Supplementary Information

*De novo* design of a four-fold symmetric TIM-barrel protein with atomic level accuracy

Po-Ssu Huang¹,²,³*, Kaspar Feldmeier¹,⁴, Fabio Parmeggiani²,³, D. Alejandro Fernandez Velasco⁵, Birte Höcker⁴*, David Baker²,³,⁶*

*Correspondence to D.B. (dabaker@uw.edu), B.H. (Birte.Hoecker@tuebingen.mpg.de) or P.-S. H. (po ssu.huang@gmail.com)

¹These authors contributed equally to this work.
²Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA
³Institute for Protein Design, University of Washington, Seattle, Washington 98195, USA
⁴Max Planck Institute for Developmental Biology, Tübingen, Germany
⁵Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, México D.F., 04510 Mexico
⁶Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195, USA

This file contains Supplementary Figures 1-9 with legends and Supplementary Table 1-5. Supplementary Figures provide additional information on computational and experimental results. Supplementary Table 1 compares the initial variants. Supplementary Table 2 lists construct sequences and notes. Supplementary Table 3 and 4 contain command lines and input files for running the design calculations. Supplementary Table 5 shows X-ray structure statistics.
Supplementary Results

Supplementary Figure 1: Heatmap of amino acid frequencies in different cycles of sequence refinement.
For each sequence refinement cycle, residue identities are restricted by the top solutions from the preceding cycle. The heatmap shows the frequencies for each amino acid at each position in a single 46 residue repeat unit. We show here heatmaps from three different rounds to illustrate the sequence convergence through the refinement cycles. At the 3rd cycle, the sequences were still diverse, but by the 8th and 10th cycles, most of the positions had amino acids assigned.
Supplementary Figure 2: CD wavelength scan spectra of the selected variants
Six variants from the panel of 22 designs showed CD traces consistent with having α/β structures, and the rest of the constructs had strong random coil signal below 208 nm and were not considered folded. A representative trace of a poorly folded protein (D9) is shown here, and all of the other poorly folded designs showed similar characteristics. Although K3 did not have a strong coil signal below 208 nm, its thermal melt (in Supplementary Fig. 4) showed no cooperative transition and was considered poorly folded.
Supplementary Figure 3: Design variants showing cooperative denaturation characters. During the explorative stage, we tested different sequences carrying a combination of design features. Out of 22 tested, five sequences showed cooperative unfolding behaviors from their thermal melts measured by CD. All five variants shared Asp1, Trp35 and Trp42, and the one variant (sTIM-1) with an additional Arg21 was found to be significantly more stable than the ones without this design feature.
Supplementary Figure 4: The accumulative effects of mutations on folding stabilities. CD temperature melts showing the contributions of the various mutations on stability. Mutating residue 1 from a lysine to an aspartate switched the structure from a molten globule to a cooperatively folded structure. Adding Arg21 increased the stability by almost 20 °C. Using a different circular permutation scheme did not significantly affect the stability.

| name                      | Tm (°C) |
|---------------------------|---------|
| K3 (Lys1)                 | N/A     |
| D3 (Asp1)                 | 51.5    |
| sTIM-1 (Asp1+Arg21)       | 71.9    |
| sTIM-7 (Asp1+Arg21+circular permutation) | 70      |
Supplementary Figure 5: Effect of two vs four-fold symmetrical barrel interior on stability. The measured thermal stabilities by CD directly correlated with hydrophobic packing (see Fig. 3 for design information); variants with the same hydrophobic interior shared similar melting transitions. The melting curves grouped in doublets: sTIM-1 and sTIM-4 (both have four isoleucines) were the most stable with melting temperatures around 72 °C; sTIM-2 and sTIM-5 (both have two isoleucines and two valines) melted around 66 °C; sTIM-3 and sTIM-6 (both have four valines) melted around 57~59 °C. Variant D12 marks the lower bound in stability without Arg21. Detailed sequences are in Supplementary Table 2.

| name | Tm (°C) |
|------|---------|
| sTIM1 | 71.9 |
| sTIM2 | 66.5 |
| sTIM3 | 56.9 |
| sTIM4 | 71.8 |
| sTIM5 | 65.8 |
| sTIM6 | 59.3 |
| D12 | 54.5 |
Supplementary Figure 6: Half-barrel construct was purified as a full-barrel. sTIM-12 was built as a half-sized barrel based on the sTIM-7 sequence, with an expected mass of 12 KDa from chemical compositions. The apparent size of sTIM-12 in solution, however, corresponds to a monodispersed 24 KDa species. Two half-sized barrels appear to self-assemble into a full barrel.
Supplementary Figure 7: Circular permutation scheme for sTIM-11.

sTIM-11 was circularly permuted in order to relocate the 6x Histidine tag used for purification from $\alpha/\beta$ loop2. Due to the perfect 4-fold symmetry in the closed circular structure, the original designs were modeled computationally as cyclic peptides, where the N terminus was jointed with the C-terminus through a virtual linkage. Constructs for experimental testing, however, have a 6x Histidine tag inserted in this region that inevitably disrupt packing of the last loop ($\alpha/\beta$ loop2 equivalent), and we tested with this circular permutation scheme to correct this issue. The inner circle shows the sequence of variant D12, and the circular permutation scheme for sTIM-11 relative to D12 is shown in the outer circle. Residue 39 on sTIM-11 is equivalent to residue 1 of non-circularly permuted designs. The new termini are located where a $\beta/\alpha$ loop was designed with minimal interaction with the rest of the core so it minimally affects packing. Neither the original non-circularly permuted variant (D12) nor the one with circular permutation (sTIM-7) yielded crystals. Only sTIM-11, which was modified from the original design with circular permutation and an additional cysteine pair, produced crystals.
Supplementary Figure 8: sTIM-11 X-ray structure colored by B-factor
High B-factors were observed from the helices near the termini and the $\beta/\alpha$ loop1 regions in the sTIM-11 X-ray structure. Thin grey lines show connectivity for the missing backbone atoms.
Supplementary Figure 9: *de novo* TIM-barrel designs do not require family specific fragments to build.

**a**, histogram of 7-residue fragments that fit the sTIM-11 model below 1Å rmsd. To analyze whether sTIM-11 structure was strongly biased by fragments from TIM-barrels, we looked up all of the 7mer peptide fragments in a non-redundant PDB set (with a homology sequence cutoff of 30%) that have rmsd to the design model of sTIM-11 at 1Å rmsd or below. Black line shows fragment counts from all of the structures in the set, and red lines shows the counts from the subset of PDBs that are TIM-barrels (classified by SCOP).

**b**, the ratios of non-redundant PDB/TIM-barrel only PDB 7mer fragments fit under 1Å rmsd. These results show that the designed *de novo* TIM-barrels did not carry structural features that require TIM-barrel specific fragments to achieve; any stretch of the designed TIM-barrel structure can be described by non-TIM-barrel fragments.
For example, the K and D series test the importance of aspartate at position 1 to set up the strand register shift.

The designed sequences test different ways of enforcing the three sequence design rules described in the text.

**Supplementary Table 1: Residues varied in initial TIM-barrel designs**

| Design name | Residue positions |
|-------------|-------------------|
|             | 1     | 7     | 14    | 21    | 30    | 35    | 36    | 42    |
| K1          | K     | A     | R     | A     | Y     | W     | R     | H     |
| K2          | K     | A     | R     | A     | Y     | W     | R     | L     |
| K3          | K     | A     | R     | A     | Y     | W     | R     | W     |
| K4          | K     | A     | R     | A     | F     | W     | R     | H     |
| K5          | K     | A     | R     | A     | F     | W     | R     | L     |
| K6          | K     | A     | R     | A     | Y     | A     | R     | W     |
| K7          | K     | A     | R     | A     | Y     | A     | R     | L     |
| K8          | K     | A     | R     | A     | F     | A     | R     | H     |
| K9          | K     | A     | R     | A     | F     | A     | R     | L     |
| D1          | D     | A     | R     | A     | Y     | W     | R     | H     |
| D2          | D     | A     | R     | A     | Y     | W     | R     | L     |
| D3          | D     | A     | R     | A     | Y     | W     | R     | W     |
| D4          | D     | A     | R     | A     | F     | W     | R     | H     |
| D5          | D     | A     | R     | A     | F     | W     | R     | L     |
| D6          | D     | A     | R     | A     | Y     | A     | R     | W     |
| D7          | D     | A     | R     | A     | Y     | A     | R     | L     |
| D8          | D     | A     | R     | A     | F     | A     | R     | H     |
| D9          | D     | A     | R     | A     | F     | A     | R     | L     |
| D10         | D     | A     | R     | A     | F     | W     | R     | W     |
| D11         | D     | L     | R     | A     | Y     | W     | K     | W     |
| D12         | D     | A     | W     | A     | Y     | W     | R     | W     |
| sTIM-1      | D     | A     | W     | R     | Y     | W     | R     | W     |

| Allowed amino acids | D/K | A/L | W/R | R/A | Y/F | W/A | R/K | W/L/H |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-------|

The designed sequences test different ways of enforcing the three sequence design rules described in the text. For example, the K and D series test the importance of aspartate at position 1 to set up the strand register shift.

*Designs in red showed cooperativity in denaturing experiments. (Supplementary Fig. 3)*
| name    | sequence                                                                 | notes                                                                 |
|---------|--------------------------------------------------------------------------|----------------------------------------------------------------------|
| sTIM-1  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | stable 4-fold TIM-barrel as described                                  |
| sTIM-2  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | same base sequence as sTIM-1, but has 2-fold barrel interior with valines and isoleucines |
| sTIM-3  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | same base sequence as sTIM-1, but has 4-fold barrel interior with valines |
| sTIM-4  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | same base sequence as sTIM-1, but has 2-fold barrel crown with half aspartate-arginine and half asparagine-threonine pairs |
| sTIM-5  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | same base sequence as sTIM-4, but 2-fold barrel interior with valines |
| sTIM-6  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | same base sequence as sTIM-4, but has 4-fold barrel interior with valines |
| sTIM-7  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | circularly permuted sTIM-1                                           |
| sTIM-8  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | sTIM-7 with one lysine; did not express                               |
| sTIM-9  | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | circularly permuted sTIM-1 with cys                                   |
| sTIM-10 | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | circularly permuted sTIM-1 with cys                                   |
| sTIM-11 | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | circularly permuted sTIM-1 with cys                                   |
| sTIM-12 | DILIVDATDKDEAWKQVEQLREGATQIAYRSDDWRLKEAWKKGAGGLGGSGLEHHHHHHH             | half-sized sTIM-7                                                      |
| name | sequence | notes |
|------|----------|-------|
| D1   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D2   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D3   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D4   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D5   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D6   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D7   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D8   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D9   | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D10  | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D11  | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |
| D12  | DILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |       |

See Article and Supplementary Table 1.
| name | sequence | notes |
|------|----------|-------|
| K1   | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKGAGA |  |
|      | LEHHHHHHH |  |
| K2   | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEALKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEALKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEALKKGA |  |
|      | LEHHHHHHH |  |
| K3   | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAWKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAWKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAWKKGA |  |
|      | LEHHHHHHH |  |
| K4   | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAYRSDDWRDLKEAHKKGA |  |
|      | LEHHHHHHH |  |
| K5   | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | LEHHHHHHH |  |
| K6   | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDWRDLKEAHKGAGA |  |
|      | LEHHHHHHH |  |
| K7   | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | WLEHHHHHH |  |
| K8   | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | WLEHHHHHH |  |
| K9   | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | KILIVDATDKDEARKQVEQLAREGATQIAAFRSDDARDLKEAHKGAGA |  |
|      | WLEHHHHHH |  |

See Article and Supplementary Table 1
### The 46 residue unit blueprint for de novo design

| Residue | A | L | E | H |
|---------|---|---|---|---|
| 1       | A | L |   |   |
| 0       | x | E | x | E |
| 0       | x | E | x | E |
| 0       | x | E | x | E |
| 0       | x | L |   |   |
| 0       | x | L | x | L |
| 0       | x | H |   |   |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | L |   |   |
| 0       | x | L | x | L |
| 0       | x | E |   |   |
| 0       | x | E | x | E |
| 0       | x | L |   |   |
| 0       | x | L | x | L |
| 0       | x | H |   |   |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | L |   |   |
| 0       | x | L | x | L |
| 0       | x | L | x | L |
| 0       | x | L | x | L |
| 0       | x | E |   |   |
| 0       | x | E | x | E |
| 0       | x | L |   |   |
| 0       | x | L | x | L |
| 0       | x | H |   |   |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | H | x | H |
| 0       | x | L |   |   |
| 0       | x | L | x | L |

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### Supplementary Table 3

#### RosettaRemodel flags

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#### Fragments only runs

- `-s` [a stub pdb of random 3 residues]
- `-remodel:blueprint TIM-barrel.blueprint`
- `-database rosetta_database`
- `-use_clusters false`
- `-overwrite`
- `-remodel:design:no_design`
- `-rg 2.0`
- `-hb_lrbb 2.0`
- `-hb_srbb 0.0`
- `-vdw 1.0`
- `-cenpack 1.0`
- `-cbeta 1.0`
- `-repeat_structure 4`
- `-randomize_loops false`
- `-num_trajectory [number of fragment sampling trajectories]`
- `-save_top [number of top pdb files to save]`
- `-remodel:quick_and_dirty`
- `-bypass_closure`

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#### Pose-relax runs

- `-s` [stub pdb file]
- `-remodel:blueprint TIM-barrel blueprint`
- `-database rosetta_database`
- `-use_clusters false`
- `-ex1`
- `-ex2`
- `-rg 2.0`
- `-hb_lrbb 2.0`
- `-hb_srbb 0.0`
- `-vdw 1.0`
- `-cenpack 1.0`
- `-cbeta 1.0`
- `-core_cutoff 17`
- `-boundary_cutoff 15`
- `-repeat_structure 4`
- `-randomize_loops false`
- `-num_trajectory [number of fragment sampling trajectories]`
- `-save_top [number of top pdb files to save]`
- `-remodel:use_pose_relax`
- `-remodel:dr_cycles 3`
- `-linmem_ig 10`
- `-cyclic_peptide`
Supplementary Table 4

== RosettaRemodel refinement flags ==

-s [pdb from a previous run]
-remodel:blueprint refinement-stage.blueprint
-database rosetta_database
-num_trajectory [number of trials to run]
-save_top [number of outputs to save]
-randomize_loops false
-repeat_structure 4
-ex1
-ex2
-no_optH false
-bypass_fragments
-hb_lrbb 2.0
-hb_srbb 0.0
-core_cutoff 16
-boundary_cutoff 15
-remodel:use_pose_relax
-remodel:dr_cycles 4
-linmem_ig 10
-overwrite
-bypass_closure
-soft_rep_design
-chain A

== sample blueprint file at iteration 3 ==

1 W L POLAR
2 A L APOLAR
3 A L APOLAR
4 G L APOLAR
5 V L APOLAR
6 L L ALLAAxc
7 A L ALLAAxc
8 D L POLAR EX 1
9 Q L PIKAA DNST
10 L L ALLAAxc
11 R L POLAR EX 1
12 K L POLAR EX 1
13 A L APOLAR
14 L L ALLAAxc
15 I L PIKAA QERKDN EX 0
16 L L ALLAAxc
17 V L APOLAR
18 R L POLAR EX 1
19 L L PIKAA DENQA
20 L L APOLAR
21 K L ALLAAxc
22 R L POLAR EX 1
23 R L POLAR EX 1
24 N L PIKAA G
25 A L APOLAR
26 R L PIKAA GNQDEKRH EX 1
27 Y L PIKAA QN
28 I L APOLAR
29 I L APOLAR
30 F L APOLAR
31 I L ALLAAxc
32 G L ALLAAxc
33 Y L POLAR EX 1
34 R L PIKAA DNST
35 A L ALLAAxc
36 E L PIKAA QERKDN EX 0
37 A L ALLAAxc
38 M L APOLAR
39 L L ALLAAxc
40 L L POLAR EX 1
41 A L APOLAR
42 L L ALLAAxc
43 K L PIKAA QERKDN EX 0
44 I L POLAR EX 1
45 G L PIKAA G
46 A L APOLAR

== sample blueprint file at iteration 8 ==

1 W L PIKAA DK
2 A L PIKAA I
3 A L PIKAA L
4 G L PIKAA I
5 V L PIKAA V
6 L L PIKAA D
7 A L PIKAA A
8 D L PIKAA T
9 Q L PIKAA DS
10 L L PIKAA ADEKRST
11 R L PIKAA DE
12 K L PIKAA DEKQR
13 A L PIKAA A
14 L L PIKAA RW
15 I L PIKAA QERKDN
16 L L PIKAA Q
17 V L PIKAA V
18 R L PIKAA E
19 L L PIKAA Q
20 L L PIKAA L
21 K L PIKAA A
22 R L PIKAA KR
23 R L PIKAA EQR
24 N L PIKAA G
25 A L PIKAA A
26 R L PIKAA K
27 Y L PIKAA Q
28 I L PIKAA I
29 I L PIKAA A
30 F L PIKAA Y
31 I L PIKAA IRST
32 G L PIKAA DS
33 Y L PIKAA D
34 R L PIKAA DS
35 A L PIKAA AW
36 E L PIKAA ERKD
37 A L PIKAA ADEQN
38 M L PIKAA L
39 L L POLAR
40 L L POLAR
41 A L PIKAA A
42 L L PIKAA HL
43 K L PIKAA RK
44 I L PIKAA K
45 G L PIKAA G
46 A L PIKAA A
Supplementary Table 5: Data collection and refinement statistics (molecular replacement)

|                          | sTIM-11 |
|--------------------------|---------|
| **Data collection**      |         |
| Space group              | P 4 1 2 1 2 |
| Cell dimensions          |         |
| a, b, c (Å)              | 50.08, 50.08, 131.28 |
| α, β, γ (°)              | 90.00, 90.00, 90.00 |
| Resolution (Å)           | 46.79 – 1.99 (2.06-1.99)* |
| R_{merge}                | 0.05804 (0.6607) |
| I / σI                   | 15.24 (1.79) |
| Completeness (%)         | 98.26 (93.20) |
| Redundancy               | 5.86 |
| **Refinement**           |         |
| Resolution (Å)           | 1.99 |
| No. reflections          | 69721 (5756) |
| R_{work} / R_{free}      | 0.2237 / 0.2607 |
| No. atoms                |         |
| Protein                  | 2836 |
| Water                    | 20 |
| B-factors                |         |
| Protein                  | 57.3 |
| Water                    | 49.9 |
| R.m.s. deviations        |         |
| Bond lengths (Å)         | 0.013 |
| Bond angles (°)          | 1.21 |

*Values in parenthesis are for highest-resolution shell.