Supporting Information

Stsiapanava et al. 10.1073/pnas.1402136111

SI Structural Features of the B Conformation of *N*-(4-oxo-4-Pyrrolidinyl-Butanoyl)-Proline in Complex with Leukotriene A_4 Hydrolase/Aminopeptidase

The chemical modification of Pro-Gly-Pro into *N*-(4-oxo-4-pyrrolidinyl-butanoyl)-proline (OPB-Pro) gives rise to the B conformation of OPB-Pro (Fig. S3) because the carbon atom lacks the charge to create the hydrogen bond with the carboxyl group of Glu296, which is required for proper substrate alignment and turnover. As a result, the carbonyl oxygen of the N-terminal proline in OPB-Pro shifts its interaction with Tyr383 in the A conformation (Fig. 2) to Glu296, creating a hydrogen bond with $O^{\varepsilon 2}$ at a distance of 2.62 Å and a coordinated bond with the zinc ion at 2.77 Å. In the B conformation, H_2O-2 substitutes the carbonyl oxygen of OPB-Pro observed in the A conformation and is stabilized by interactions with Tyr383 (2.41 Å) and the zinc ion (1.85 Å). The distance between H_2O-2 and nearest atom of OPB-Pro B was calculated as 1.8 Å. This distance is longer than that between H_2O-1 and OPB-Pro A, but it is still too short for two separate molecules.

SI Materials and Methods

Enzyme Expression and Purification. Human recombinant leukotriene (LT) A_4 hydrolase/aminopeptidase (LTA4H) was expressed in *Escherichia coli* JM101 and purified as described (1). Protein purity was checked by SDS/PAGE electrophoresis (Pharmacia Phast System) with a 10–15% gradient gel after every purification step. Protein concentration was determined by UV absorbance at 280 nm (Cary 300 UV-Vis; Varian) using an extinction coefficient of 104,905 $M^{-1}{\cdot}cm^{-1}$ for human LTA4H.

Enzyme Activity Assays. The epoxide hydrolase activity of LTA4H was assayed essentially as described (2). In brief, 3 μg of enzyme in 100 μL of 25 mM Tris·HCl (pH 7.8) was incubated with 10 μM LTA_4 for 1 min. The reaction was stopped by addition of 2 vol of MeOH followed by 1 vol of water, and 300 pmol of prostaglandin B_1 was added as an internal standard. After acidification to pH 3 with 10 mM HCl, samples were analyzed by reverse-phase HPLC on a 3.9 × 150-mm column (C_{18}; Nova-Pak Waters) eluted with acetonitrile/methanol/water/acetic acid at a ratio of 30:35:35:0.1 (vol/vol) and at a flow rate of 0.7 mL/min. Absorbance was monitored at 270 nm.

The aminopeptidase activity of LTA4H was assessed with *p*-nitroanilide derivatives of Ala and Val as substrates. Aliquots of enzyme (1.25 μg) in 250 μL of 10 mM Tris·HCl (pH 7.8), containing 100 mM KCl, were incubated at room temperature for 10 min in a 96-well plate with 150 μL of 1 mM substrate in assay buffer. The absorbance was measured at 405 nm using a Multiscan spectrophotometer (Labsystems). An extinction coefficient of 9,500 $M^{-1}{\cdot}cm^{-1}$ for 4-nitroaniline was used to calculate the activity of the enzyme.

Isolation of Polymorphonuclear Neutrophils and Analysis of LTB_4 Synthesis. Human polymorphonuclear neutrophils (PMNs) were isolated from freshly prepared buffy coats (Karolinska Hospital blood bank) by dextran sedimentation, gradient centrifugation on Ficoll–Paque (Amersham Biosciences), and hypotonic lysis of erythrocytes. PMNs were suspended at a density of 10×10^6 PMNs per milliliter in Dulbecco's PBS (Gibco Invitrogen). PMN purity (>95%) and viability (>98%) were determined using Hemacolor (J. T. Baker) and Trypan blue (Sigma Chemical Co.) staining, respectively. PMNs (10×10^6 PMNs per milliliter) were pretreated with 4-(4-benzylphenyl)thiazol-2-amine (ARM1; 0, 0.5, 5 or 50 μM) for 10 min, followed by incubation with calcium ionophore A23187 (2.5 μM) for 5 min at 37 °C, and were then quenched with an equal volume of methanol. After acidified to pH 3–4, the samples were purified by solid-phase extraction (OASIS cartridge; Waters) and analyzed by reverse-phase HPLC to quantify LTB_4, as described above.

1. Rudberg PC, Tholander F, Thunnissen MMGM, Haeggström JZ (2002) Leukotriene A4 hydrolase/aminopeptidase. Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms. *J Biol Chem* 277(2):1398–1404.

2. Wetterholm A, et al. (1991) Recombinant mouse leukotriene A_4 hydrolase: A zinc metalloenzyme with dual enzymatic activities. *Biochim Biophys Acta* 1080(2):96–102.

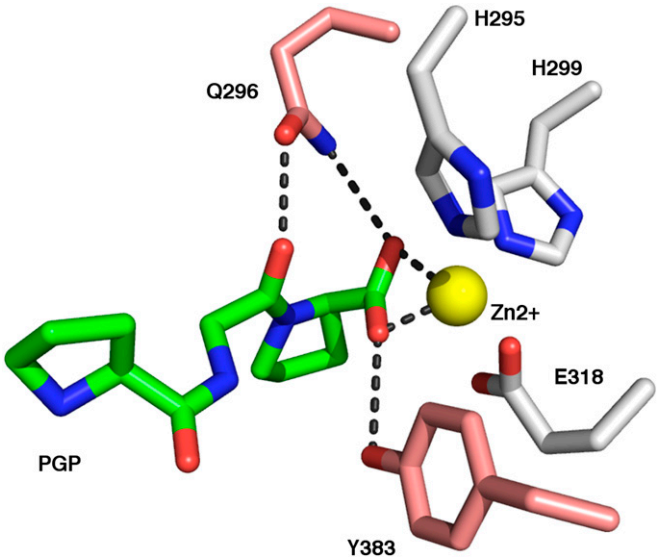


Fig. S1. Stick representation of Pro-Gly-Pro (PGP) substrate in the active site of E296Q LTA4H. The zinc-binding residues are shown in gray, Tyr383 and mutated Gln296 are shown in pink, PGP is shown in green, and the yellow sphere represents a zinc ion.

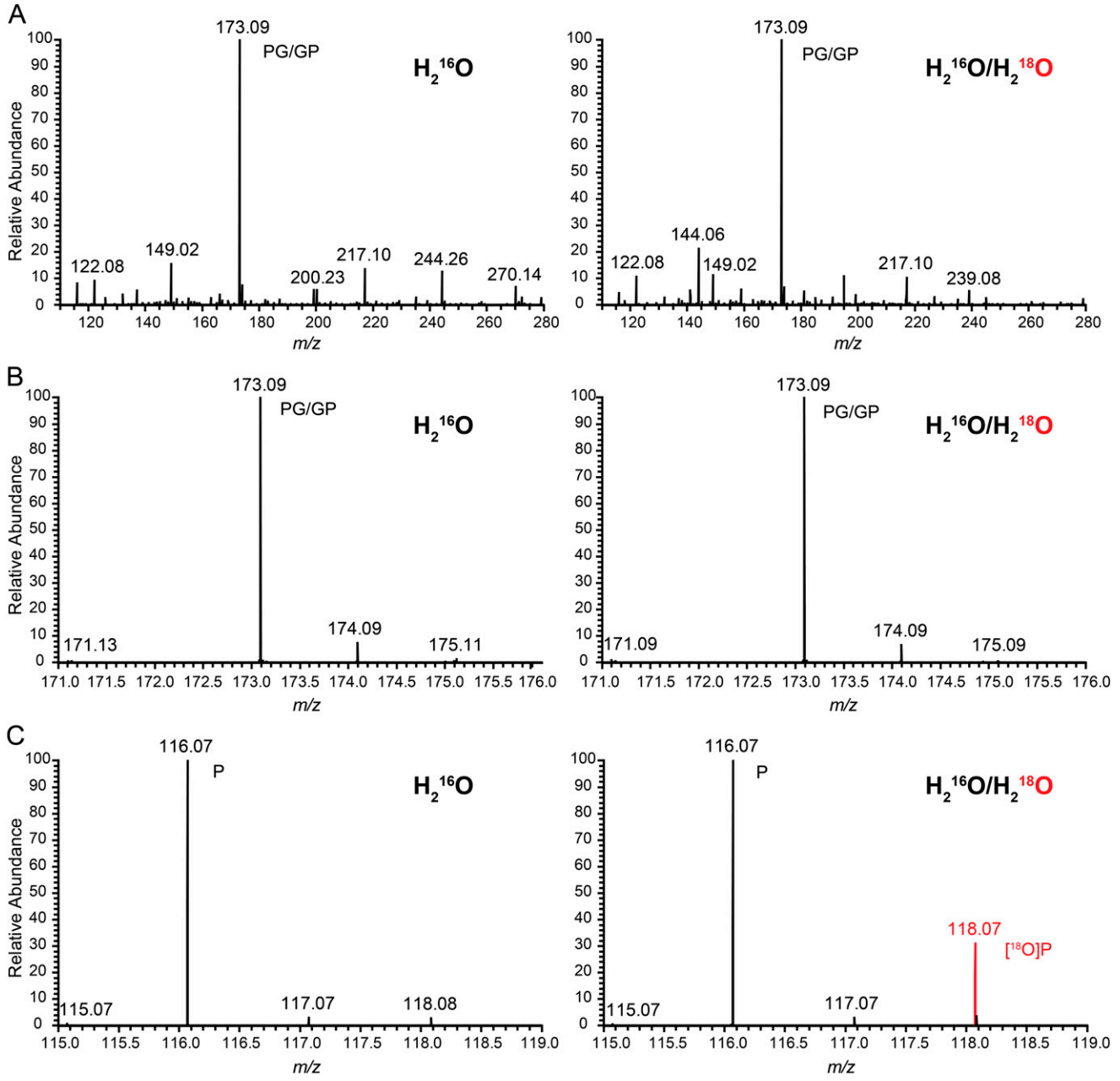


Fig. S2. Mass spectrometric analysis of Pro-Gly-Pro hydrolysis products obtained in ^{18}O water. (*A*) Mass spectrum of products obtained in ^{16}O water (*Left*) compared with the spectrum of products obtained in a 1:1 mixture of ^{16}O and ^{18}O water (*Right*). (*B*) Same analysis as in *A* in the mass range of *m/z* 171.0–176.0; the dipeptides Pro-Gly (PG) and Gly-Pro (GP) appear at *m/z* = 173.09. (*C*) Same analysis as in *A* in the mass range of *m/z* 115.0–119.0; free Pro (P) appears at *m/z* = 116.07. Pro with incorporation of ^{18}O appears at *m/z* = 118.07.

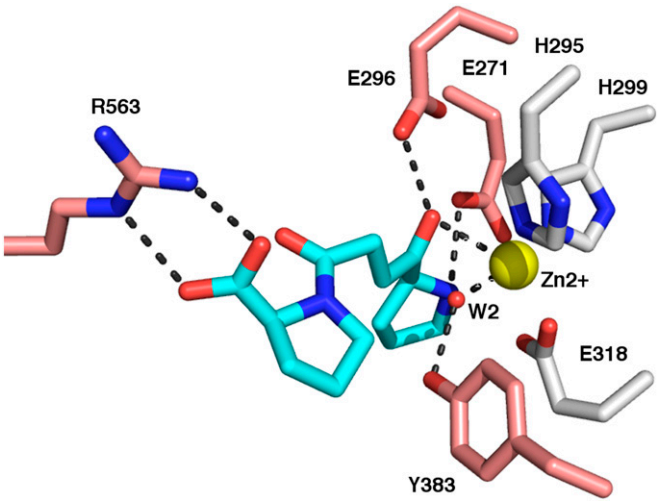


Fig. S3. Crystal structure of the active site of LTA4H in complex with the substrate analog OPB-Pro in the B conformation. Active site residues and OPB-Pro are shown in stick representation. The zinc-binding residues are colored in gray, the amino acid residues of peptidase active site are shown in pink, OPB-Pro is shown in cyan, the yellow sphere represents a zinc ion, and the red sphere represents the oxygen atom of a water molecule (W2).

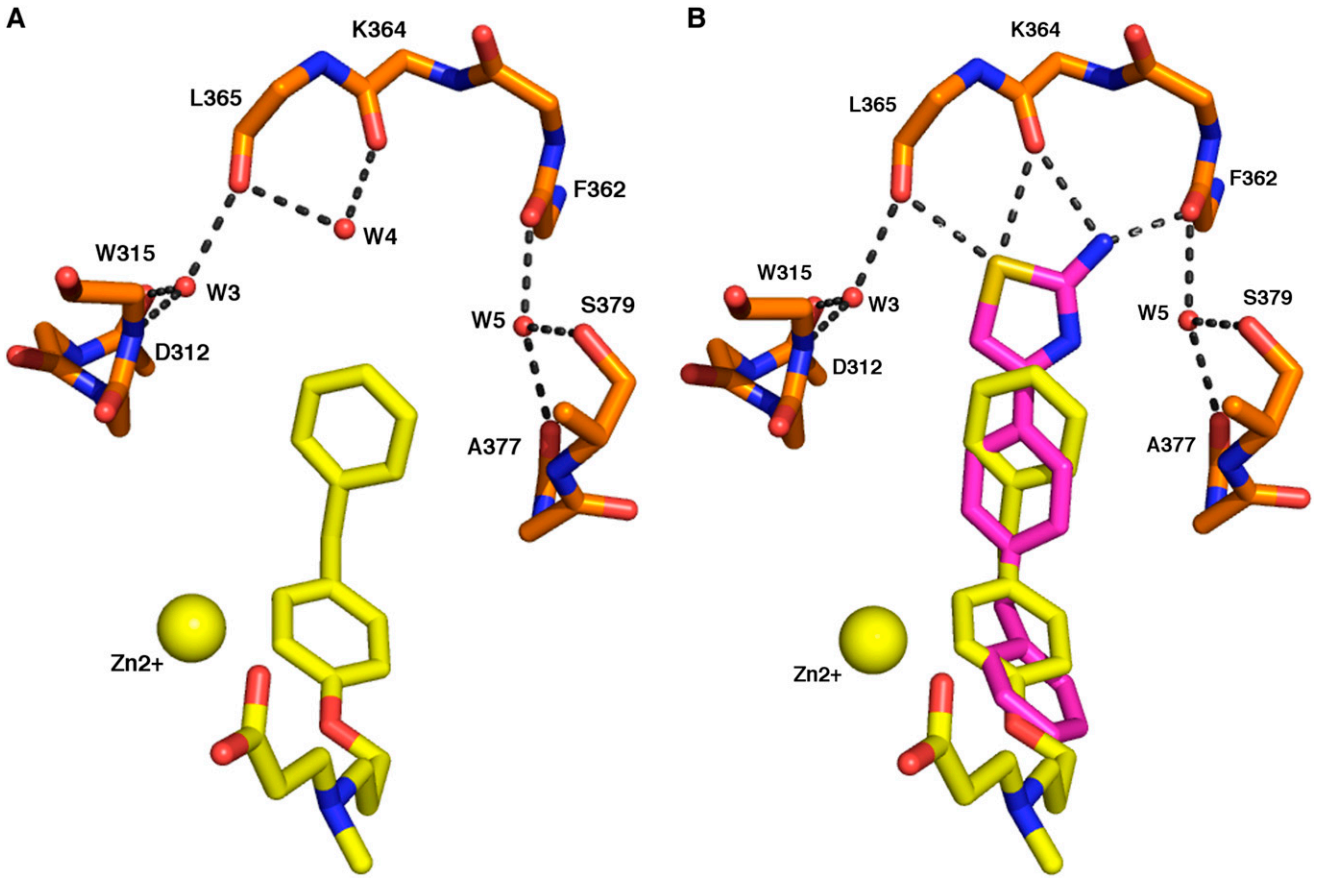


Fig. S4. (*A*) Crystal structure of LTA4H complexed with *N*-[3-(4-benzylphenoxy)propyl]-*N*-methyl-β-alanine (SC-57461A) inhibitor. The SC-57461A inhibitor is shown in yellow stick representation, and the main chain residues Asp312, Trp315, Phe362, Lys364, and Leu365, as well as Ser379 of the epoxide hydrolase pocket, are shown in orange. The zinc ion is presented as a yellow sphere, and red spheres indicate oxygens of three water molecules (W3, W4, and W5) at the bottom of the pocket. The first water molecule, denoted W3, interacts with the main chain carbonyl oxygen of Asp312 (hydrogen bond distance of 2.79 Å) and Leu365 (2.73 Å), and the amine nitrogen of Trp315 (2.86 Å). W4 is fixed through hydrogen bonds with the carbonyl oxygens of Arg364 (2.92 Å) and Leu365 (2.90 Å). The third water molecule, W5, is coordinated by interactions with the main chain carbonyls of Phe362 (2.84 Å) and Ala377 (2.76 Å), and with the hydroxyl group of Ser379 (2.63 Å). (*B*) Superposition of LTA4H containing ARM1 (in purple stick representation) with the structure complexed with SC-57461A. The sulfur of ARM1 substitutes one of the waters at the end of the hydrophobic cavity.

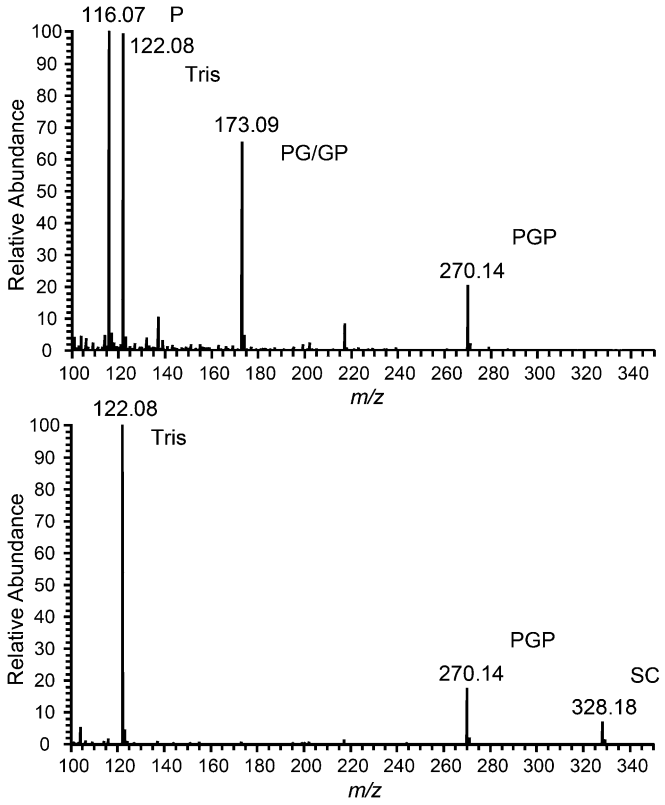


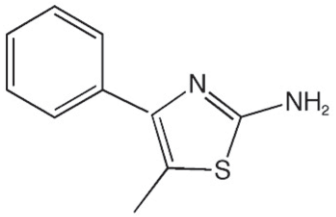
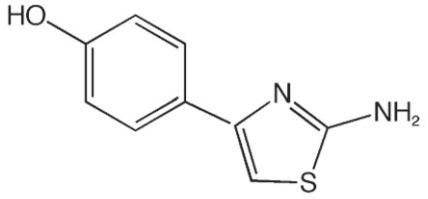
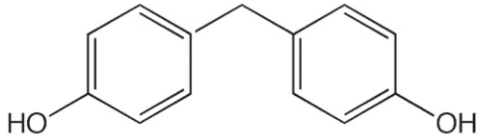
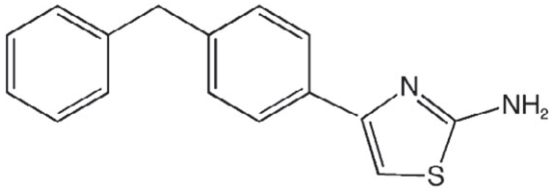
Fig. S5. Mass spectrometric verification of LTA4H-catalyzed hydrolysis of Pro-Gly-Pro in the presence of 4-(4-benzylphenyl)thiazol-2-amine (ARM1). LTA4H (0.01 mg/mL) was incubated for 1 h on ice with 200 μM Pro-Gly-Pro in the presence of 100 μM ARM1 or SC 57461A. After incubation the assay mixture was filtered to remove the enzyme. Mass spectrometric analysis demonstrated cleavage of PGP into Pro (*m/z* = 116.07) and Gly-Pro (*m/z* = 173.09) in the sample containing ARM1 (*Upper*) but not that containing SC-57461A (*Lower*).

Table S1. Fragment ions of Gly-Pro

	No.	A-ion	B-ion	C-ion	X-ion	Y-ion	Z-ion	No.
Gly	1	*30.03442*	*58.02933*	*75.05588*	—	173.0927	156.0661	2
Pro	2	127.0872	155.0821	—	142.0505	*116.0712*	*99.04465*	1

The fragment ions that could be detected (from different fragmentation modes) are underlined, and the ions that are characteristic (unique) for the fragmentation of Gly-Pro are italicized. The mass calculations were done using the Fragment Ion Calculator from the Institute for Systems Biology (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html).

Table S2. Compounds designed to inhibit epoxide hydrolase activity of LTA4H

Compound ID no.	Chemical structure	Chemical name	K_i, mM
1		5-methyl-4-phenylthiazol-2-amine	>1
2		4-(2-aminothiazol-4-yl)-phenol	>1
3		4,4′-methylenediphenol	>1
4 (ARM1)		4-(4-benzylphenyl)-thiazol-2-amine	0.002

ARM1, 4-(4-benzylphenyl)thiazol-2-amine.

Table S3. Data collection, refinement, and model building statistics of LTA4H in complex with OPB-Pro, ARM1, and both OPB-Pro and ARM1

Dataset	OPB-Pro	ARM1	OPB-Pro and ARM1
Data collection			
Wavelength, Å	0.976	0.918	0.918
Resolution range, Å	42.18–1.72 (1.77–1.72)	42.18–1.65 (1.75–1.65)	43.79–1.62 (1.72–1.62)
Unit-cell parameters, Å	a = 76.766, b = 87.483, c = 98.964 $\alpha = \beta = \gamma = 90°$	a = 66.171, b = 76.701, c = 67.215 $\alpha = \gamma = 90$, $\beta = 98.43°$	a = 76.809, b = 87.588, c = 100.056 $\alpha = \beta = \gamma = 90°$
Space group	*P*212121	*P*12121	*P*212121
Observed reflections	519,269	660,502	571,177
Unique reflections	71,210	77,641	86,391
Completeness, %	99.7 (99.6)	96.8 (91.9)	99.7 (98.8)
$\langle I/\sigma(I)\rangle$	10.09 (1.83)	26.03 (9.24)	13.51 (1.96)
R_{merge},* %	18.1 (156)	6.1 (24.3)	9.8 (100.0)
$CC_{1/2}$†	99.6 (76.4)	99.9 (98.2)	99.8 (76.4)
Average multiplicity	7.3	8.51	6.61
Wilson B-factor, Å^2	20.24	11.47	17.95
Refinement			
Reflections used in working set	67,568	69,877	82,070
Reflections used in test set	3,605	3,882	4,320
Maximum resolution, Å	1.72	1.65	1.62
R_{work}‡/R_{free}§, %	17.5/21.5	14.2/17.5	15.6/19.3
No. of non-H atoms	5,380	5,375	5,544
No. of protein atoms	4,860	4,852	4,860
No. of ligand atoms, ions	33	36	49
No. of water molecules	487	487	635
Average B-factor, Å^2	22.947	12.953	20.921
rmsd from ideal¶			
Bond lengths, Å	0.018	0.023	0.021
Bond angles, °	1.929	2.162	2.072
Ramachandran statistics of ϕ/ψ angels‖, %			
Preferred regions	97.52	97.85	98.1
Allowed regions	100	100	100
Outliers	0.0	0.0	0.0
PDB ID code	4MS6	4L2L	4MKT

Values for the highest resolution shell are given in parentheses.

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

†From (1).

‡$R = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$.

§R_{free} (2) was monitored with 5% of the reflection data excluded from refinement.

¶From Engh and Huber (3).

‖As determined by MolProbity.

1. Karplus PA, Diederichs K (2012) Linking crystallographic model and data quality. *Science* 336(6084):1030–1033.
2. Brunger AT (1993) Assessment of phase accuracy by cross validation: The free R value. Methods and applications. *Acta Crystallogr D Biol Crystallogr* 49(Pt 1):24–36.
3. Engh RA, Huber R (1991) Accurate bond and angle parameters for X-ray protein-structure refinement. *Acta Crystallogr A* 47(Pt 4):392–400.