SUPPLEMENTARY INFORMATION

Analysis of nucleotide binding to p97 reveals the properties of a tandem AAA hexameric ATPase

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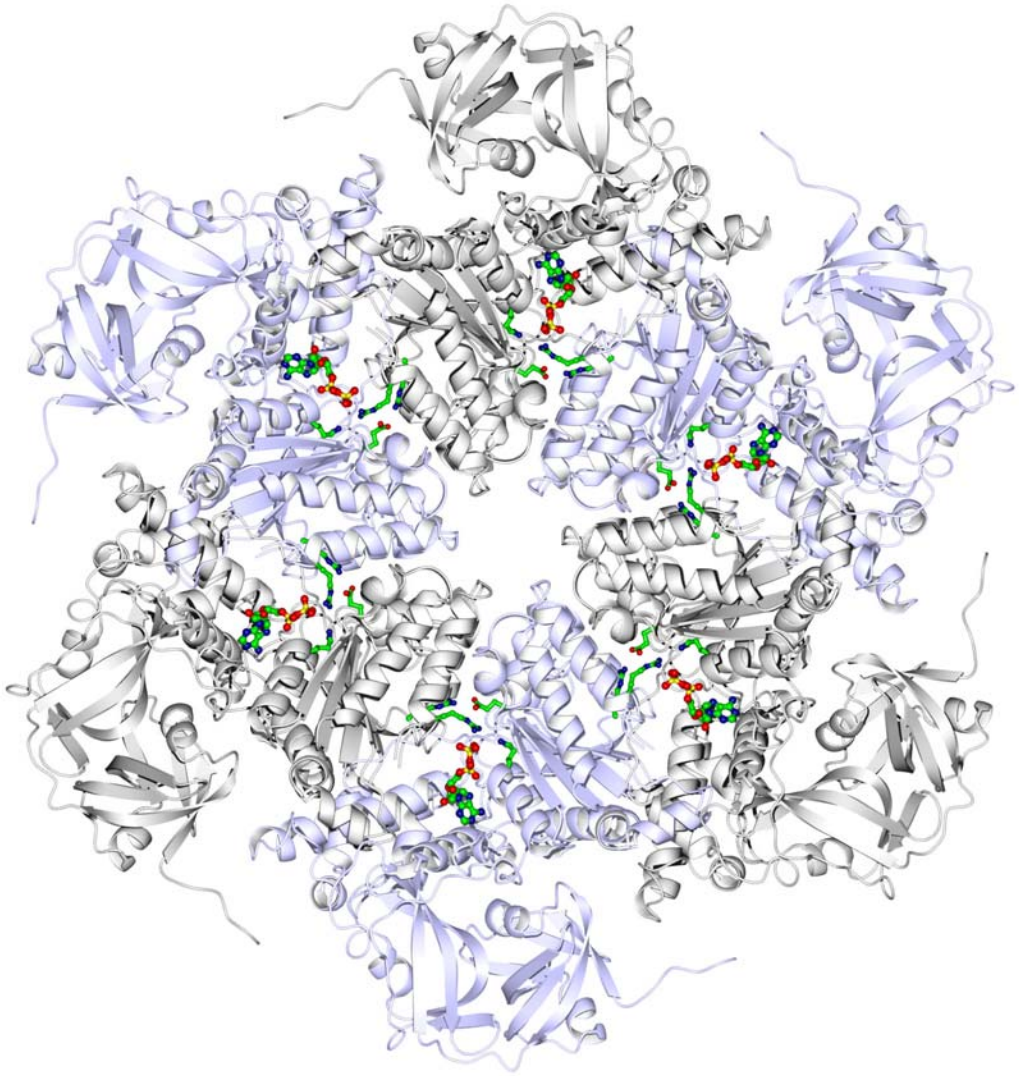
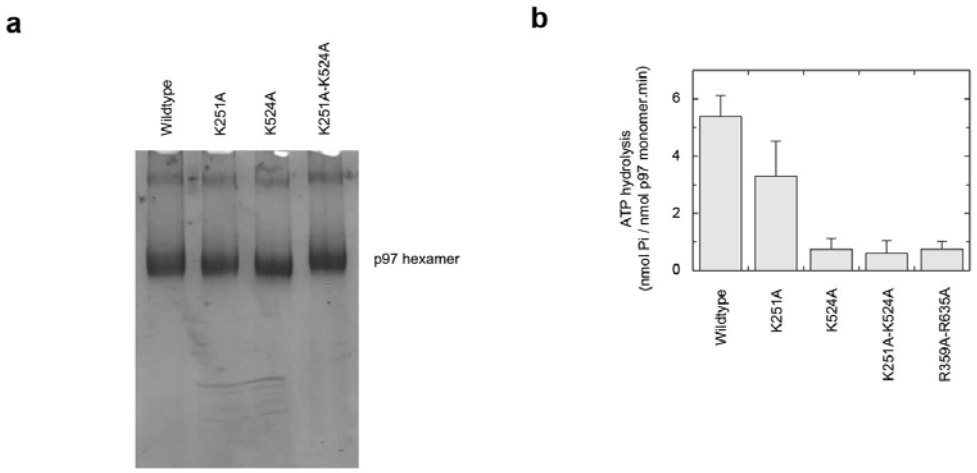
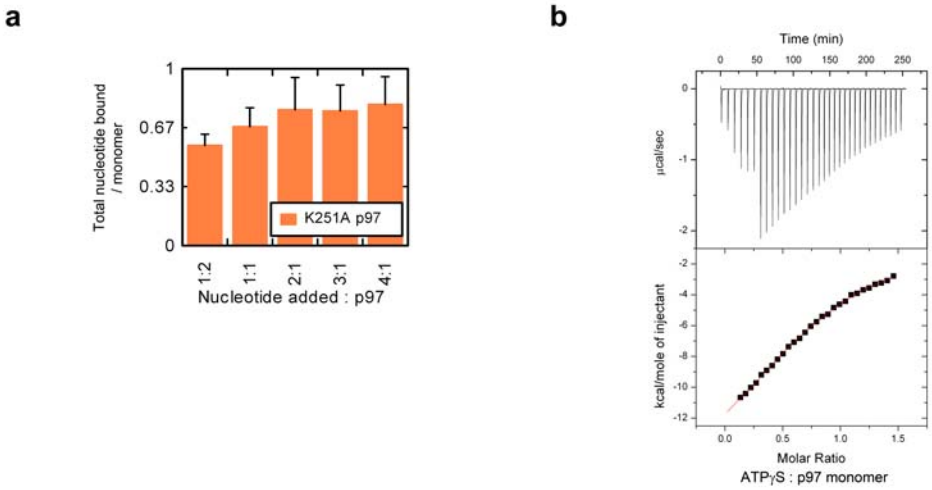


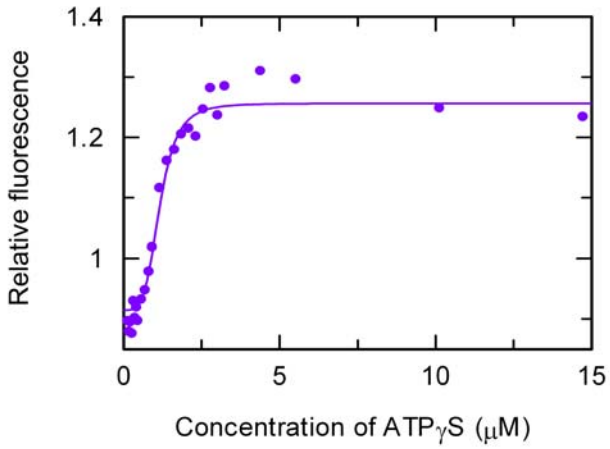
Supplementary Figure 1. The crystal structure of p97 hexamer. This view of the hexameric ring shows the D1 domains positioned in the centre of the ring making close inter-monomer contacts and the N domains at the periphery, protruding away from the ring. The D2 domains have similar structure to the D1 domains and are stacked behind the D1 domains in this orientation. The D1 active site is positioned at the monomer interface and is shown bound to ADP. The side chains of residues K251, E305, R359, R362 are also highlighted in ball and stick form.



Supplementary Figure 2. Characterization of mutant p97 preparations. a. Non-denaturing gel demonstrating that mutations of the Walker A motif do not alter the oligomeric state of p97, as mutants have the same mobility as wildtype p97. The major band represents hexameric p97 and the faint higher band interpreted as a small population of p97 aggregates. **b.** The ATPase activity of wildtype p97 and Walker A and SRH mutants. When compared to wt p97, mutation of the D1 Walker A motif (K251A) decreased the activity by a third whereas mutation of the D2 Walker A motif (K524A and K251A-K524A) decreased the activity to below a fifth of wildtype p97. Mutation of an arginine residue of the SRH in D1 and D2 (R359A-R635A) similarly decreased ATPase activity suggesting that these residues are also key for ATP hydrolysis.



Supplementary Figure 3. ATPγS binding to Walker A mutants of p97.
a. Modified heat denaturation experiments when ATPγS is added show that p97 mutated in the D1 Walker A motif is likely to be saturated by 0.75 molecules nucleotide per monomer (4.5 molecules nucleotide per hexamer). This potentially represents ATPγS binding to the D2 domain and a small amount of ADP pre-bound to the mutated D1 domain. **b.** ITC data of ATPγS titrated against K251A-K524A p97 (625 µM and 100 µM respectively). The resulting diagonal ITC profile demonstrates that ATPγS binding was of low affinity due to Walker A mutation of both domains.



Supplementary Figure 4. Tryptophan fluorescence of rat p97 when ATPγS is added. ATPγS induced tryptophan fluorescence changes of p97 purified from rat tissue show a sigmoidal binding curve. Fitting to the cooperative binding equation indicates that the affinity of ATPγS binding is similar to recombinant p97 and the Hill coefficient indicates that cooperativity involves at least 4 monomers.

Supplementary Table 1. **Results from fits to ITC data.** ITC data was fitted to two site and competition models listed and the values derived from the best fits (lowest reduced Chi^2) to multiple ITC experiments (n) were averaged (mean, standard deviations shown as +/-). The range of Chi^2 values indicates the spread of best fits obtained for the multiple experiments averaged for each protein/ligand combination. To constrain fits to low affinity sites (e.g. wildtype binding ADP and K251A binding ATPγS) the stoichiometry is fixed at 1.

p97	Ligand	Model		N	+/-	K_a (M^{-1})	+/-	K_d (μM)	+/-	ΔH (kcal.mol^{-1})	+/-	n	Chi^2 Range
Wildtype	**ADP**	Two site	**D1 ADP**	0.2	0.05	1.55E6	1.3E6	1.1	0.8	-9.2	3.5	9	700 – 39200
			D2 ADP	1	-	1.59E4	9.3E3	86	51	-6.5	2		
K524A	**ATPγS & ADP**	Competition	**D1 ADP**	1	-	1.74E6	4.6E4	0.6	0.15	-11.8	1.4	4	7300- 48700
			D1 ATPγS	1.05	0.04	5.04E5	1.3E5	2.1	0.6	-12.9	0.8		
K251A	**ATPγS**	Two site	**D2 ATPγS**	1	0.2	4.5E5	2.4E5	3	2	-9.9	2.1	3	2900 – 9480
			D1 ATPγS (K251A)	1	-	1.5E4	6.1E3	74	34	-3.5	1.2		
Wildtype	**ATPγS & ADP**	Competition	**D1 ADP**	1	-	1.2E6	-	0.85	-	-11	1.8	4	3360 – 61480
			D1 & D2 ATPγS	1.59	0.16	1.05E6	1.2E6	1.7	0.9	-9.2	1		
R359A-R635A	**ATPγS & ADP**	Competition	**D1 ADP**	1	-	2E6	-	0.5	-	-59	4.2	3	23240-42350
			D1 & D2 ATPγS	1.66	0.07	1.3E5	2.9E4	7.8	1.6	-15.5	4.4		

Supplementary Table 2. Details of tryptophan fluorescence fitting data. Data from multiple tryptophan fluorescence titrations (n) were fitted to a cooperative binding equation (one site binding equation in the case of R359A-R635A) and the mean and standard deviations (+/-) shown here. Values presented for K524A and K251A-K524A were calculated from fits to titrations of up to 1 mM ATPγS (experimental data not shown).

p97	K_d (μM)	+/-	*h*	+/-	F_{max}	+/-	n
Wildtype	1	0.2	2.8	0.3	0.49	0.08	4
Wildtype (apyrase treated)	1.3	0.3	3.6	0.8	0.49	0.12	3
Wildtype (rat liver)	1.1	0.2	4	0.1	0.36	0.03	2
K251A	0.8	0.4	2.7	0.3	0.43	0.21	4
K524A	235	122	1	0.1	0.28	0.03	4
K251A-K524A	164	60	1	0.3	0.25	0.03	4
R359A-R635A	1.9	0.6	-	-	0.32	0.1	3

Supplementary Table 3. A summary of the properties of p97 and mutants of the Walker A and SRH motifs. Ranges given indicate the spread of results from multiple techniques and * indicates values fixed in fitting.

p97	Mutation effect		Pre-bound ADP	ADP		ATPγS		Trp Fluorescence		ATPase
			(ADP/monomer)	Kd (μM)	N	Kd (μM)	N	Kd (μM)	*h*	Specific activity
Wildtype	n/a	**D1**	0.9	0.5 - 1.5	1	2	1	-	-	5.4
		D2	-	90	1*	2	0.5 - 0.6	1 - 1.3	2.8 - 4	
K251A	Walker A Reduces nucleotide binding affinity in D1	**D1**	0.2	-	-	74	1*	-	-	3.3
		D2	-	-	-	3	0.65 - 1	0.8	2.7	
K524A	Walker A Reduces nucleotide binding affinity in D2	**D1**	1.1	0.6	1*	2.1	1.05	-	-	0.7
		D2	-					235	1	
K251A-K524A	Walker A Reduces nucleotide binding affinity in D1 and D2	**D1**	0.2	-	-	-	-	-	-	0.6
		D2	-	-	-	-	-	164	1	
R359A-R635A	SRH No cooperative conformational change in AAA ring	**D1**	0.9	0.5	1*	7.8	1*	-	-	0.7
		D2	-	-	-	7.8	0.66	1.9	-	

SUPPLEMENTARY METHODS

ATPase activity assay

Inorganic phosphate produced by ATP hydrolysis was detected by a colorimetric assay as described previously [1,2]. Briefly, a 1 ml reaction mixture of 1 μM of p97 monomers was equilibrated at 37 °C and 3 mM ATP was added to start the reaction. 100 μl aliquots were removed at time points over 30 min and mixed with 900 μl colour mixture (6 mM Ammonium Molybdate, 450 mM H_2SO_4, 0.06% Polyvinyl Alcohol, 120 μM Malachite Green). Colouration of each aliquot was allowed to develop over 20 min and then stabilised with 100 μl 3.4% Citric Acid. Absorbance measurements at 600 nm were taken and plotted against time. The gradient of the linearly related points (abs/min) was converted to moles phosphate (Pi) by a standard curve, factored for dilution and concentration to give nmol Pi/(nmol p97 monomer.min).

Purification of rat liver p97

Rat p97 was purified as previously described [3] concentrated up to ~10 g/l using Centricon-30 (Amicon), and dialyzed against the following buffer: 50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 5% glycerol, pH 8. No nucleotide was added into the buffers during the above purification steps and binding experiments were carried out in the same buffer as other experiments.

REFERENCES

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2. Cogan, E.B., Birrell, G.B. & Griffith, O.H. A robotics-based automated assay for inorganic and organic phosphates. *Anal Biochem* 271, 29-35 (1999).
3. Rabouille, C., Levine, T.P., Peters, J.M. & Warren, G. An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell* 82, 905-14 (1995).