeCap complex**

AnCE was cloned and expressed in *Pichia pastoris* as described previously [22]. In brief, AnCE was purified to homogeneity from culture media using hydrophobic interaction chromatography and size-exclusion chromatography. The crystals of the AnCE complex with SeCap were grown at 21 °C by the hanging drop vapour diffusion method. AnCE protein (10 mg·mL$^{-1}$ in 50 mM Hepes, pH 7.5) was preincubated with SeCap (1 mM) and 10 $\mu$M zinc acetate, on ice for 3 h before crystallization. Preincubated sample (2 $\mu$L) was mixed with the reservoir solution consisting of 1.3 M sodium citrate, 100 mM Hepes, pH 7.5 and suspended above the well. Diffraction quality of cocrystals appeared after ~1 week. X-ray diffraction data for the AnCE–SeCap complex were collected on the PX station I24 at Diamond Light Source. A total of 170 images were collected using a PILATUS 6M detector (Dectris, Baden, Switzerland). No cryoprotectant was used. Raw data images were indexed and scaled with XDS [30] and the CCP4 program SCALA [31]. Initial phasing for structure solution was obtained using the molecular replacement routines of the program PHASER [32]. The atomic coordinates of native AnCE [22] (PDB code 2X8Y) were used as a search model. The resultant model was refined using REFMACS [33] and adjustment of the model was carried out using COOT [34]. Water molecules were added at positions where $F_o - F_c$ electron-density peaks exceeded 3$\sigma$ and potential H-bonds could be made. Based on electron-density interpretation, the inhibitor and sugar moieties were added in the complex structure and further refinement was carried out. The coordinate and parameter files for SeCap were generated using SKETCHER [31]. Validation was conducted with the aid of MOLPROBITY [35]. Figures were drawn with PYMOL. Hydrogen bonds were verified with the program HBPLUS [25]. The detailed refinement statistics for the complex structure are given in Table 1.

**Acknowledgements**

This work was supported by the Medical Research Council (UK) through a project grant (number G1001685), Wellcome Trust (UK) equipment grant (number 088464) and a Royal Society (UK) Industry Fellowship to KRA; the Wellcome Trust (UK) through a Senior International Research Fellowship (number 070060), the National Research Foundation of South Africa, the Ernst and Ethel Erikson Trust, the Deutscher Akademischer Austausch Dienst (DAAD) and the University of Cape Town to EDS; the Department of Science and Technology (India), Ramanna and Swarnajayanti fellowships to GM; BJB thanks the Council of Scientific and Industrial Research (India) and Indian Institute of Science Bangalore for a research fellowship. We thank the scientists at stations I02 and I24 of Diamond Light Source (Didcot, UK) for their support during X-ray diffraction data collection.

**References**

1 Ehlers MR & Riordan JF (1989) Angiotensin-converting enzyme: new concepts concerning its biological role. Biochemistry 28, 5311–5318.

2 Unger T (2002) The role of the renin–angiotensin system in the development of cardiovascular disease. *Am J Cardiol* 89, 3A–9A.

3 Eriksson U, Daničczyk U & Penninger JM (2002) Just the beginning: novel functions for angiotensin-converting enzymes. *Curr Biol* 12, R745–R752.

4 Turner A & Hooper NM (2002) The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol Sci* 23, 177–183.

5 Acharya KR, Sturrock ED, Riordan JF & Ehlers MR (2003) Ace revisited: a new target for structure-based drug design. *Nat Rev Drug Discov* 2, 891–902.

6 Sturrock ED, Natesh R, van Rooyen JM & Acharya KR (2004) Structure of angiotensin I converting enzyme. *Cell Mol Life Sci* 61, 2677–2686.

7 Watermeyer JM, Kröger WL, Sturrock ED & Ehlers MR (2009) Angiotensin-converting enzyme − new insights into structure, biological significance and
prospects for domain-selective inhibitors. *Curr Enzym Inhib* 5, 134–147.

8 Erdos EG & Yang HYT (1967) An enzyme in microsomal fraction of kidney that inactivates bradykinin. *Life Sci* 6, 569–574.

9 Yang HYT & Erdös EG (1967) Second kininase in human blood plasma. *Nature* 215, 1402–1403.

10 Yang HYT, Erdös EG & Levin YJ (1971) Characterization of a dipeptide hydrolase (kininase II: angiotensin I converting enzyme). *Pharmacol Exp Ther* 177, 291–300.

11 Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Gregear G & Corvol P (1988) Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci USA* 85, 9386–9390.

12 Ehlers MR, Fox EA, Strydom DJ & Riordan JF (1989) Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proc Natl Acad Sci USA* 86, 7741–7745.

13 Alexander RW (1995) Hypertension and the pathogenesis of atherosclerosis – oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* 25, 155–161.

14 Kunsh C & Medford RM (1999) Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 85, 753–766.

15 Mugesh G, du Mont WW & Sies H (2001) Chemistry of biologically important synthetic organoselenium compounds. *Chem Rev* 101, 2125–2179.

16 Kumar S, Engman L, Valgimigli L, Amorati R, Fumo MG & Pedulli F (2007) Antioxidant profile of ethoxyquin and some of its S, Se, and Te analogues. *J Org Chem* 72, 6046–6055.

17 Ondetti MA, Rubin B & Cushman DW (1977) Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. *Science* 196, 441–444.

18 Cushman DW, Cheung HS, Sabo EF & Ondetti MA (1977) Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry* 16, 5484–5491.

19 Bhuyan BJ & Mugesh G (2011) Synthesis, characterization and antioxidant activity of angiotensin converting enzyme inhibitors. *Org Biomol Chem* 9, 1356–1365.

20 Houard X, Williams TA, Michaud A, Dani P, Isaac RE, Shirras AD, Coates D & Corvol P (1998) The *Drosophila melanogaster*-related angiotensin I-converting enzymes Acer and Ance – distinct enzymic characteristics and alternative expression during pupal development. *Eur J Biochem* 257, 599–606.

21 Kim HM, Shin DR, Yoo OJ, Lee H & Lee JO (2003) Crystal structure of *Drosophila* angiotensin I-converting enzyme bound to captopril and lisinopril. *FEBS Lett* 538, 65–70.

22 Akif M, Georgiadis D, Mahajan A, Dive V, Sturrock ED, Isaac RE & Acharya KR (2010) High-resolution crystal structures of *Drosophila melanogaster* angiotensin-converting enzyme in complex with novel inhibitors and antihypertensive drugs. *J Mol Biol* 400, 502–517.

23 Natesh R, Schwager SLU, Sturrock ED & Acharya KR (2003) Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. *Nature* 421, 551–554.

24 Natesh R, Schwager SLU, Evans HR, Sturrock ED & Acharya KR (2004) Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. *Biochemistry* 43, 8718–8724.

25 McDonald IKM & Thornton JM (1994) Satisfying hydrogen bonding potential in proteins. *J Mol Biol* 238, 777–793.

26 Semianario-Vidal L, van Nesden C, Mugesh G & Lazarowski ER (2010) Ebselen is a potent non-competitive inhibitor of extracellular nucleoside diphosphokinase. *Purinergic Signal* 6, 383–391.

27 Wiles JA, Phadke AS, Bradbury BJ, Pucci MJ, Thanassi JA & Deshpande M (2011) Selenophene-containing inhibitors of type IIA bacterial topoisomerases. *J Med Chem* 54, 3418–3425.

28 Dalkas GA, Marchand D, Gallelyand JC, Martinez J, Spyroulias GA, Cordopatis P & Cavelier F (2009) Study of a lipophilic captopril analogue binding to angiotensin I converting enzyme. *J Peptide Sci* 16, 91–97.

29 Gordon K, Redelingshuys P, Schwager SLU, Ehlers MR, Papageorgiou AC, Natesh R, Acharya KR & Sturrock ED (2003) Deglycosylation, processing and crystallization of human testis angiotensin-converting enzyme. *Biochem J* 371, 437–442.

30 Kabsch W (2010) Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr* 66, 133–144.

31 CCP4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr* 50, 760–763.

32 McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC & Read RJ (2007) Phaser crystallographic software. *J Appl Crystallogr* 40, 658–674.

33 Murshudov GN, Vagin AA & Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr* 53, 240–255.

34 Emsley P & Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr* 60, 2126–2132.

35 Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB, Snieckus Y, Richardson JS et al. (2007) MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* 35, 375–383.