Supplemental Information inventory

Supplemental Figures and Tables.

Figure S1. ClpX hexamer structures; W-W-W^{TT} activity (related to Figure 1 and Table S1).

(**A**) Top and side views of structures (protein in cartoon representation; nucleotide in CPK representation) of a 4L:2U hexamer (left), the modeled 5L:1U hexamer (middle), and the 6L hexamer (right). The 4L:2U and 5L:1U structures are asymmetric, whereas the 6L structure is essentially 6-fold symmetric. (**B**) Tethered ClpX W-W-W^{TT} trimers (0.3 μM pseudo hexamer) containing the TT modifications (D76C^{TAMRA}; K330C^{TAMRA}) had activities similar to the parental W-W-W enzyme (0.3 μM pseudo hexamer) in hydrolyzing 4 mM ATP (left panel) and in supporting degradation of 10 μM cp7-GFP-ssrA by 0.5 μM ClpP (right panel). Data are shown as mean ± SD.

Figure S2. ADP binding and gel filtration; activity of covalent hexamers with one mutant subunit (related to Figure 2).

(**A**) Binding of ADP to W-VI-W assayed by isothermal titration calorimetry. **(B)** Binding of ADP to W_6 ClpX assayed by isothermal titration calorimetry. In panels A and B, the initial concentrations in pseudo hexamer equivalents of the ClpX variants were 15.8 μM (W-VI-W) and 54.0 μM (W_6). Binding isotherms were fit to a one-site model using MicroCal Origin software. Data also fit well two a two-site model (W-VI-W; site 1: K_D = 3 ± 1 μM, N = 2.0 ± 0.6; site 2: K_D = 26 ± 10 μM, N = 1.3 ± 0.8; W6; site 1: K_D = 5 ± 2 μM, N = 2.0 ± 1.1; site 2: K_D = 46 ± 20 μM, N = 2.0 ± 0.8). **(C)** W-VI-W and W-W-W chromatographed at positions expected for pseudo hexamers on a Superose 6 gel-filtration column (loading concentration 15 μM). **(D)** ATP dependence of the rate of cp7-CFP-ssrA (20 μM) degradation by covalent ClpX hexamers (0.2 μM pseudo hexamer; 0.5 μM $ClpP_{14}$) containing all wild-type subunits or a single nucleotide-binding-deficient VI, VIE, or VIK subunit. The VIK subunit contains V78A/I79A and K125M, which alters the conserved lysine of the Walker-A motif and also prevents hydrolysis in the subunit bearing this substitution. The data for W-W-W-W-W-W and W-W-W-W-W-VIE are reproduced from Fig. 2F. The lines are fits to the Hill equation. Fitted parameters are listed in Table S2. Data are shown as mean ± SD.

Figure S3. Metal dependence of ClpX and ClpP activity; ATP hydrolysis and nucleotide binding of nCoMET variants (related to Figure 3).

(**A**) Mg^{2+} and Co^{2+} supported activity of W-W-W ClpX (1 μM pseudo hexamer) in unfolding 10 μM cp7-CFP-ssrA. **(B)** Mg^{2+} and Co^{2+} supported activity of W-W-W (0.3 μM pseudo hexamer) in hydrolysis of ATP in the presence of 10 μM cp7-CFP-ssrA. **(C)** Mg^{2+} and Co^{2+} supported activity of W-W-W (1 μM pseudo hexamer) in hydrolysis of ATPγS. In panels A-C, assays were performed in PD buffer supplemented with the appropriate divalent metal (10 mM) at room temperature. **(D)** Co^{2+} inhibits ClpP cleavage. The rate of cleavage of a succinyl-Leu-Tyr-AMC dipeptide (50 μM) by $ClpP_{14}$ (1 μM) was assayed by changes in fluorescence (excitation 345 nm; emission 440 nm) in PD buffer supplemented with 10 mM $MgCl_2$ or 10 mM $CoCl_2$. Rates were normalized to the rate with 10 mM $MgCl_2$. **(E)** Rates of hydrolysis of ATP (5 mM) by W-W-W and the Oregon-Green labeled W-W-W* variant (0.3 μM pseudo hexamer) in the presence of cp7-CFP-ssrA (10 μM). **(F)** ADP binding to pseudo hexamers (0.1 μM) of W*-VI-W, W-VI*-W, and W-VI-W* assayed by nCoMET. The lines are fits to a hyperbolic equation. K_{app} values are listed in Table S3. **(G)** Nucleotide binding to W-W-W* (0.1 μM pseudo hexamer for ATPγS and ADP titrations; 0.5 μM pseudo hexamer plus 10 μM cp7-CFP-ssrA for ATP titration) assayed by nCoMET. Lines are fits to a hyperbolic function. K_{app} values are listed in Table S3. Data are represented as mean ± SD.

Figure S4. Activity and nucleotide dependence of cCoMET variants (related to Figure 4).

(**A**) Tethered ClpX trimers (0.3 μM pseudo hexamer) containing the cCoMET (§) modifications ($K330C^{TAMRA}$; H68Q/N72H/D76H) were active in hydrolyzing 4 mM ATP in the presence or absence of Ni^{2+}-NTA (500 μM). **(B)** Modified variants were also active in supporting degradation of 10 μM cp7-GFP-ssrA by 0.5 μM ClpP. Same conditions as panel A. **(C)** ADP-dependent changes in the conformations of subunits containing cCoMET probes were assayed

for pseudo hexamers (0.3 μM) of $W^{§}$-VI-W, W-$VI^{§}$-W, and W-VI-$W^{§}$. Lines are fits to a Hill equation. The values of K_{app} and n were 11 ± 1 μM and 1.1 ± 0.1 for $W^{§}$-VI-W, 270 ± 8 μM and 2.2 ± 0.1 for W-$VI^{§}$-W, and 6 ± 2 μM and 1.1 ± 0.2 for W-VI-$W^{§}$. (**D**) Nucleotide-dependent changes in the rightmost subunit of W-W-$W^{§}$ (0.3 μM pseudo hexamer for ATPγS and ADP titrations; 0.3 μM pseudo hexamer plus 10 μM cp7-CFP-ssrA for ATP titration) assayed by cCoMET. Lines are fits to a Hill equation. K_{app} and n values were 66 ± 5 μM and 1.4 ± 0.1 for ADP, 150 ± 5 μM and 1.6 ± 0.1 for ATP, and 27 ± 1 μM and 1.6 ± 0.1 μM for ATPγS. Data are represented as mean ± SD.

Figure S5. L-lock variants bind ClpP (related to Figure 5).

Rates of cleavage of a fluorescent decapeptide (15 μM) by a cysteine-free $ClpP_{14}$ variant (50 nM) were determined in the presence of different ClpX variants (0.2 μM pseudo hexamer) and 1 mM ATPγS. The disulfide-bonded L-lock enzymes bind ClpP and enhance peptide cleavage, although they do not support degradation of protein substrates. Data are represented as mean ± SD.

Figure S6. Specificity of nCoMET quenching; cCoMET controls (related to Experimental Procedures).

(A) Specificity of nCoMET quenching. In PD buffer plus 10 mM $CoCl_2$, ATP reduced the fluorescence of W-W-W*, but GTP or buffer with no nucleotide did not result in quenching. Data are shown as mean ± SD. **(B)** In the absence of Ni^{2+}-NTA, titration of ATP against W-W-$W^{§}$ (0.3 μM) did not result in quenching. **(C)** Titration of Ni^{2+}-NTA against W-W-$W^{§}$ (0.1 μM) gave ~30% quenching The line is a single hyperbolic fit with K_{app} = 25 ± 2 μM. **(D)** ATP dependence of cCoMET quenching for a W-W-W variant containing TAMRA-labeled S389C in the small AAA+ domain of the second subunit and the His^{72}-X_3-His^{76} mutations in the large domain of the third subunit. This cCoMET pair spans a single rigid-body unit. The line is a single hyperbolic fit with K_{app} = 30 ± 9 μM. At nucleotide concentrations at which the low-affinity sites are occupied and ATP hydrolysis occurs, no major changes were observed indicating that the conformational changes monitored with other cCoMET pairs, which correlate with ATP hydrolysis, involve changes across the hinged interfaces of ClpX rather than the rigid-body interfaces.

Table S1. Crystallographic statistics (related to Figure 1).

ClpX variant	E-E-ER	W-W-W	W-W-W	W-W-R	E-R	W-W-W
PDB code	4I81	4I4L	4I34	4I5O	4I63	4I9K
tether length	20	20	20	20	20	0
bound nucleotide	ATPγS	ADP	none	none	none	none
crystallization well solution	*a*	*a*	*a*	*b*	*b*	*c*
Data collection						
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P6_3$
unit-cell lengths (a, b, c) (Å)	57.9 199.2 211.9	55.9 181.9 201.4	58.3 199.6 203.4	55.2 199.9 222.3	55.2 201.2 222.6	119.4 119.4 111.7
unit-cell angles (α,β,γ) (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 120
resolution (Å)	50.0 – 3.8	50 – 3.7	50.0 – 4.1	50.0 – 4.5	50.0 – 5.7	60.0 – 4.5
R_{sym} (%)	7.4 (84.3)	7.7 (30.9)	5.9 (29.4)	7.7 (27.8)	9.6 (35.2)	18.6 (27.2)
(I)/sig(I)	24.7 (1.6)	16.9 (3.1)	24.8 (3.8)	17.1 (4.3)	20.7 (2.9)	5.5 (4.1)
redundancy	6.7 (6.5)	3.8 (3.4)	5.7 (4.3)	4.0 (3.2)	8.5 (5.0)	6.4 (6.6)
completeness (%)	98.8 (95.5)	93.9 (95.3)	97.2 (84.1)	92.2 (80.4)	99.4 (96.2)	94.6 (95.7)
Refinement						
resolution (Å)	41.0 – 3.9	45.4 – 3.7	38.5 – 4.1	48.3 – 4.5	49.1 – 5.7	49.1 – 5.0
R_{work}/R_{free} (%)	26.2/29.6	27.5/33.4	27.4/30.8	28.6/32.3	30.7/31.5	32.2/35.2
rmsd bond angles (°)	0.452	0.508	0.499	0.452	0.449	0.613
rmsd bond lengths (Å)	0.002	0.003	0.003	0.002	0.002	0.004
allowed Ramachandran (%)	100	100	100	100	100	100

Well solution *a* is 1.9 M ammonium sulfate, 75 mM sodium acetate (pH 4.8). Well solution *b* is 2.2 M ammonium sulfate, 0.2 M ammonium bromide, 0.1 M bicine (pH 9.0). Well solution *c* is 2 M ammonium sulfate, 0.15 M potassium sulfate, 4 mM ATP, 4 mM $MgCl_2$ chloride, and 50 mM EDTA.

Table S2. Nucleotide-interaction parameters obtained from activity assays (related to Figure 2).

variant	nucleotide	assay	K_M or $K_{1/2}$ (μM)	Hill constant	R^2
W-W-W	ATP	hydrolysis	230 ± 3	1.3 ± 0.1	0.999
		unfolding	410 ± 23	1.4 ± 0.1	0.998
	ATPγS	hydrolysis	9 ± 1	1.3 ± 0.2	0.998
W-VI-W	ATP	hydrolysis	3900 ± 350	not determined	0.998
		unfolding	3300 ± 210	1.6 ± 0.1	0.999
	ATPγS	hydrolysis	470 ± 37	0.9 ± 0.1	0.999
W-VIE-W	ATP	hydrolysis	79 ± 7	0.9 ± 0.1	0.996
W-W-W-W-W-W	ATP	degradation	22 ± 2	1.4 ± 0.2	0.985
W-W-W-W-W-VI	ATP	degradation	40 ± 3	1.5 ± 0.2	0.987
W-W-W-W-W-VIE	ATP	hydrolysis	67 ± 11	1.3 ± 0.2	0.979
		degradation	79 ± 14	0.8 ± 0.1	0.987
W-W-VIE-W-W-W	ATP	hydrolysis	86 ± 16	1.1 ± 0.2	0.976
		degradation	98 ± 10	1.3 ± 0.1	0.993
W-W-W-W-W-VIK	ATP	degradation	400 ± 100	1.2 ± 0.2	0.972

VI – V78A/I79A
VIE – V78A/I79A/E185Q
VIK – V78A/I79A/K125M

Table S3. Nucleotide-interaction parameters obtained from nCoMET assays of binding (related to Figure 3).

variant	nucleotide	K_{app} (μM)	R^2
W-W-W*	ADP	25 ± 4	0.990
	ATP	79 ± 8	0.996
	ATPγS	17 ± 2	0.994
W-VI-W*	ADP	1.8 ± 0.1	0.998
	ATP	14 ± 1	0.996
	ATPγS	13 ± 1	0.998
W*-VI-W	ADP	10 ± 2	0.982
	ATP	68 ± 14	0.984
	ATPγS	59 ± 8	0.992
W-VI*-W	ADP	not detected	
	ATP	not detected	
	ATPγS	430 ± 200	0.947

VI – V78A/I79A

Extended Experimental Procedures

Crystallization and structure determination

Crystallized proteins included a tethered E-R dimer and tethered W-W-W, W-W-R and E-E-ER trimers, where ER designates E185Q/R370K subunits (Table S1). These proteins did not contain the C169S mutation. Polypeptide tethers were twenty (T_{20}) or zero (T_0) residues and compatible with ClpX function (Glynn et al., 2012). Variants were crystallized at room temperature by hanging-drop vapor diffusion after mixing 1 µL of well solution with 1 µL of protein solution (~40 µM pseudo hexamer). The composition of well solutions are listed in Table S1. The nucleotide-bound form of W-W-W with T_{20} tethers was obtained using a soaking procedure similar to one described previously (Glynn et al., 2009) but limited to 5 min. To obtain the structure of E-E-ER bound to ATPγS, nucleotide-free crystals were soaked in 3.4 M sodium malonate, 75 mM sodium acetate (pH 4.8), 4 mM ATPγS, and 4 mM $MgCl_2$ for approximately 2 h. All crystals were cryo-protected by coating in Paratone-N (Hampton Research) and flash-frozen in liquid nitrogen. Data were collected at the 24-ID-C beamline of the Advanced Photon Source, Argonne National Laboratories. Unit-cell volumes indicated that crystals with the space groups $P2_12_12_1$ and $P6_3$ contained six and two subunits in the asymmetric unit, respectively. Data collection and refinement statistics are listed in Table S1.

Diffraction data were integrated and scaled using HKL2000 (Otwinowski and Minor, 1997), TRUNCATE (Winn et al., 2011), MOSFLM (Leslie and Powell, 2007) and SCALA (Winn et al., 2011). The structures of the large and small AAA+ domains of *E. coli* ClpX (Glynn et al., 2009) were used as molecular-replacement search models in PHASER (McCoy et al., 2007). Each domain was placed independently to avoid bias towards previously observed conformations. Manual model building and real-space refinement were carried out in COOT (Emsley et al.,

2010). Rigid-body refinement, TLS refinement, and grouped atomic displacement parameter refinement were performed using PHENIX (Adams et al., 2010). Individual large and small AAA+ domains were defined as rigid bodies and TLS groups, with either individual domains or individual residues defined as atomic displacement parameter groups based on the resolution and quality of the data.

For crystals soaked in nucleotide, examination of calculated mF_o-DF_c maps revealed strong peaks of positive density in the nucleotide-binding pockets of loadable subunits. The single bound nucleotide in the ATPγS-soaked W-W-W (T_{20}) structure could not be determined unambiguously, probably because of mixed occupancy by nucleotide and sulfate ions, and ADP provided the best fit for the electron density. For the E-E-ER crystal soaked in sodium malonate and ATPγS, the bound nucleotides were unambiguously modeled as ATPγS.

Structural validation was performed using MolProbity (Chen et al., 2010). Superposition of structures was carried out using LSKQAB (Winn et al., 2011). The atomic coordinates for all structures have been deposited in the Protein Data Bank, with accession codes listed in Table S1.

Supplemental References

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