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Supplemental Data

ATP Binding and ATP Hydrolysis Play Distinct Roles in the Function of 26S Proteasome

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Supplemental Experimental Procedures

Purification of 26S Proteasome

Fresh bovine blood was collected at a local abattoir in the presence of 150 mM sodium citrate. The blood was washed repeatedly with phosphate buffered saline containing 5 mM glucose. Cells were lysed in 50 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol, 2 mM ATP and 10 mM $MgCl_2$. The lysate was centrifuged at 15,000 x g for 60 mins. The supernatant was removed and the pellet was re-extracted with an equal volume of lysis buffer. Supernatants were combined and glycerol was added to a final concentration of 10% (V/V). Solid ammonium sulfate was added to the combined supernatants to 40% saturation, and the precipitated material was collected by centrifugation at 15,000 x g for 60 mins. The pellet was washed with lysis buffer containing 40% saturated ammonium sulfate and recentrifuged. The washed pellet was resuspended in Buffer A (20 mM Tris-HCl, pH 7.6 at $4^{o}C$, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 5 β-mercaptoethanol , and 10% glycerol), and dialyzed overnight against several changes of the same buffer. The dialyzed material was centrifuged to remove red cell ghosts and any undissolved material and applied to a column of DEAE Affi-Gel Blue. The column was washed thoroughly to remove unbound protein. Bound protein was eluted with a linear gradient of 70-350 mM NaCl in Buffer A. Proteasome activity was determined by hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC. The fractions with peak activity

were pooled and concentrated by positive-pressure filtration on an Amicon XM300 membrane. The concentrated sample was subjected to preparative glycerol density gradient centrifugation. Gradients were 12.5-38% glycerol in Buffer A and centrifuged for 17 hrs at 28,000 rpm in a Beckman Sw28 rotor. Proteasome activity was assayed as above. Proteasome protein was determined by Coomassie blue staining of SDS-PAGE and western blotting using antibodies against selected subunits of the 26S proteasome. Fractions with peak proteasome content were pooled, dialyzed against Buffer A, concentrated to approximately 1 mg/ml and quickly frozen for storage at - 80^{O}C until use.

Purified 26S proteasome contained deubiquitylating proteins Rpn11/S13 and Uch37 as constituent subunits, and stoichiometric levels of Usp14, a reversibly associating deubiquitylating protein. A detailed description of 26S proteasome purification and subunit composition will be provided in a subsequent report (D. Thompson and G.N. DeMartino, manuscript in preparation).

Glycerol Density Gradient Centrifugation

Density gradient centrifugation was carried out as described previously (Wojcik and DeMartino, 2002). Linear glycerol gradients (12.5-40% glycerol) were made in 20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM β–mercaptoethanol. This fractionation procedure separates 20S proteasome from 26S proteasome.

Measurement of ATPγS Hydrolysis

The standard colorimetric assay for measurement of ATP hydrolysis was not suitable for measurement of ATPγS hydrolysis. Therefore, we utilized a procedure involving thin layer chromatography to compare rates of ATP and ATPγ S hydrolysis by the proteasome. ATP-[γ– ^{32}P] and ATP-[γ–^{35}S] (200 μM) were incubated with and without

proteasome, as described below. At various times during incubation, 3 μl samples were spotted on a prescored Baker-flex cellulose Pei-F thin layer chromatography plate (Baker) and developed using a mobile phase of 0.4 M LiCl and 1.5 M formic acid. The plates were dried, and placed in a cassette with a Phosphor screen (Amersham Biosciences). After exposure and processing on a Storm ™ imager (Amersham Biosciences), densitometry was performed on non-hydrolyzed nucleotide and hydrolyzed produces. Quantification was accomplished using ATP-[γ– ^{32}P] and ATP-[γ–^{35}S] standards.

Depletion of ATP by Apyrase

ATP was depleted from 26S proteasome samples by treatment of samples with apyrase (8 U/μl) at 30^{o} C as described for specific experiments. Verification of ATP depletion was accomplished by measuring ATP, as described below. This treatment reduced ATP to less than nM concentrations.

Measurement of ATP

ATP was measured by standard methods using a firefly luciferase bioluminescent assay kit (Sigma) according to the manufacturer's instructions. To measure ATP bound to the proteasome before and after apyrase treatment, proteasome samples were subjected to successive centrifugations through Micro Biospin 30 columns (Biorad). Proteasome-free ATP-containing buffers were subjected to the same procedure and served as controls. This procedure removed 99.99% of soluble ATP from the buffer. Isolated proteasome was heated to 90^{o}C for 10 mins and centrifuged for prior to determination of bound ATP.

Supplemental Results

Table S1. Activation of 20S Proteasome

Condition	Proteasome activity
Control	1.1 ± 0.3
PA28	28.5 ± 1.5
SDS	24.6 ± 0.9
PA700 (+ATP)	23.3 ± 1.1
PA700 (-ATP)	1.9 ± 0.5

20S proteasome (20 nM) was incubated alone (Control) or with PA28 (200 nM), SDS (0.03%), or PA700 (200 nM, with either no ATP or 200 μM ATP) and assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, as described in Experimental Procedures. Proteasome activity is expressed as arbitrary fluorescent units ± SEM of triplicate assays. Similar results were obtained in three independent experiments.

Table S2. 26S Proteasome and PA700 Do Not Hydrolyze ATPγS

ATP		ATP γ S	
PA700	**26S**	**PA700**	**26S**
pmoles nucleotide hydrolyzed /min/pmol			
5.1 ± 0.4	6.0 ± 0.3	0.03 ± 0.05	0.07 ± 0.02

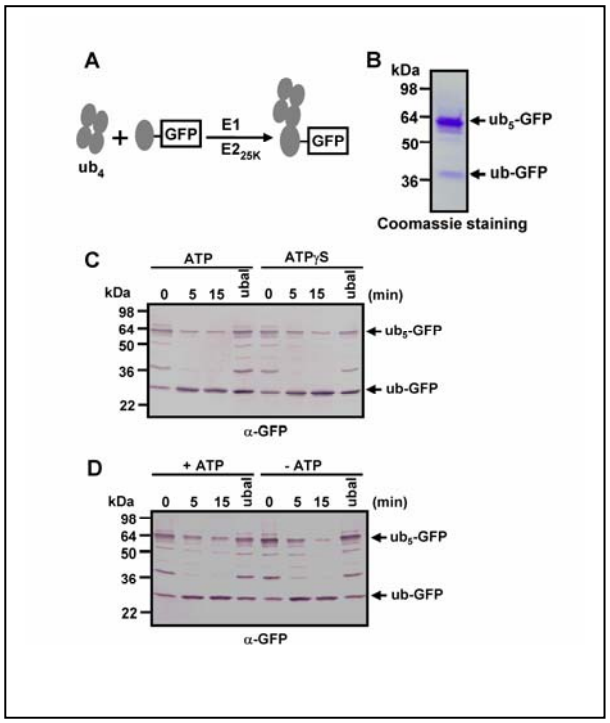
To verify that effects of ATPγ S reflect only a requirement for ATP binding, we measured relative rates of ATP and ATPγ S hydrolysis by the thin layer chromatographic method described in Experimental Procedures. ATP hydrolysis was detected readily by this method although calculated rates were several-fold lower than those calculated using the standard colorimetric assay (Van Veldoven and Mannaerts, 1987). Nevertheless, no significant hydrolysis of ATPγ S was detected. We estimate that our assay was sufficiently sensitive to detect rates of nucleotide hydrolysis at least 100-fold slower than those measured here. These results strongly indicate that the effects promoted by ATPγ S reflect those that depend on ATP binding and not hydrolysis. Data represent mean values of triplicate assays ± SEM. Similar results were obtained in two independent experiments.

Table S3. Apyrase Efficiently Removes ATP from Solution and from the Proteasome

Buffer - Apyrase	Buffer +Apyrase	PA700 -Apyrase	PA700 +Apyrase
pmoles ATP/pmol PA700			
0	0	6.8 ± 0.5	0.2 ± 0.1

Apyrase rapidly depleted soluble ATP from ATP-containing buffers (from 200-1000 μM ATP to less than nM ATP). Therefore, all reported experiments involving apyrase-treated 26S contained ATP concentrations at least two orders of magnitude lower than those required for any ATP effect. To verify that apyrase treatment also efficiently removed any bound ATP from the proteasome we incubated PA700 and control PA700-free buffer containing 1 mM ATP for 5 min with apyrase. These solutions were subjected to centrifugation through buffer exchange columns and assayed for ATP levels, as described above. The data show that ATP was readily detected with isolated, untreated PA700 but not with isolated, apyrase-treated PA700. No ATP was detected in either treated or untreated PA700-free buffers after a comparable buffer exchange. These results indicate that apyrase effectively removes ATP from proteasome samples. Data are expressed as pmoles ATP/pmol PA700; for PA700-free buffers, equivalent samples were processed and assayed. Data represent mean values of triplicate assays ± SEM. Similar results were obtained in two independent experiments.

Figure S1. Polyubiquitin Chain Engagement and Deubiquitylation Do Not Require ATP Hydrolysis



(A) Scheme for preparation of Ub_5-GFP. Ub_4 was conjugated to Ub (G76)-GFP as described under Experimental Procedures. (B) Ni-NTA purified Ub_5-GFP (2 μg) was subjected to 10% SDS-PAGE and visualized by Coomassie blue staining. Deubiquitylation of 100 n M Ub_5-GFP by 40 nM 26S proteasome (C) or 80 nM PA700 (D) after indicated times of incubation was monitored by with an anti-GFP antibody. Effect of ubiquitin aldehyde (2 μM) was assessed after 15 minutes of incubation where indicated (Ubal).