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A spectrophotometric assay for conjugation of ubiquitin and ubiquitin-like proteins¹

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Abstract

Ubiquitination is a widely-studied regulatory modification involved in protein degradation, DNA damage repair and the immune response. Conjugation of ubiquitin to a substrate lysine occurs in an enzymatic cascade involving an E1 ubiquitin activating enzyme, and E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase. Assays for ubiquitin conjugation include electrophoretic mobility shift assays and detection of epitope-tagged or radiolabeled ubiquitin, which are difficult to quantitate accurately and are not amenable to high throughput screening. We have developed a colorimetric assay that quantifies ubiquitin conjugation by monitoring pyrophosphate released in the first enzymatic step in ubiquitin transfer, the ATP-dependent charging of the E1 enzyme. The assay is rapid, does not rely on radioactive labeling, and requires only a spectrophotometer for detection of pyrophosphate formation. We show that pyrophosphate production by E1 is dependent on ubiquitin transfer and describe how to optimize assay conditions to measure E1, E2, or E3 activity. The kinetics of polyubiquitin chain formation by Ubc13-Mms2 measured by this assay are similar to those determined by gel assays, indicating that the data produced by this method are comparable to methods that measure ubiquitin transfer directly. This assay is adaptable to high-throughput screening of ubiquitin and ubiquitin-like conjugating enzymes.

Keywords

Ubiquitin Conjugation; Ubiquitin-like proteins; Ubc13-Mms2; ubiquitin-activating enzyme

Introduction

Protein ubiquitination is a reversible post-translational modification that regulates diverse pathways in eukaryotic cells by controlling protein stability, activity, binding and intracellular targeting [1; 2]. Biological processes that are regulated by ubiquitination include proteasomal protein degradation, DNA damage signaling, the inflammatory response and transcription [2; 3; 4]. Ubiquitin, and ubiquitin-like modifiers (Ubl²) such as SUMO and NEDD8, are small proteins that are attached to substrates via an isopeptide linkage between the C-terminus of ubiquitin (or the Ubl) and a substrate lysine [1; 2].

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Substrates can be modified by a single ubiquitin or by a polyubiquitin chain, in which one ubiquitin is covalently linked to the next via one of its seven surface lysines [5; 6; 7]. Ubiquitin and Ubls are conjugated to substrate lysines by the sequential activity of three enzymes: an E1 activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ligase [2; 8]. The human genome encodes several E1 enzymes, 35 E2 enzymes, and more than 500 E3 enzymes [3; 4; 9; 10]. Our understanding of which E2 and E3 enzymes work in concert to conjugate ubiquitin to specific substrates is incomplete and remains an active area of research [4; 9; 11].

The first step in the ubiquitination cascade is the ATP-dependent charging of the E1 activating enzyme (Figure 1). The E1 uses ATP to adenylate the C-terminus of ubiquitin, forming ubiquitin-AMP and pyrophosphate. The ubiquitin-AMP ester bond is then cleaved by an active site cysteine within E1, resulting in a thioester bond between the C-terminus of ubiquitin and the sulfhydryl of cysteine [12; 13; 14]. Ubiquitin is then transferred from the active site cysteine of the E1 to an active site cysteine in the E2 [2; 4]. The E3 ligase then binds to both the E2 and the substrate, promoting isopeptide bond formation with a substrate lysine [3]. Members of the HECT family of E3 ligases first transfer the ubiquitin from the E2 to an active site cysteine in the E3 before conjugating the ubiquitin to the substrate, while the RING domain E3 ligases simply target E2 to the substrate [3; 8]. Substrate specificity is thought to be intrinsic to the specific combination of E2 and E3, although the number of functional combinations of E2s and E3s is not known. Studies of the mechanism and specificity of ubiquitin and Ubl transfer enzymes are important for understanding ubiquitin and Ubl signaling in the cell, and for identifying targets for therapeutic intervention.

The last ten years have seen growing interest in the ubiquitin conjugation machinery and in the proteasome as targets for drug discovery. The efficacy of the proteasome inhibitor, Bortezomib, in treating multiple myeloma and mantle lymphoma has spurred efforts to target upstream events in the ubiquitin pathway by inhibiting E1, E2 and E3 enzymes [15]. Nutlin, an inhibitor of the RING E3, Mdm2, appears to be effective in the treatment of multiple myeloma [16]. Recently, an inhibitor of the E1 activating enzyme for the ubiquitin-like molecule, Nedd8, entered Phase I trials for the treatment of cancer [9]. These two examples offer the promise of success in targeting the ubiquitin conjugation pathway by therapeutics. However, progress in developing and screening small molecule inhibitors of E1, E2 and E3 has been limited by the lack of a simple, high throughput assay for ubiquitination using native substrates [16].

The most widely used activity assay for ubiquitin or Ubl transfer is the band shift assay, in which ubiquitinated and unmodified proteins are separated by gel electrophoresis and detected by one of a variety of methods, including staining or Western blot [17; 18; 19]. However, these assays make it difficult to quantitate protein modification accurately because of variability or non-linearity in the signal from stained or antibody-bound proteins. The attachment of epitopes or fluorescent tags to ubiquitin or substrates permits accurate quantification and enhances sensitivity; however, adding an epitope tag such as hexahistidine, glutathione-S-transferase (GST), green fluorescent protein (GFP), Myc or HA to ubiquitin or to the substrate has the potential to disrupt interactions between the substrate and the transfer enzymes [20; 21; 22]. Ubiquitin radiolabeled with ^{125}I or ^{32}P is also used [23; 24; 25], but requires special handling due to the hazards of working with radioactivity, particularly gamma emitters like ^{125}I . In addition, radiolabeling must be performed regularly because of the short half-lives of these isotopes. None of the gel shift assays are

²Abbreviations used: Ub, ubiquitin; Ubl, ubiquitin-like protein; FRET, Forster Resonance Energy Transfer; DTT, Dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; ATP, Adenosine Triphosphate; AMP, adenosine monophosphate; BSA, Bovine serum albumen; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

amenable to high throughput formats, both because of the time needed to perform the assay and the variability in signal between gels. Ubiquitination and Ubl transfer assays that rely upon Förster resonance energy transfer (FRET) between GFP or fluorescently labeled ubiquitin and Ubls have also been developed for real-time, quantitative measurement of ubiquitination [22; 26; 27; 28; 29; 30]. However, the FRET assay requires attaching a fluorophore to ubiquitin or to the substrate, which may interfere with the function of ubiquitin transfer enzymes and may preclude the use of native substrates [21]. In addition to methods that directly measure the ubiquitinated product, coupled enzyme methods that monitor ATP cleavage by E1 have used radiolabeled ATP and quantitated the phosphate released [31; 32]. However, none of the above methods are amenable to large-scale or high-throughput screening.

We have developed a spectrophotometric method for measuring ubiquitin and Ubl transfer that does not require tagged substrates or gel electrophoresis and can be used to quantitate each step within the ubiquitin transfer cascade. The assay couples ubiquitin transfer by E2 and E3 to the formation by E1 of pyrophosphate, a product of the charging reaction, which is broken down to phosphate by pyrophosphatase. The inorganic phosphate is quantitated by measuring the absorbance of molybdenum blue, a reduced complex of phosphate and molybdate that absorbs visible light. All reagents required for the assay are easily obtainable and the assay only requires a spectrophotometer capable of measuring absorbance between 600 nm and 850 nm. With the use of a plate reader, the assay can be readily applied to high-throughput screening of compounds that target ubiquitin or Ubl transfer enzymes.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, US) and used without further purification. Inorganic pyrophosphatase from *E. coli* (I5907) was purchased from Sigma-Aldrich and resuspended in 20 mM HEPES, pH 7.5, 100 mM NaCl then aliquoted, frozen and stored at -20°C . We note that, while pyrophosphatase from *S. cerevisiae* can be substituted for the bacterial enzyme, the yeast enzyme has measurable ATPase activity and produces higher background absorbance values in the molybdenum blue assay compared to the bacterial enzyme (data not shown). We therefore recommend using the bacterial enzyme.

Protein Expression and Purification

S. cerevisiae Ubc13 and Mms2 were expressed in *E. coli* and purified as previously described by Eddins, et al. [33]. Hexahistidine tagged, human E1 enzyme was expressed in *E. coli* using a pET32 vector. Cells were grown at 37°C to an O.D. of 0.3, shifted to 16°C and induced with IPTG overnight. Cells were lysed and then loaded onto a nickel affinity column and eluted with imidazole. Peak fractions were pooled, dialyzed into buffer containing 50 mM NaCl, loaded onto a mono Q column (GE Healthcare) and eluted with a 0 – 400 mM NaCl gradient. Peak fractions were pooled, concentrated and purified by size exclusion chromatography on a Superdex 200 column to 20 mM Tris, pH 7.8, 100 mM NaCl, and 7.5 mM β -mercaptoethanol (GE Healthcare). Fractions containing E1 were pooled, concentrated and stored at -80°C until use. A gel showing the purity of recombinant E1 is shown in Supplemental Figure 1

Wild type and mutant human ubiquitin were expressed in *E. coli* and purified following the protocol of Pickart and Raasi [20; 34]. Ubiquitin mutants are denoted in the text with superscripts; Ub^{K63R} or Ub ^{Δ G75, Δ G76}, for example, indicate mutation of lysine 63 to arginine or the deletion of glycine 75 and glycine 76 from ubiquitin.

S. cerevisiae Rad5 RING domain (residues 846 - 1196) was expressed in *E. coli* from a pMalc2xHV plasmid containing an N-terminal hexahistidine tag followed by a tobacco etch virus (TEV) protease cleavage site. The RING domain was purified by nickel affinity chromatography then dialyzed with 1 mg of TEV protease overnight to remove the affinity tag. Dialyzed protein was then concentrated and further purified by size exclusion chromatography on a Superdex 75 column in 20 mM Tris, pH 7.5, 100mM NaCl, and 5 mM β -mercaptoethanol. Fractions containing the Rad5 RING domain were pooled, concentrated, and stored at -80°C until use.

Solutions for Molybdenum Blue Assay

The quench and developer solutions were modified from conditions described in Gawronski and Benson [35] and McCain and Zhang [36]. The quench solution contains 8% L-ascorbic acid and 0.67% ammonium molybdate in 0.67 N HCl. This solution is made fresh from a 2:3 dilution of a solution of 12% ascorbic acid in 1 N HCl with 2% ammonium molybdate in water. The ammonium molybdate/ascorbic acid solution turns yellow upon mixing with ammonium molybdate and is stable for only a few days in the dark at 4°C (the solution will eventually turn green or brown and signal will deteriorate significantly). The developer solution contains 2% sodium citrate-trihydrate, 2% sodium (meta)arsenite in 2% acetic acid. Acetic acid is added last. This solution is stable for about a week at room temperature.

Generating a Standard Curve

A 1 mM sodium phosphate stock solution used for standards was made in buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl. This stock is diluted to a final phosphate concentration between 0 and 200 μM in 20 mM HEPES, pH 7.5, 100 mM NaCl in a final volume of 50 μL . To each standard, 150 μL of the quench solution is added and the quenched reactions are incubated at room temperature. After 5 minutes, 150 μL of the developer solution is added and the absorbance of the sample is measured at 850 nm. The absorbance of each standard is then plotted as absorbance versus the concentration of phosphate in mM or μM . The data are then fitted to a linear equation to obtain the slope in units of $\text{abs} \cdot \text{mM}^{-1}$ or $\text{abs} \cdot \mu\text{M}^{-1}$. Absorbance values from ubiquitination reactions are then converted to μM phosphate using the slope of the phosphate standard curve (Eq. 1).

$$\text{Absorbance}_{850\text{nm}}/\text{slope of std. curve (abs}/\mu\text{M)} = \mu\text{M Phosphate formed} \quad (\text{Eq. 1})$$

Since there are two phosphates generated from every pyrophosphate molecule, the concentration of pyrophosphate formed (usually in μM) is then divided by 2 (Eq. 2).

$$\mu\text{M Phosphate formed}/2 = \mu\text{M Pyrophosphate formed} = \mu\text{M Ubiquitin transferred} \quad (\text{Eq. 2})$$

It is important to generate the standard curve using the same instrument as that used to conduct the experiments, since instrument-to-instrument variations, measurement path length, and other factors can affect the absolute absorbance measurements.

Molybdenum Blue Assay of ubiquitin conjugation

For the assays described in this paper, reactions contained 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM MgCl_2 , 500 U inorganic pyrophosphatase, 0.05 to 0.6 μM E1 enzyme, 0.1 to 4 μM Ubc13-Mms2 enzyme, 1 μM Rad5 RING domain, 80 μM Ub^{K63R} and 0 to 1 mM $\text{Ub}^{\Delta\text{G75}}$, ΔG76 or Ub^{d77} . Reactions were initiated by the addition of 2 mM ATP. All reactions were performed at 37°C . At the designated time points, a 50 μL aliquot of the reaction was removed and quenched with 150 μL of the quench solution. After 5 minutes at room temperature (23°C to 26°C), 150 μL of the developer solution was added and the absorbance of the solution was then measured at 850 nm in a Cary 50 Bio UV-Vis

spectrometer (Agilent Technologies, Santa Clara, CA) or by recording a spectrum from 500 nm to 900 nm in a POLARStar Omega plate reader (BMG LABTECH GmbH, Offenburg, Germany). If necessary, the volume of the reaction aliquot can be decreased and the ratios of the aliquot to quench and developer proportionally reduced. The absorbance at 850 nm was used to quantitate activity; however any wavelength between 600 nm and 875 nm can be used for measurement. The amount of pyrophosphate formed should be equivalent to the amount of ubiquitin transferred by E1 to E2. The quantity of pyrophosphate formed is also equivalent to the amount of ubiquitin conjugated to substrate if either E2 or E3 is the rate limiting enzyme (see Results and Discussion). Each new E2 or E3 enzyme to be assayed will require optimization of conditions (see Results and Discussion for more details).

Reaction rates in μM per minute were determined from linear fits of μM pyrophosphate formed versus time. The rate of pyrophosphate formation was then plotted as a function of ubiquitin concentration and fit to the Michaelis-Menten equation to determine kinetic constants (Eq. 3):

$$v = (k_{\text{cat}} \times [E]_{\text{t}} \times [S]) / (K_{\text{M}} + [S]) \quad (\text{Eq. 3})$$

where v is the initial velocity at a given substrate concentration, k_{cat} is the apparent first order rate constant of the enzyme, $[E]_{\text{t}}$ = the total concentration of enzyme, $[S]$ is the total concentration of substrate, K_{M} is the concentration of substrate required to reach one-half the maximum velocity of the enzyme.

Fluorescent-Ubiquitin assays

Ubiquitin^{K63C} was labeled with fluorescein-5-maleimide (Thermo Scientific, Pittsburgh, PA) in PBS buffer for 2 hours at room temperature in the dark. Labeling reactions were dialyzed overnight into 2 L of 20 mM HEPES pH 7.5, 1 mM EDTA. Ubiquitin was further purified by cation exchange chromatography as described [20]. Fractions that contained ubiquitin were pooled and dialyzed overnight into 2 L of 20 mM HEPES pH 7.5. The concentration of ubiquitin was determined using the BCA assay kit (Thermo Scientific, Pittsburgh, PA) following manufacturer's protocol. Labeled ubiquitin was stored at 4 °C wrapped in foil.

Band shift assays using 10 μM fluorescently labeled ubiquitin (labeled at cysteine 63) with an additional 70 μM ubiquitin^{K63R} (total donor ubiquitin concentration = 80 μM) were performed using the assay conditions and reagents described above for the molybdenum blue assay. At designated time points, reactions were stopped by the addition of 4X NuPAGE SDS-PAGE loading buffer with dye (NP0007 Invitrogen, Carlsbad, CA). Reactions were separated by SDS-PAGE and the bands visualized on a Typhoon 9410 phosphorimager (GE Healthcare, Waukesha, WI). Band intensity was measured using Quantity One version 4.6.7 (Bio-Rad, Hercules, CA). Fluorescence intensity was converted to concentration by dividing the band intensity by the slope obtained from a calibration curve of fluorescein ubiquitin standards of known concentration on the same gel. Data were plotted and fitted as described above to determine kinetic constants.

Results and Discussion

Assay Description

We set out to develop a quantitative assay that could be used with native substrates for both general activity screening assays and mechanistic studies. Activation of ubiquitin or an Ubl by E1, the first step in the ubiquitin transfer cascade (Figure 1), involves formation of ubiquitin-AMP and pyrophosphate from ubiquitin and ATP. The ubiquitin adenylate is then cleaved by the active site cysteine of the E1, resulting a thioester bond between E1 and the

C-terminus of ubiquitin and AMP. The ubiquitin is then transferred to the active site cysteine of the E2 in a transthiolation reaction (Figure 1). The E3 ligase binds to the E2~ubiquitin conjugate and promotes transfer of the ubiquitin or Ubl to the substrate lysine. The release of pyrophosphate is therefore, in principle, directly related to the amount of ubiquitin transferred because E1 must transfer ubiquitin to E2 before it can undergo another round of ATP cleavage. It is therefore possible to use the rate of pyrophosphate production by E1 as a measure of ubiquitin transfer. Pyrophosphate is measured by first hydrolyzing it with the enzyme, pyrophosphatase, to produce inorganic phosphate, which is then complexed with molybdate in the presence of ascorbic acid to produce a blue color, sometimes referred to as molybdenum blue [34; 35; 36; 37]. The relative absorbance of visible or infrared light by phosphomolybdate is proportional to the amount of phosphate generated, and hence to the number of times the E1 enzyme is charged with ubiquitin. The absorbance of phosphomolybdate compounds has been used to assay the activity of phosphatases, synthetases, and other enzymes that release inorganic phosphate [35; 36]. We therefore investigated whether this method could be adapted to measuring substrate ubiquitination by monitoring pyrophosphate release by E1.

To assay ubiquitination using the phosphomolybdate assay, all proteins are first mixed in the absence of ATP and equilibrated at 37 °C. The ubiquitination reaction is then initiated with the addition of ATP. At designated times, an aliquot of each reaction is removed and diluted into a solution of ammonium molybdate, ascorbic acid and hydrochloric acid as described in Methods. Hydrochloric acid lowers the pH of the solution, which decreases the catalytic activity of the ubiquitin conjugation enzymes and effectively quenches the reaction [38; 39]. Molybdate forms a complex with phosphate, which is reduced by ascorbic acid and gives rise to the blue color [35; 40]. Phosphomolybdate formation is then stopped after 5 minutes by the addition of a citrate/arsenite solution, which competes for free molybdate and enhances the color stability of the phosphomolybdate [41]. The phosphomolybdate formed at each time point was then quantified by measuring the absorbance at 850 nm. The absorbance is divided by two, since two phosphates are produced for every ubiquitin transferred because ATP is cleaved to AMP and pyrophosphate. These numbers are corrected for background ATP hydrolysis that is not dependent on the activity of E1 or E2. The concentration of phosphate is then determined from the corrected absorbance by comparison with the standard curve (see Methods).

We developed the assay using the enzymes that synthesize K63-linked polyubiquitin, which is synthesized in yeast by the E2, Ubc13, in complex with Mms2, a non-catalytic subunit that confers the specificity for K63 of the acceptor ubiquitin [21; 33; 42; 43]. Ubc13-Mms2 can synthesize free polyubiquitin chains in the absence of the RING E3 ligase, Rad5 [42; 44; 45]. When added to the *in vitro* reaction in the absence of other substrates, Rad5 stimulates the rate of free chain synthesis by Ubc13-Mms2 [45]. It is therefore possible to monitor the rate of the unstimulated E1/E2 reaction, as well as the E3-stimulated reaction.

We first determined whether the rate of ATP cleavage by E1 was increased during free chain synthesis by Ubc13-Mms2, which would confirm that ATP cleavage by E1 is coupled to transfer of ubiquitin to E2. We carried out the reactions under conditions that would lead to formation of diubiquitin only by using 80 μM of Ub^{K63R}, which can only serve as a donor ubiquitin, and saturating amounts of Ub^{D77}, which can only serve as an acceptor because D77 prevents the ubiquitin from binding to and reacting with the E1 [20]. Figure 2 shows the time course of pyrophosphate produced during diubiquitin formation by Ubc13-Mms2, which is repeatedly charged by E1 for each ubiquitin transferred. A control reaction containing the E1, ubiquitin, pyrophosphatase, and ATP, which corrects for background ATP hydrolysis that is not due to ubiquitin transfer to E2, is nearly 100-fold lower than with Ubc13-Mms2 (1.2 $\mu\text{M}/\text{min}$ vs. 0.014 $\mu\text{M}/\text{min}$). We verified that pyrophosphatase was

saturating by assaying the rate of pyrophosphate formation over a broad range of pyrophosphatase concentrations and found that it did not change significantly at pyrophosphatase concentrations greater than 20 Units (Supplemental Figure 2). We note that there is significant ATPase activity at pyrophosphatase concentrations greater than 500 Units (data not shown) and recommend controlling for this activity by assaying phosphate formation in the absence of E1.

We next measured the rate of pyrophosphate production in the presence of a RING domain E3 ligase to show that pyrophosphate production can reflect the activity of an E3. In the presence of the RING domain of yeast Rad5, we measured a rate of 2.9 $\mu\text{M}/\text{min}$, a 3-fold stimulation in the rate of pyrophosphate production (Figure 2). This is comparable to a previously reported 4-fold stimulation in the rate of ubiquitin chain formation by Ubc13/Mms2 and intact Rad5, which was assayed using band shift assays [45]. These data indicate that measuring phosphate production is a viable assay of E2 and RING E3 activity.

Different sets of enzymes involved in ubiquitin and Ubl modification require distinct conditions for assaying activity. In order to determine whether some of these conditions might affect molybdenum blue color formation, we determined how different assay buffers, salts and additives affect assay results, thereby establishing the range of conditions under which the assay can be used. Absorbance of a phosphomolybdate standard was compared in the presence of a variety of buffers, salts, reducing agents and reaction additives such as BSA and EDTA, and the absorbance was compared over a broad range of concentrations (Supplemental Figure 3). Phosphomolybdate absorbance was unaffected by most reaction components tested except for phosphate buffers, high concentrations of DTT (>20 mM), HEPES buffer (>100 mM), and bovine serum albumin (>3 mg/mL; this also showed significant precipitation). For best results, phosphate standards should be obtained using buffers that are identical to reaction conditions.

Phosphomolybdate absorbs most strongly between 600 nm and 900 nm; therefore any spectrophotometer or plate reader that can measure absorbance between these wavelengths can be used for this assay (Figure 3a). We find that the absorbance of phosphomolybdate at 600 nm, 710 nm, and 850 nm is linear and highly reproducible from 5 μM to 200 μM phosphate (Figure 3b). Phosphomolybdate absorbs most strongly around 850 nm, but any wavelength between 600 nm and 875 nm is suitable for assaying ubiquitin transfer and produces similar results (Figure 3c). The blue color is stable at all three wavelengths for greater than four hours in the presence of ubiquitin, with a ~3% loss in signal (data not shown).

Application of the molybdenum blue assay to high-throughput investigations of ubiquitin conjugation

The molybdenum blue assay is easily adapted to a multi-well plate format for high-throughput measurement of ubiquitin conjugation using a plate reader. Possible applications include screening for activity by different E2-E3 pairs, potential substrates or the effect of small molecule inhibitors of E1, E2, or E3 enzyme activity. The assay is particularly well suited to screening for E2-E3 pairs and ubiquitin/Ubl targets because it can be used with native enzymes and substrates.

To demonstrate the application of the molybdenum blue assay to high throughput screening, we used the assay in a plate format to measure the effects of several different ubiquitin mutations on K63-linked polyubiquitin formation by Ubc13-Mms2. Assays containing 0.5 μM E1, 2 μM E2 (Ubc13-Mms2) and 100 or 500 μM of each mutant ubiquitin were incubated at 37°C for 60 minutes (Figure 4). For each mutant ubiquitin, control reactions measured pyrophosphate production by E1 plus ubiquitin and ubiquitin alone in separate

wells in the plate. Reactions were quenched after 60 minutes and the absorbance was measured in a plate reader as described in Methods. The results in Figure 4 show that Ubc13-Mms2 formed ubiquitin chains with all ubiquitin mutants tested except the K63R substitution, which cannot serve as an acceptor ubiquitin for K63-linked polyubiquitin chain synthesis [21]. Substitutions at residues 8, 20, 42, 44, or 57 of ubiquitin decreased chain formation by factors ranging from 3 to 10-fold as compared to wild-type ubiquitin. Importantly, the relative effects of each ubiquitin mutation as assayed by the phosphomolybdate plate assay agree well with band shift assays of ubiquitin chain formation (Supplemental Figure 4). The effects of mutations at residues 8, 42, and 44 of ubiquitin agree with structural studies showing that these residues are important for acceptor ubiquitin binding to Mms2 [33; 46]. Residues 20 and 57 of ubiquitin are proximal to the acceptor ubiquitin binding surface in Ubc13 suggesting that these substitutions disrupt this interaction [33]. These data show that it is possible to rapidly screen the effects of ubiquitin mutations on ubiquitin transfer using the molybdenum blue assay.

Establishment of the rate-limiting enzyme

For quantitative investigations using the molybdenum blue assay, it is essential to carry out the assay under conditions in which the enzyme of interest (E1, E2 or E3) is rate-limiting. Under these conditions, the rate of pyrophosphate formation will reflect the rate-limiting step in the enzymatic cascade. To address whether isopeptide bond formation by Ubc13-Mms2 was the rate-limiting step in our assays, we determined how the rate of pyrophosphate formation changed with increasing concentration of E1 and E2. Reaction rates are dependent both on the concentration of substrate and the concentration of enzyme [47]. Therefore, if E2 is rate limiting, increasing the concentration of E2 two-fold will increase the rate of pyrophosphate formation by two-fold as well. A smaller change would indicate that E1 or, in the molybdenum blue assay, pyrophosphatase is rate limiting. Since the rates of catalysis will likely be different for each combination of E1, E2, and E3 enzymes, it is therefore important to determine the concentration range where the activity of the enzyme of interest is rate-limiting if the molybdenum blue assay is to be used to determine kinetic constants. Once the concentrations at which E2 is the rate-limiting step have been established, the steady-state kinetics of ubiquitin transfer can be measured with the confidence that the measured rates of phosphate production correlate with isopeptide bond formation catalyzed by E2. To maximize signal-to-noise and, hence, accuracy, assays should utilize the maximum enzyme concentration that is still rate-limiting.

As an example of how the rate-limiting concentration of E2 is determined, we measured the rate of pyrophosphate formation at concentrations of E2 ranging from 0 to 4 μM and at two different concentrations of E1. Figure 5 shows a plot of the rate of pyrophosphate formation as a function of Ubc13/Mms2 concentration. The rate of pyrophosphate formation varies linearly with E2 concentration between 0 and 2 μM Ubc13-Mms2 at both 0.3 μM and 0.6 μM E1 (shown by the gray box in Figure 5), indicating that ubiquitin conjugation by Ubc13-Mms2 is the rate-limiting step under these conditions. At concentrations of Ubc13-Mms2 greater than 2 μM , however, this relationship no longer strictly holds, suggesting that the E2 is no longer entirely rate limiting.

Steady-State Kinetics of Diubiquitin Formation by Ubc13-Mms2

In order to compare the results of the molybdenum blue assay with a conventional ubiquitin conjugation assay, we compared the steady-state kinetics of diubiquitin formation by Ubc13-Mms2 as measured using a band shift assay and the molybdenum blue assay. Formation of diubiquitin only was ensured by utilizing a donor ubiquitin with a K63R substitution, which blocks chain formation by Ubc13-Mms2, and an acceptor ubiquitin lacking the two C-terminal glycine residues, which cannot be activated by E1. The rate of

diubiquitin formation was assayed by the two methods at several concentrations of the acceptor ubiquitin. In the molybdenum blue assay, absorbance measurements were corrected for background ATP hydrolysis by subtracting from each measurement the absorbance in a matched reaction lacking E2. Data were plotted as the rate (in s^{-1}) versus concentration of ubiquitin and fit to the Michaelis-Menten equation to calculate kinetic constants. The saturation plot shown in Figure 6 displays the curves from the molybdenum blue and gel assays along with the calculated rate constants. The turnover number for E2 and Michaelis constant determined from the molybdenum blue ($k_{cat} = 0.033 \pm 0.004 s^{-1}$ and $K_M = 243 \pm 63 \mu M$, $R^2 = 0.95$) and gel shift assay ($k_{cat} = 0.034 \pm 0.005 s^{-1}$ and $K_M = 271 \pm 93 \mu M$, $R^2 = 0.94$) are in very good agreement. These values are comparable to those previously reported by Carlile, *et al.* ($k_{cat} = 0.02 s^{-1}$ and $K_M = 212 \mu M$) using a gel assay with $^{125}[I]$ -ubiquitin [45]. Taken together, our results indicate that kinetic data obtained using the molybdenum blue assay are comparable to kinetic data obtained by other methods.

Assay Controls and Caveats

The key to successfully measuring ubiquitin conjugation in a molybdenum blue assay is to control for background ATPase activity that could artificially inflate or deflate the apparent rate of ubiquitin transfer. While we have explained the importance of determining the rate-limiting enzyme when using the molybdenum blue assay, good laboratory practice and additional controls may be necessary to increase accuracy. The background level of phosphate released can be affected by the purity of the enzymes, substrates, and reagents used in the assay. In general, a background value or rate reflecting ATP cleavage by solvent, pyrophosphatase and E1 (if E1 is not the enzyme under investigation) should be subtracted from all measurements. This value can be from a single reaction which measures the combined enzymatic and non-enzymatic ATP cleavage rates or from separate reactions independently measuring the rate of ATP cleavage by solvent, pyrophosphatase, and E1. Fresh ATP stocks should be used to reduce the background phosphate signal that is due to the slow hydrolysis of ATP during prolonged storage. Signs of ATP degradation will initially appear as elevated background and can lead to lowered rates because E1 is inhibited by AMP [12]. We typically make a large 100 mM stock of ATP, which is aliquoted and stored at $-20^\circ C$ and discarded after six months. For highly quantitative measurements, the E1, E2 and E3 enzymes and ubiquitin should be $>95\%$ pure to reduce the likelihood of contaminating ATPase activity. The background rate of phosphate formation in the absence of substrate due to a contaminate can be subtracted from the overall rate of phosphate formation or this activity can be removed through further purification. For a more qualitative activity screen, for example in high-throughput screens of different E2-E3 pairs, it is possible to use partially purified proteins provided that the appropriate controls for the ATPase activity of the contaminants are performed.

The molybdenum blue assay correlates pyrophosphate production with total ubiquitin transferred. Therefore, any transfer of ubiquitin, including auto-modification of E3 enzymes, modification of multiple sites within the same substrate and ubiquitin chain formation, will be counted in the final absorbance measurement. If measurement of heterogeneous modifications is not desired, it is important to establish the order of modification (i.e. substrate $>$ E3 $>$ chains) and the timescale in which the desired modification occurs. In the simplest case, in which the substrate is the first protein modified, the assay length can be kept sufficiently short such that nearly all the ubiquitin conjugation events involve the product of interest. In cases where the substrate is not preferentially modified first or where mixed modification is unavoidable, we recommend combining the molybdenum blue assay with a band shift assay. Aliquots of the reaction should be taken before initiation and after the last time point for separation by SDS-PAGE. The gel should be stained for total protein or blotted for the ubiquitin or Ubl to identify the modified proteins. For each protein, the

percent of the product of interest is determined and used to partition the total rate of ubiquitin transfer measured in the molybdenum blue assay into the component rates of modification.

In addition to background formation of ATP, any reagents added to the reaction must not act by altering the activity of pyrophosphatase or affecting the chemical reaction to form phosphomolybdate. This is especially important when screening chemical libraries for inhibitors or activators of ubiquitin conjugation. Since this interference with the assay is not a likely event, follow-up controls can be carried out only on compounds that show an effect in the initial high-throughput screen.

Conclusions

We have developed a straightforward, colorimetric assay for ubiquitin conjugation that can be used in kinetic studies as well as in high-throughput screens for the effects of mutations, small molecules, different E2-E3 pairs and the presence of different potential substrates. The assay couples release of pyrophosphate by E1 to the transfer of ubiquitin by E2 and/or E3. The pyrophosphate is quantitated by breaking it down with pyrophosphatase into phosphate, which is combined with molybdate to form a blue phosphomolybdate complex, sometimes referred to as molybdenum blue. The assay is rapid, simple, safe and as inexpensive to perform as gel shift assays, while as quantitative as radioactive and FRET assays that have been previously used to investigate ubiquitin conjugation [22; 23; 26; 27; 28; 29; 45; 48]. Additional advantages of the assay are the ability to use native, untagged ubiquitin as well as native substrates. Since ATP cleavage by an E1 is universal for activation of ubiquitin and ubiquitin-like proteins such as SUMO and NEDD8, the molybdenum blue assay is applicable to these systems, as well. This assay will open the door to in-depth screening of therapeutics targeting ubiquitin conjugation and mechanistic studies of E2 and E3 enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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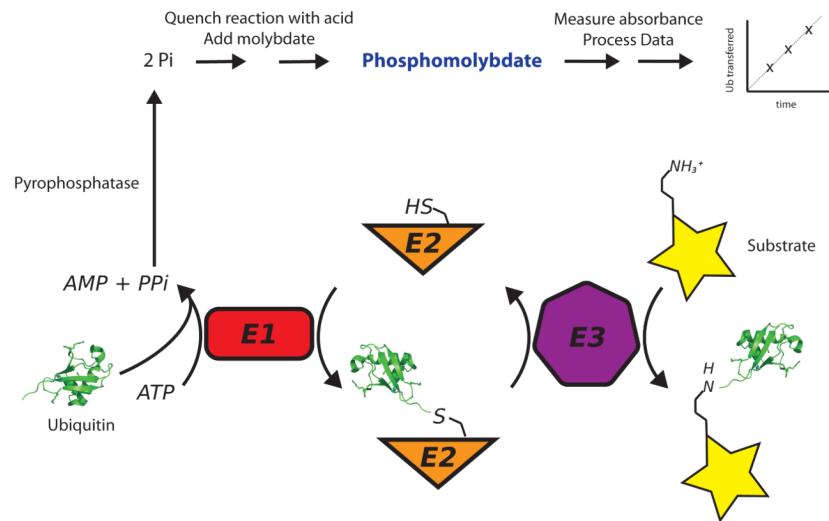


Figure 1. Schematic of Molybdenum Blue Assay

The ubiquitination cascade (bottom) involves the sequential action of E1, E2 and E3 enzymes, leading to ubiquitination of the substrate lysine. In the first step, the ubiquitin activating enzyme, E1, adenylates ubiquitin, forming ubiquitin-AMP and releasing pyrophosphate. To assay ATP consumption (top), the pyrophosphate product is broken down by pyrophosphatase into two phosphate molecules. Addition of ascorbic acid and ammonium molybdate in hydrochloric acid halts the reaction progress and initiates phosphomolybdate formation. The solution is then developed by addition citric acid and sodium arsenite and the phosphomolybdate formed measured by visible spectroscopy.

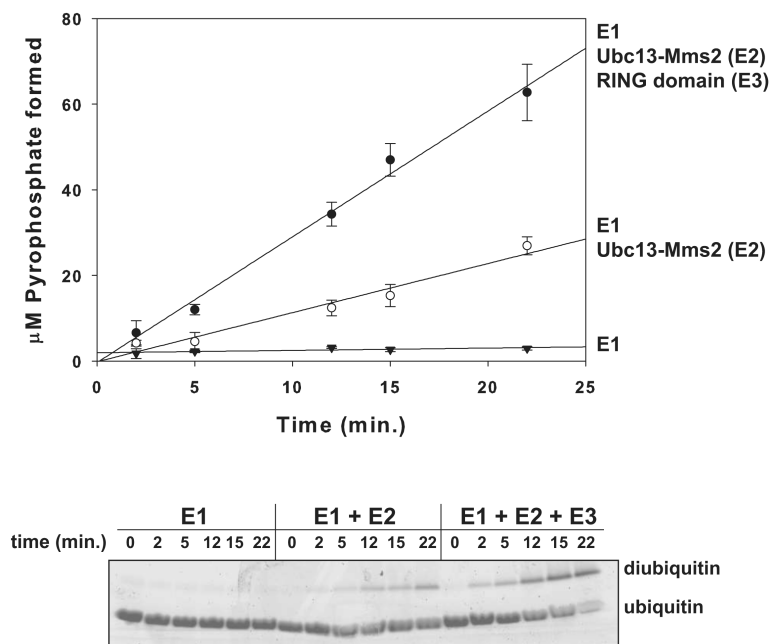


Figure 2. Pyrophosphate production during diubiquitin synthesis

The plot shows pyrophosphate formation during diubiquitin synthesis by Ubc13-Mms2 in the absence (open circles) and presence (black circles) of the RING domain of the Rad5 E3. The control (black triangles) shows the background signal in the absence of Ubc13-Mms2. Reactions contained 0.6 μM Ubc13-Mms2, 1 μM Rad5 RING domain, 0.3 μM E1, 80 μM ubiquitin^{K63R} (the donor ubiquitin), and 800 μM ubiquitin^{D77} (the acceptor ubiquitin). -Data points are an average of three independent measurements and the error bars are the standard deviation of the experiments. Lower panel shows a gel of diubiquitin synthesis using fluorescently labeled ubiquitin.

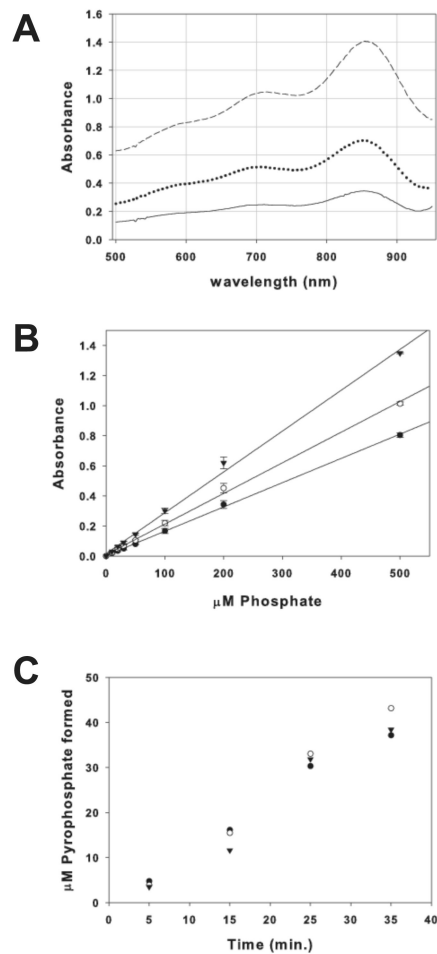


Figure 3. Absorbance of phosphomolybdate

(A) Absorbance spectrum of phosphomolybdate from 100 μM (solid line), 200 μM (dotted line), and 500 μM (dashed line) sodium phosphate in 20 mM HEPES, pH 7.5 and 100 mM NaCl. Spectra were taken of 200 μL solutions in a 96-well plate using a POLARStar Omega plate reader (B) Linearity of phosphomolybdate absorbance at 600 nm (filled circles), 710 nm (open circles), and 850 nm (filled triangles). (C) Molybdenum blue assay of time course of Ubc13-Mms2 ubiquitin chain formation measured at 600 nm (filled circles), 710 nm (filled triangles), and 850 nm (open circles).

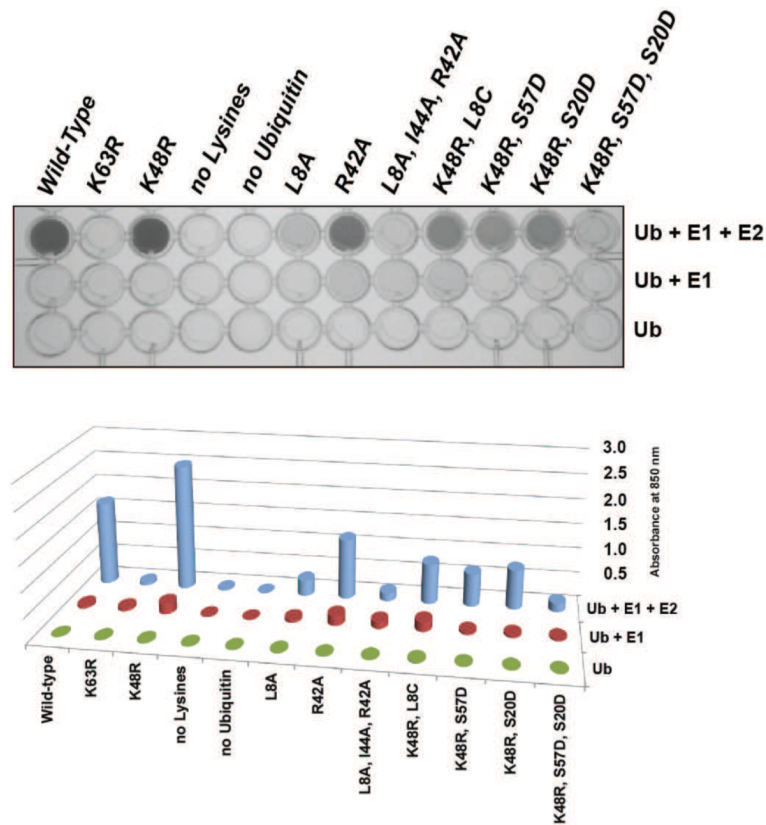


Figure 4. High throughput screen for the effect of ubiquitin mutations on polyubiquitin chain formation

The effect of ubiquitin mutations on polyubiquitin chain formation by Ubc13-Mms2. Wells in row 1 contain E1, Ubc13-Mms2 and ubiquitin, row 2 contains E1 and ubiquitin, and row 3 contains ubiquitin alone. Protein concentrations used were 2 μ M Ubc13-Mms2, 0.5 μ M E1, 500 μ M ubiquitin (except for no-lysine ubiquitin which was at 100 μ M). Reactions were incubated for 1 hour at 37 $^{\circ}$ C, quenched and the absorbance measured. Assay was performed in duplicate, with representative data shown.

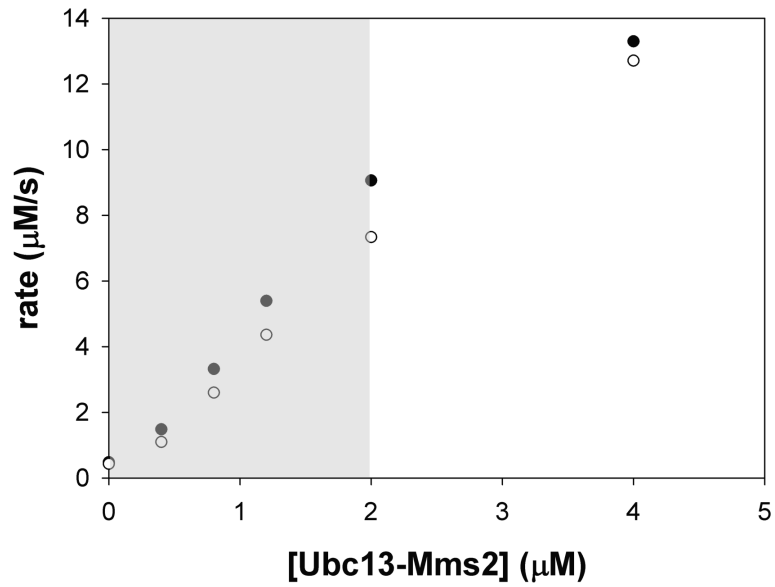


Figure 5. Rate-limiting concentration range of Ubc13-Mms2

Rate of pyrophosphate formation in $\mu\text{M}/\text{sec}$ at 0, 0.4, 0.8, 1.2, 2, and 4 μM Ubc13-Mms2 at 0.3 μM E1 (filled circles) and 0.6 μM E1 (open circles). Grey box indicates concentrations where ubiquitin transfer by Ubc13-Mms2 is directly correlated with pyrophosphate production by E1.

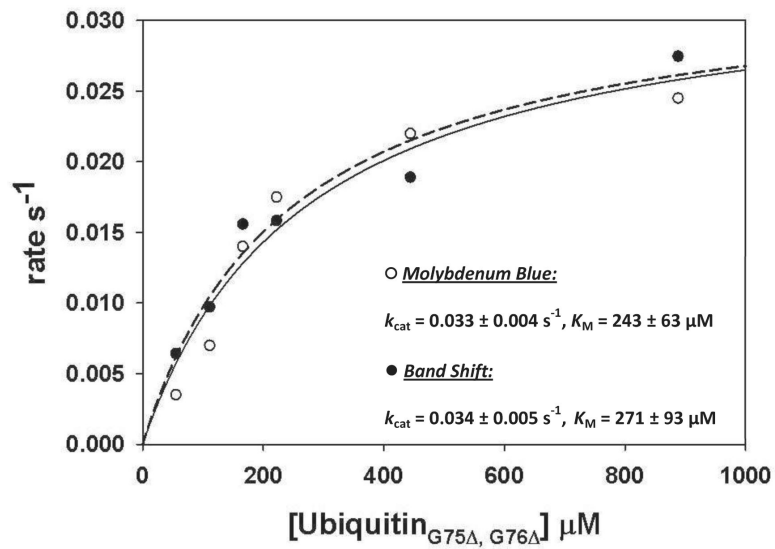


Figure 6. Ubiquitin^{ΔG75,ΔG76} saturation curves for Ubc13-Mms2

Reactions contained 0.3 μM Ubc13-Mms2, 0.2 μM E1, 0 to 950 μM ubiquitin^{ΔG75,ΔG76}, and 80 μM ubiquitin K63R. For molybdenum blue assay reactions (black circles), 500 Units of pyrophosphatase was present and for the band shift assay (open circles), 10 μM ubiquitin labeled with fluorescein at position 63 and with 70 μM unlabeled ubiquitin^{K63R}. Each rate is an average of 3 to 5 separate experiments. Error bars are not shown for clarity.