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Supplementary Materials for

Elucidation of master allostery essential for circadian clock oscillation in cyanobacteria

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Supplementary Text

Selection of non-phosphorylatable amino acids upon designing phospho-mimicking mutants

Phosphorylatable amino acids, such as serine and threonine, are often substituted with alanine to investigate the functional effects of those phosphoryl modifications. A S431A/T432A KaiC double mutant (KaiC-AA) was used as a phospho-mimic mutant of KaiC-ST (*21*). However, as reported previously (*46*), alanine substitutions at both or either of the dual phosphorylation sites (S431A or T432A) resulted in much higher ATPase activity (25.7 ATP d^{-1} for KaiC-AA) than that observed for fully dephosphorylated KaiC-ST (13.6 ± 1.4 ATP d^{-1}) (fig. S2C). By contrast, KaiC-SV is designed to reduce these unwanted side effects by selecting valine instead of alanine because valine has the closest volume (fig. S2A) and topology (fig. S2B) to threonine. KaiC-SV, which has a helical PSw (upper left panel of **Fig. 2**) and similar ATPase activity (13.3 ± 0.7 ATP d^{-1}) to KaiC-ST (fig. S2C), exhibits temperature-compensated ATPase- (**Fig. 4B**) and mono–P-cycles (**Fig. 4A**) that are in sharp contrast to the arrhythmicity observed for KaiC-SA and KaiC-AT (fig. S2C). KaiC-CT was designed using the same logic described above to generate a mutant with ATPase activity (9.9 ± 0.7 ATP d^{-1}) that does not exceed that of KaiC-ST but also exhibits arrhythmicity (**Fig. 4A**). The present results clearly indicate that S431 is the primary residue responsible for CI–CII allostery affecting rhythmicity and that special care must sometimes be taken when replacing phosphorylatable amino acids with non-phosphorylatable ones.

Assembly and disassembly dynamics for the KaiA/KaiB/KaiC-pSV ternary complex

After reconstructing the *in vitro* P-cycle for KaiC-SV (fig. S3A), every aliquot taken at a time interval of 13 h was used for size-exclusion chromatography and RALS analyses. When maximally phosphorylated (fig. S3B), KaiC-pSV weakly binds to KaiB. The KaiA/KaiB/KaiC-pSV ternary complex (*8, 9*) begins to form at a dephosphorylating state (fig. S3C), and its accumulation peaks at the maximally dephosphorylated state (fig. S3D). The populated ternary complex is disassembled at a phosphorylating state (fig. S3E). KaiC-SV exhibits minimal allostery to achieve system-level synchronization via KaiA sequestration (*22, 24*) and to drive the assembly–disassembly cycle of the Kai proteins.

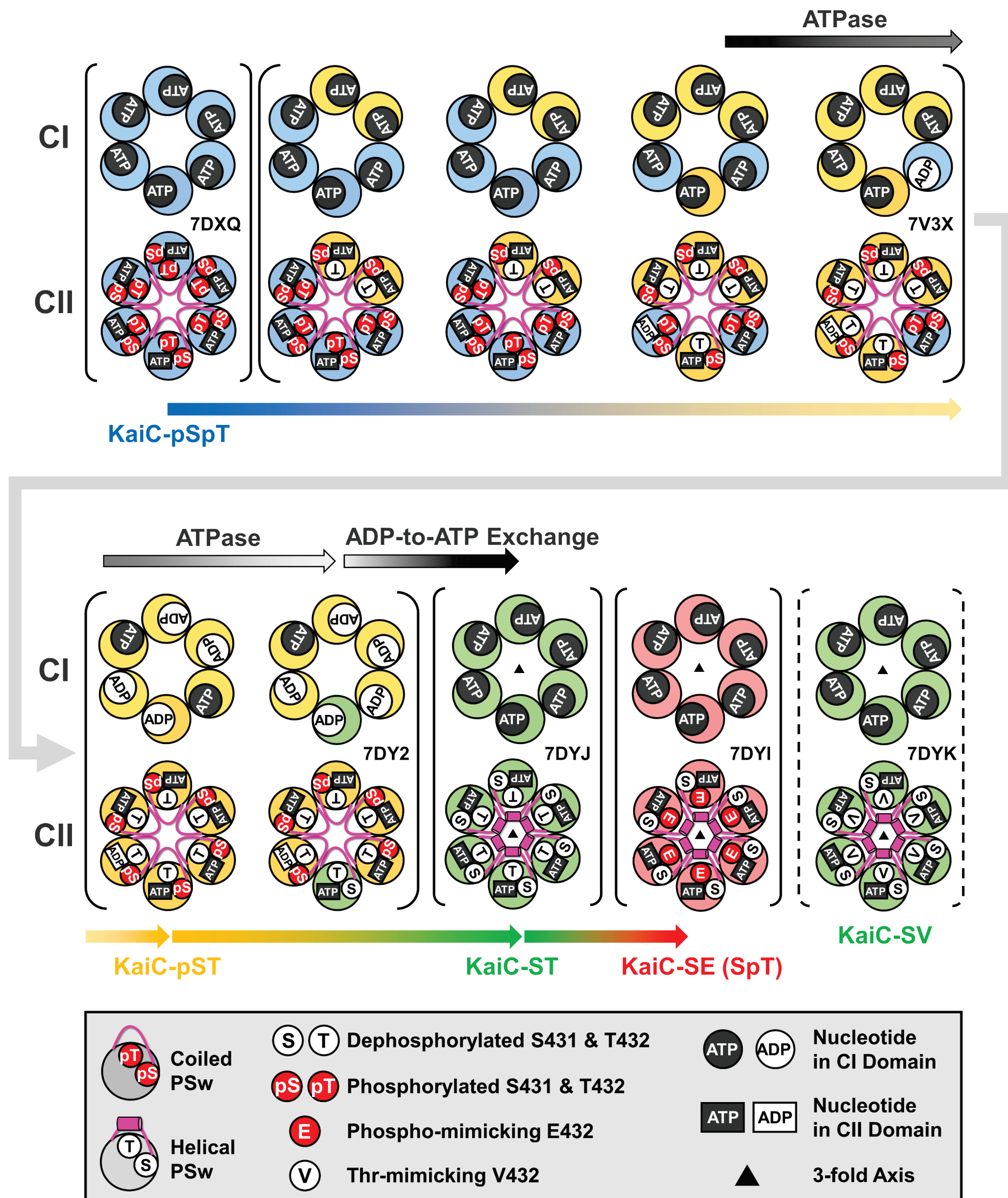

Fig. S1. Schematic drawing of the crystal structure library.

Five crystallographic data sets (in solid parentheses) are aligned in the following order: 7DXQ (a hexamer of KaiC-pSpT in $P2_12_12_1$), 7V3X (four hexamers of partial KaiC-pSpT and KaiC-pST in $P2_1$), 7DY2 (two hexamers of KaiC-pST and partial KaiC-ST in $P2_1$), 7DYJ (a hexamer of KaiC-ST reconstructed in $P6_3$), and 7DYI (a hexamer of KaiC-SE reconstructed in $P6_3$) with annotations in the box below the alignment. KaiC-SV, a mono–P-cycle oscillator, is coded as

7DYK and drawn as a hexamer according to the $P6_3$ space group in dashed parentheses. The filled triangles in 7DYJ, 7DYI, and 7DYK indicate the crystallographic three-fold axes present at the centers of the hexamers. Arrows above and below the hexameric rings represent biochemical events in the CI domain and phosphorylation-state transitions in the CII domain, respectively.

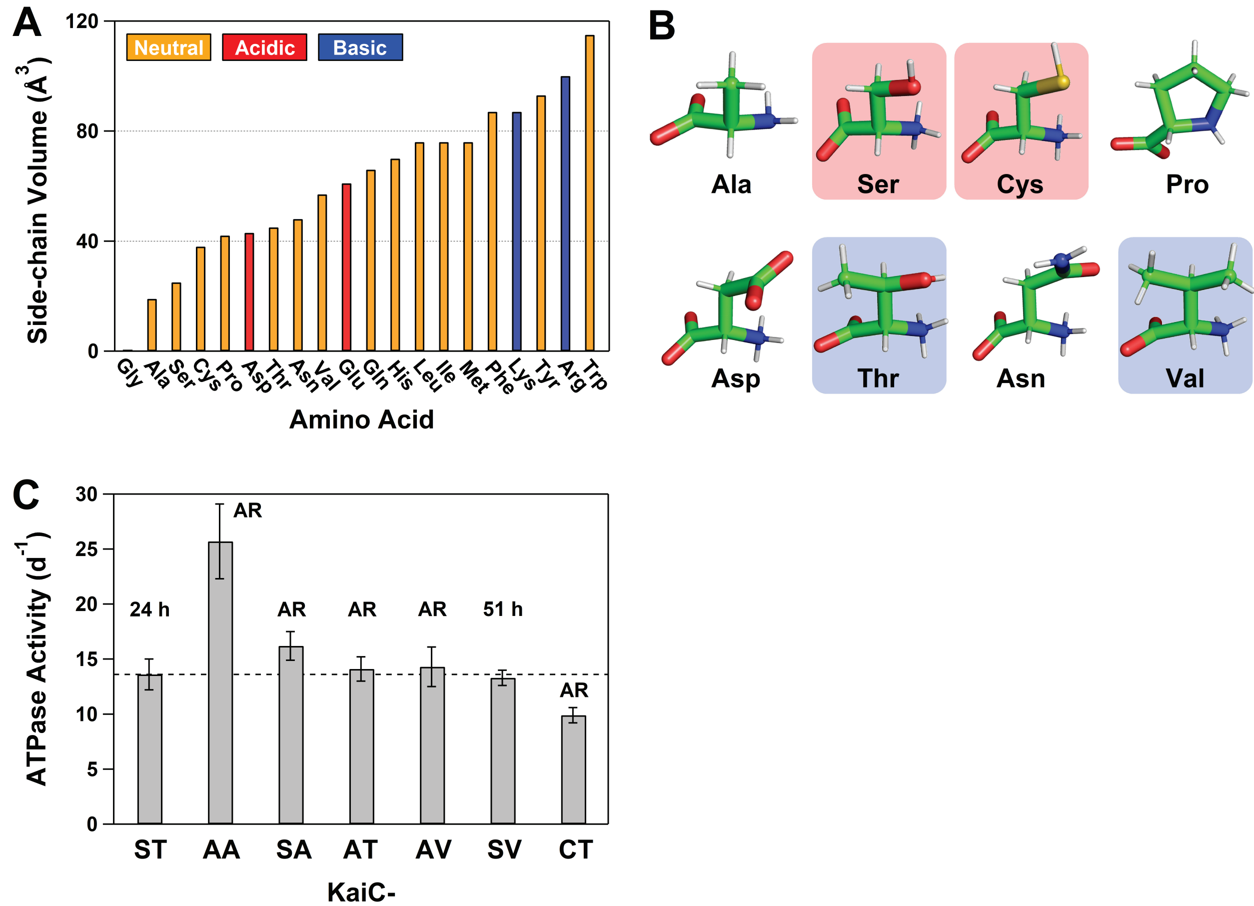

Fig. S2. Candidate amino acid substitutions at dual phosphorylation sites.
(**A**) Side-chain volume and (**B**) topology of amino acid residues. (**C**) Steady-state ATPase activity at 30°C. Values and AR above the error bars indicate the period length or arrhythmicity, respectively, for the *in vitro* P-cycle in the presence of KaiA and KaiB at 30°C.

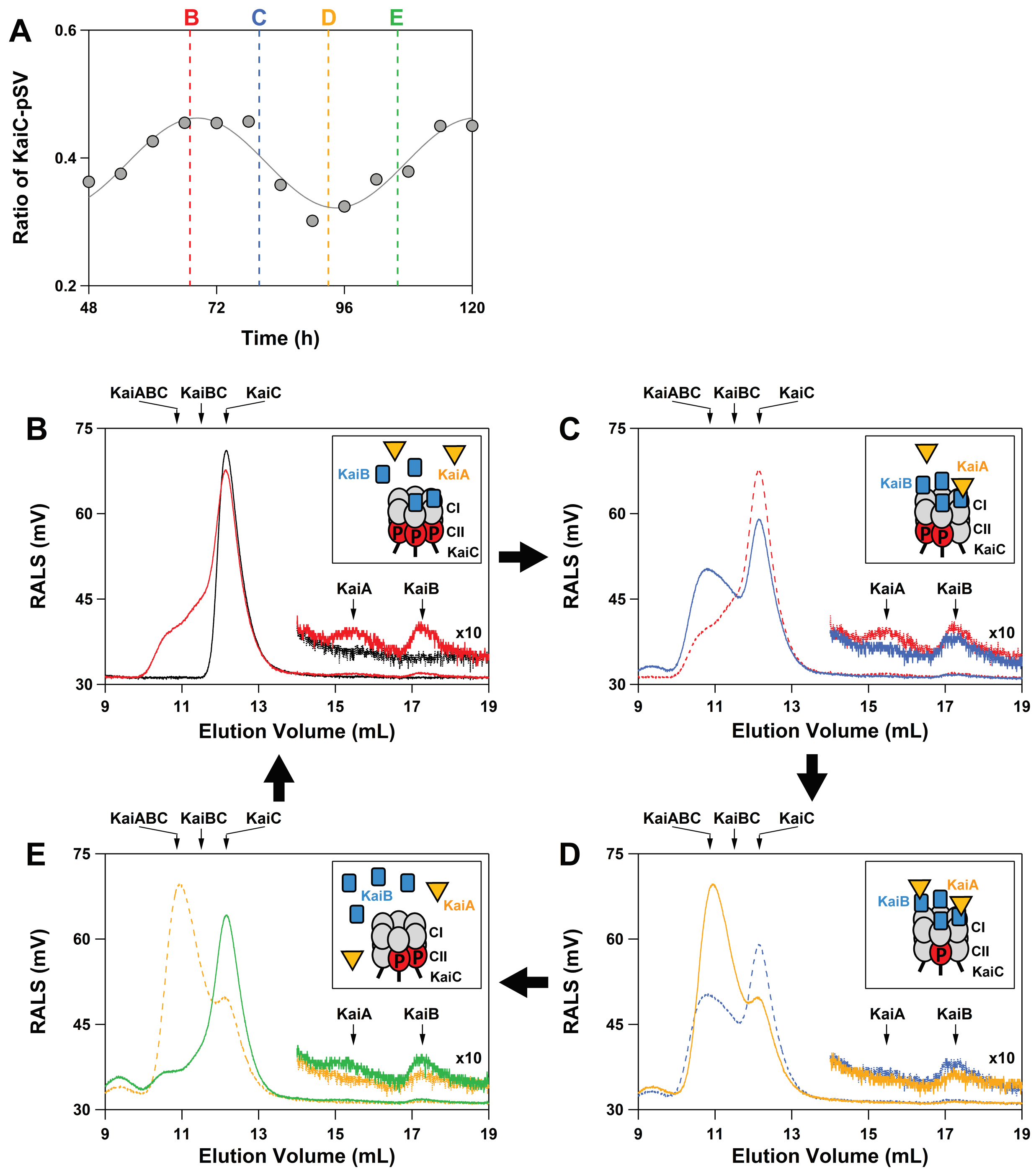

Fig. S3. Assembly and disassembly cycle of the KaiA/KaiB/KaiC-SV ternary complex. (**A**) P-cycle for KaiC-SV at 30°C. (**B**) Red, (**C**) blue, (**D**) orange, and (**E**) green solid lines correspond to the elution curves for aliquots taken at 67, 80, 93, and 106 h, respectively. Molecular masses of proteins in the eluted fractions were analyzed using right-angle light scattering (RALS). The black solid line depicts the elution curve for KaiC-SV alone. For reference, elution curves for the aliquots taken at 67, 80, and 93 h are shown using red, blue, and orange dotted lines, respectively. The insets correspond to schematic illustrations of the assembled and disassembled states of the ternary complex.

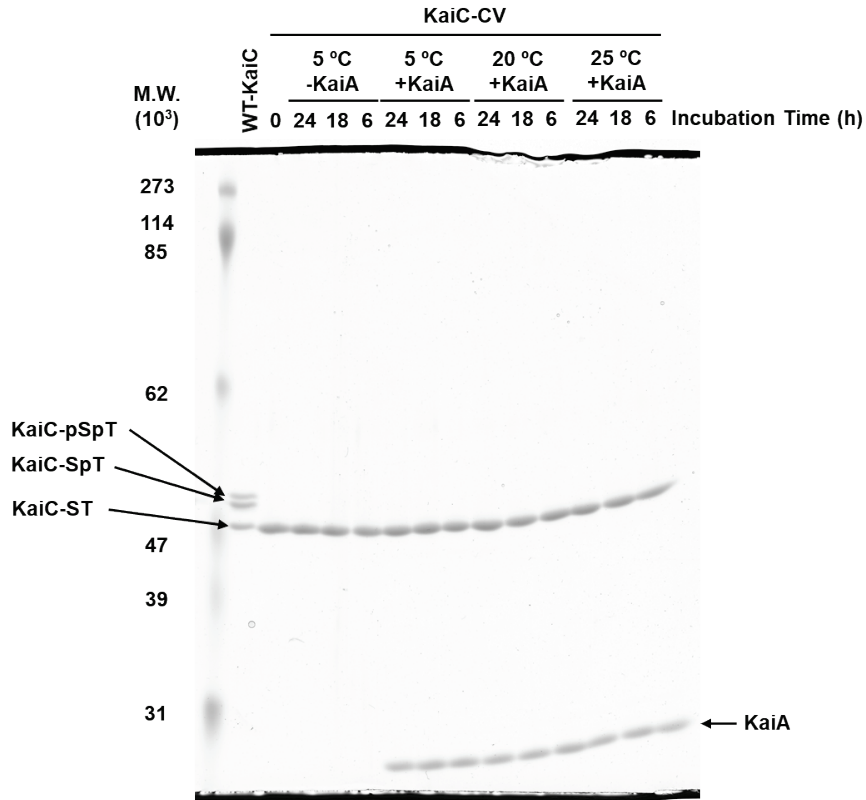

Fig. S4. SDS-PAGE analysis of the S431C/T432V mutant (KaiC-CV) at 5, 20, and 25 °C with or without KaiA.

The possibility of KaiC-CV (0.2 mg/mL, 3.4 μM) being auto-phosphorylated was examined by lowering temperature and by increasing the concentration of KaiA. Note that the final concentration of KaiA (0.12 mg/mL, 3.7 μM) was three-fold higher than the standard concentration (0.04 mg/mL) for the *in vitro* P-cycle assay.

Table S1. Data collection and refinement statistics.

Protein	KaiC-pSpT	KaiC-pSpT&pST	KaiC-pST	KaiC-ST	KaiC-SE	KaiC-SV
Data Collection						
Space group	$P2_12_12_1$	$P2_1$	$P2_1$	$P6_3$	$P6_3$	$P6_3$
Unit cell parameters						
a, b, c (Å)	92.4, 159.9, 207.24	185.5, 205.8, 186.2	92.8, 206.6, 168.4	94.6, 94.6, 179.9	94.3, 94.3, 180.6	94.9, 94.9, 180.6
α, β, γ (º)	90, 90, 90	90, 115.1, 90	90, 94.7, 90	90, 90, 120	90, 90, 120	90, 90, 120
Wavelength (Å)	0.9	0.9	0.9	0.9	0.9	0.9
Resolution range (Å)[a]	30-2.80	50-3.10	50-3.04	30-2.40	48.5-2.64	48.5-3.00
	(2.90-2.80)	(3.15-3.10)	(3.15-3.04)	(2.44-2.40)	(2.77-2.64)	(3.11-3.00)
Total reflections	561605	883423	457569	507210	280978	191896
Unique reflections	75955 (7451)	228464 (11426)	120308 (12016)	35578 (1775)	26712 (3551)	19313 (1788)
Redundancy	7.4 (7.3)	3.9 (3.8)	3.8 (3.8)	14.3 (14.1)	10.5 (10.4)	10.4 (10.8)
Completeness (%)	99.9 (100)	99.9 (100)	99.9 (100)	100 (99.8)	99.9 (99.9)	99.7 (98.2)
R_{merge} (%)[b]	9.2 (>100)	10.2 (74.4)	13.3 (89.9)	27.0 (>100)	10.9 (>100)	13.8 (96.2)
(I)/sigma(I)	21.4 (2.0)	13.8 (2.1)	15.6 (2.0)	36.4 (2.7)	13.2 (2.1)	12.9 (2.8)
Model building						
Molecular replacement	2GBL	2GBL	2GBL	2GBL	2GBL	7DYJ
Total atoms	21020	78739	39277	6837	6669	6705
Protien	20538	77135	38547	6653	6510	6564
Ligands	384	1512	712	128	128	128
Water	98	92	18	56	31	13
R_{work} (%)[c]	25.0	27.5	26.3	26.7	29.4	27.4
R_{free} (%)[c]	31.8	34.0	32.6	31.0	33.1	32.8
R.M.S.D. from ideality						
Bond length (Å)	0.002	0.006	0.002	0.002	0.002	0.002
Bond angles (º)	1.2	1.5	1.2	1.25	1.2	1.2
Average B factors (Å^2)	45.7	58.0	47.0	58.4	70.2	72.7
Rmachandran plot						
Most favored (%)	82.6	92.2	82.3	86.9	84.5	84.2
Allowed (%)	16.8	6.3	17.5	12.8	15.6	15.6
Disallowed (%)	0.6	1.4	0.2	0.2	0.0	0.1
PDB code	7DXQ	7V3X	7DY2	7DYJ	7DYI	7DYK

[a]Values in parentheses are for the highest-resolution shell.
[b]$R_{merge} = \Sigma|I-\langle I\rangle|/\Sigma I$, where I corresponds to the observed intensity of reflections.
[c]$R_{work, free}=\Sigma|F_{obs}|-|F_{calc}|/\Sigma|F_{obs}|$. R_{free} is the cross-validation of the R-factor using the test reflections, 5% of the data, not included in the refinements.

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