2

Purification of E1 and E1-Like Enzymes

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Summary

Ubiquitin-activating enzyme is the archetype for a family of enzymes catalyzing the ATP-coupled activation of ubiquitin and other class 1 ubiquitin-like proteins required for their subsequent conjugation to cellular targets. The general physical and mechanistic features of the E1 family appear well conserved. Formation of an obligatory E1–ubiquitin thiol ester intermediate forms the basis of a one-step covalent purification of the enzyme on ubiquitin-linked affinity columns that has been adapted for the isolation of E1 paralogs. We describe the facile purification of active E1 from outdated human red blood cells in yields (2–4 nmol/U of blood) that make this an attractive alternative to expression of the proteolytically labile recombinant protein. In addition, two stoichiometric activity assays are described that rely on formation of the E1 ^{125}I-ubiquitin thiol ester and ubiquitin [2,8-^{3}H]adenylate intermediates.

Key Words: Affinity chromatography; AppBp1; E1; Nedd8; purification; Uba3; ubiquitin; ubiquitin-like protein.

1. Introduction

The ubiquitin-activating enzyme (E1/Uba1) catalyzes the first step in the conjugation of ubiquitin to protein targets and serves as the archetype for paralogous enzymes catalyzing the activation of other class 1 ubiquitin-like polypeptides including Sumo, Nedd8, ISG15, Hub1, FAT10, and Apg12. The E1 catalytic cycle yields a ternary enzyme complex comprising stoichiometric amounts of a ubiquitin carboxyl terminal thiol ester to an absolutely conserved active site cysteine (Cys^{632}, human Uba1a numbering) and a tightly bound ubiquitin adenylate mixed anhydride that serves as the immediate precursor of the thiol ester ***(1,2)*** (*see* **Fig. 1**). Translation from alternative start sites of the E1 mRNA, transcribed from the single gene encoding the enzyme, yields nuclear (Uba1a) and cytoplasmic (Uba1b) isozymes of 117,789 Da and 113,740 Da ***(3,4)***, respectively, that are otherwise functionally indistinguishable with respect to E2 thiol ester formation ***(5)***. The half-reactions of E1-catalyzed ubiquitin activation and E3 ligase-catalyzed isopeptide bond formation are linked through a superfamily of

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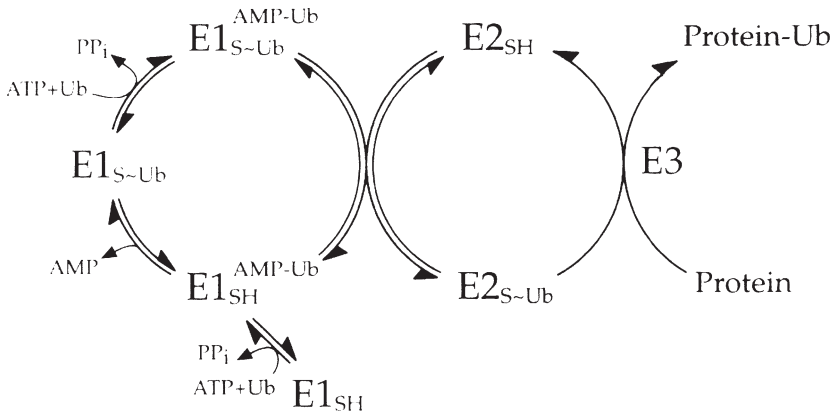

Fig. 1. The mechanism of ubiquitin conjugation. In the presence of ATP and ubiquitin, E1 forms a ternary complex composed of ubiquitin thiol ester and ubiquitin adenylate intermediates. The former intermediate is transferred to E2 to form the corresponding E2 thiol ester. The E3 ligase catalyzes the conjugation of ubiquitin by aminolytic cleavage of the cognate E2–ubiquitin thiol ester.

cognate E2/Ubc isoforms that transfer the polypeptide as an E2–ubiquitin thiol ester ***(6,7)*** (*see* **Fig. 1**). The hierarchical architecture of ubiquitin conjugation accounts for the broad substrate specificity and evolutionary plasticity of this posttranslational modification that is shared with paralogous pathways for ligation of the ubiquitin-like proteins, reviewed in **refs. *6–8***.

In vitro reconstitution of ubiquitin ligation requires the presence of sufficient E1 and E2, determined empirically, to render the overall process rate limiting with respect to E3-catalyzed conjugation in order to yield unambiguous information regarding substrate specificity and function ***(6,9,10)***. The relatively small size of the E2 isoforms (14–35 kDa) favors their expression in high yield within *Escherichia coli*. Small amounts of recombinant human GST–E1 can be similarly expressed ***(11)***; however, the proteolytic instability frequently observed when expressing large recombinant proteins precludes yields sufficient to serve as a practical source of reagent-grade quantities of activating enzyme ***(5)***. Early work demonstrated that ubiquitin-linked affinity columns afford a facile method for isolating E1 from cell extracts from which free ubiquitin has been removed by anion-exchange chromatography ***(1,12)***. In the presence of ATP, Mg^{2+}, and a suitable ATP-regenerating system, E1 forms a covalent thiol ester with column-bound ubiquitin that can be specifically eluted by forcing the reaction in reverse on addition of AMP and PP_i. Subsequent elution with dithiothreitol (DTT) at alkaline pH yields a mixture of endogenous E2 isoforms, a small fraction of E1 noncovalently bound to E2, and other ubiquitin interacting proteins that can be resolved further by anion-exchange FPLC ***(13,14)***. The relative simplicity of this affinity method makes cell-free extracts an attractive source of E1. However, the presence of a proteolytic activity in cell extracts that inactivates ubiquitin by limited diges-

tion of the C-terminal glycine dipeptide complicates the general use of ubiquitin affinity methods ***(15)***. We have found that human erythrocytes are devoid of this proteolytic activity and contain significant amounts of active E1 ***(9,10)***. We describe a protocol for the facile affinity purification of human E1 from outdated red blood cells that has been refined from earlier methods ***(1,14)***. Because reconstitution of in vitro conjugation requires an accurate knowledge of E1 concentrations, we also provide two alternative methods for quantitating the active enzyme that relies on the stoichiometric formation of the ubiquitin adenylate and thiol ester intermediates ***(1,2)***.

2. Materials

2.1. Ubiquitin Affinity Column

1. Affi-Gel 10 activated affinity support (Bio-Rad).
2. Bovine ubiquitin (Sigma).
3. 0.1 *M* Sodium bicarbonate, pH 9.0, at room temperature.
4. Glass-fritted (medium) Büchner funnel and side-arm flask.
5. 0.1 *M* Ethanolamine-HCl, pH 8.0, at room temperature.
6. 50 m*M* Phosphate-buffered saline (PBS), pH 7.4, at room temperature.
7. 0.1 *M* Tris-HCl, pH 9.0, at room temperature.
8. 50 m*M* Tris-HCl, pH 7.5, at room temperature.
9. Bovine serum albumin (BSA) (Sigma).

2.2. Preparation of Human Erythrocytes

1. 5 U of outdated packed human erythrocytes.
2. Surgical gloves, mask, and face shield (for use while harvesting cells from the blood bank bags).
3. 50 m*M* Potassium PBS, pH 7.4, at room temperature.
4. Krebs–Ringer phosphate: 0.1 *M* sodium phosphate buffer, pH 7.4, 120 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgCl_2$ at 37°C.
5. 20 m*M* 2,4-Dinitrophenol.
6. 0.5 *M* 2-Deoxyglucose.
7. 0.1 *M* DTT.
8. DEAE-52 (Whatman) anion-exchange resin (prehydrated).
9. 0.3 *M* Potassium phosphate buffer, pH 7.0, at 4°C.
10. 25 m*M* Potassium phosphate buffer, pH 7.0, containing 1 m*M* DTT at 4°C.
11. 25 m*M* Potassium phosphate buffer, pH 7.0, containing 25 m*M* KCl and 1 m*M* DTT at 4°C.
12. 25 m*M* Tris-HCl, pH 7.2, containing 0.5 *M* KCl and 1 m*M* DTT at 4°C.
13. Solid ammonium sulfate.
14. 50 m*M* Tris-HCl, pH 7.2, containing 1 m*M* DTT at 4°C.

2.3. Affinity Isolation of E1

1. 50 m*M* Tris-HCl, pH 7.5, containing 2 m*M* ATP and 10 m*M* $MgCl_2$ at room temperature.
2. 0.1 *M* ATP.
3. 1.0 *M* $MgCl_2$.
4. 0.5 *M* Creatine phosphate.
5. Creatine phosphokinase (Sigma) at 10^3 IU/mL in 50 m*M* Tris-HCl, pH 7.5, and 1 m*M* DTT.
6. 50 m*M* Tris-HCl, pH 7.5, at room temperature.

7. 50 m*M* Tris-HCl, pH 7.5, containing 0.2 *M* KCl at room temperature.
8. 25 m*M* Tris-HCl, pH 7.5, containing 2 m*M* AMP and 2 m*M* inorganic pyrophosphate (PP_i) at room temperature.
9. 0.1 *M* DTT.
10. 0.1 *M* Tris-HCl, pH 9.0, containing 10 m*M* DTT at room temperature.
11. HR 5/5 Mono Q anion-exchange FPLC column (Pharmacia).
12. Mono Q FPLC buffers: Buffer A: 50 m*M* Tris-HCl, pH 7.5, containing 1 m*M* DTT at 4°C. Buffer B: 50 m*M* Tris-HCl, pH 7.5, containing 1.0 *M* NaCl and 1 m*M* DTT at 4°C.
13. Superose 12 buffer: 50 m*M* Tris-HCl, pH 7.5, containing 50 m*M* NaCl and 1 m*M* DTT at 4°C.

2.4. E1 Quantitation

1. Sodium ^{125}I (carrier-free) or [2,8-^3H]ATP (30–50 Ci/mmol; Amersham Biosciences).
2. 1 *M* Tris-HCl, pH 7.5, at room temperature.
3. 10 mg/mL of chloramine-T (make fresh).
4. 10 mg/mL of sodium bisulfite (make fresh).
5. 10 m*M* KI.
6. 50 m*M* Tris-HCl, pH 7.5, at room temperature.
7. 10% (w/v) Trichloroacetic acid (TCA) at 4°C.
8. 0.2 *M* Triethylamine-HCl, pH 9.0, at room temperature.

3. Methods

3.1. Preparation of a Ubiquitin Affinity Column

Any activated affinity support can be used to prepare the ubiquitin affinity column; however, we prefer Affi-Gel 10 (Bio-Rad) because the *N*-hydroxysuccinimide ester chemistry consistently yields a >90% coupling efficiency when used with compatible nonamine buffers and care is taken to minimize hydrolysis of the activating group prior to coupling ***(2)***. The latter point requires all reagents to be prepared beforehand. The following protocol assumes a column of 10-cc bed volume and having a ubiquitin concentration of approx 2 mg/mL (234 μ*M*), which is sufficient for processing fraction II cell extract equivalent to 1 U of packed human erythrocytes yielding 2–4 nmol of active E1. The protocol can be readily adapted to prepare similar affinity columns containing recombinant ubiquitin-like proteins ***(16)*** or E2/Ubc paralogs ***(9,10)***.

1. Prepare the coupling solution by dissolving 20 mg of bovine ubiquitin in 10 mL of 0.1 *M* sodium bicarbonate buffer, pH 9.0, equilibrated to room temperature (*see* **Note 1**). Because of the high coupling efficiency, the volume and ligand concentration of the coupling solution should approximate the bed volume and final ligand concentration of the column. Save a 50-μL aliquot of the coupling solution for later determination of the ligand concentration on the column (*see* **Note 2**).
2. Thoroughly suspend the Affi-Gel 10 by vigorous shaking and then pour 1.25 times the desired bed volume into a 15-mL glass-fritted (medium) Büchner funnel attached to a side-arm flask. Using low vacuum, wash the affinity support with five bed volumes each of ice-cold distilled water and 0.1 *M* sodium bicarbonate, pH 9.0. Draw the buffer layer down to the top of the affinity support but DO NOT draw to dryness because the support is difficult to rehydrate. Immediately transfer the desired amount of equilibrated affinity support by spatula to a 50-mL Erlenmeyer flask (*see* **Note 3**).
3. Add the coupling solution to the equilibrated affinity support and incubate at room temperature for 1 h with gentle mixing on a rotary shaker.

4. Block any remaining unreacted activated groups on the affinity support by adding 1 mL of 0.1 *M* ethanolamine-HCl, pH 8.0, and incubating an additional hour at room temperature.
5. Pour the coupled affinity support into a 1.5 × 10 cm column. Collect the coupling solution for determining the amount of bound ligand on the column. Successively wash the column with five bed volumes each of 0.1 *M* sodium bicarbonate, pH 9.0, 50 m*M* PBS, pH 7.4, containing 5 mg/mL of BSA to block nonspecific protein binding sites on the affinity support, 50 m*M* PBS, pH 7.4, then 0.1 *M* Tris-HCl, pH 9.0, to remove noncovalently bound protein that may otherwise elute during the final pH 9 DTT wash of the column (*see* **Subheading 3.2.2.**), and 50 m*M* Tris-HCl, pH 7.5. The column should be stored at 4°C in 50 m*M* Tris-HCl, pH 7.5, containing 0.1% (w/v) sodium azide to retard bacterial growth.

3.2. Isolation of Human Ubiquitin E1

Although the majority of cellular proteins present in their nucleated progenitor cells are lost during the terminal differentiation of erythrocytes, low amounts of many enzymes remain in the soluble fraction, making these cells an attractive source from which to purify human E1 and other enzymes of the ubiquitin pathway. Erythrocyte fraction II is prepared from the 10^5g supernatant of red cell lysate by DEAE fractionation at pH 7.0. Free ubiquitin and the ubiquitin-like proteins fail to bind to the anion-exchange matrix and appear in the unadsorbed fraction (fraction I) while E1 is retained in the absorbed fraction, which is subsequently eluted in 0.5 *M* KCl ***(17,18)***. In addition, this step removes hemoglobin, the major protein within erythrocyte cytosol. Erythrocyte fraction II can also be used for the purification of the AppBp1-Uba3 heterodimeric Nedd8 activating enzyme by Nedd8-AffiGel 10 covalent affinity chromatography ***(16)***. Although not tested to date, fraction II presumably also contains the human Aos1-Uba2 heterodimeric Sumo activating enzyme ***(19,20)***.

3.2.1. Preparation of Human Erythrocyte Fraction II

1. Obtain 5 U of outdated packed human red blood cells (preferably leukocyte free; *see* **Note 4**). Carefully remove the cells from their storage bags by cutting open with scissors and pool.
2. Collect the cells by centrifuging at 4500*g* for 15 min. Remove the plasma by aspiration, and then gently resuspend the cells in twice their volume of 50 m*M* PBS, pH 7.4. Centrifuge again and remove the supernatant by aspiration.
3. Resuspend the cells in an equal volume of Krebs–Ringer phosphate and adjust to 0.2 m*M* 2,4-dinitrophenol and 20 m*M* 2-deoxyglucose. Incubate the cell suspension with gentle shaking for 90 min at 37°C to deplete cellular ATP (*see* **Note 5**).
4. Collect the cells by centrifugation for 15 min at 4500*g*. Remove the supernatant by aspiration and pool with the prior washes for decontamination. Wash the cell pellet three times in an equal volume of 50 m*M* PBS, pH 7.4, removing the supernatant each time by aspiration. Use care during washing and resuspending the cells because ATP depletion makes the cells fragile.
5. Lyse the cells by adding 1.6 pellet volumes of ice-cold distilled water. Immediately add DTT to a final concentration of 1 m*M*. Allow the lysate to stand on ice for 30 min.
6. Centrifuge the lysate at 10^5g for 1 h at 4°C. Collect the supernatant and adjust to pH 7.2 with 1 *M* sodium phosphate buffer, pH 7.2, if necessary. The 10^5g pellet is soft and easily disturbed; therefore, the supernatant should be removed by aspiration. If desired, lysates

can be divided into five equal aliquots and stored at –80°C because they are stable for at least a year without appreciable loss of E1 activity. The subsequent steps can be performed with individual lysate aliquots to avoid processing large volumes. *(The remaining steps assume the processing of a volume of fraction II equal to one unit of packed erythrocytes.)*

The following steps are performed at 4°C.

1. Prepare a DEAE column (Whatman DE-52) having a bed volume equal to 50% of the 10^5g supernatant volume to be processed (*see* **Note 6**). Add bulk prehydrated DE-52 to a beaker to give a gently packed approximate volume equaling 25% more than needed in order to allow for loss while removing "fines." The DE-52 should be equilibrated in twice the volume of 0.3 *M* potassium phosphate buffer, pH 7.0, 4°C, and the pH adjusted as necessary. After most of the DE-52 has settled out by gravity, "fines" suspended in the buffer should be removed by aspiration. Resuspend in an equal bed volume of 0.3 *M* potassium phosphate buffer, pH 7.0, 4°C, and allow to settle again. Remove "fines" by aspiration; repeat a third time. Suspend the DE-52 in an equal bed volume of 25 m*M* potassium phosphate buffer, pH 7.0, 4°C, containing 1 m*M* DTT and adjust the pH as necessary. Pour the column to the desired bed volume and then equilibrate the column with three bed volumes of the same buffer by gravity flow. Check the pH of the buffer eluting from the column. If the pH is not 7.0, continue washing the column.
2. Load the 10^5g supernatant onto the DE-52 column and begin collecting the unadsorbed fraction immediately as the color begins to elute from the column (*see* **Note 7**). Wash the column with five bed volumes of column buffer or until the OD_{280nm} does not change. Wash the column with three bed volumes of 25 m*M* potassium phosphate buffer, pH 7.0, 4°C, containing 25 m*M* KCl and 1 m*M* DTT to remove residual weakly adsorbed free ubiquitin. Elute the erythrocyte fraction II from the column with three bed volumes of 25 m*M* Tris-HCl, pH 7.2, containing 0.5 *M* KCl and 1 m*M* DTT.
3. Fraction II proteins eluted at 0.5 *M* KCl are concentrated by ammonium sulfate precipitation. With a magnetic stirrer set to a low speed, slowly add solid ammonium sulfate to the 0.5 *M* KCl eluate to achieve 85% saturation at 4°C (610 g/L of eluate). After all of the crystals have dissolved, allow the stirring to continue for at least 1 h at 4°C.
4. Centrifuge at 14,000*g* for 20 min. Resuspend the ammonium sulfate pellet in 5% of the equivalent lysate volume of 50 m*M* Tris-HCl, pH 7.2, containing 1 m*M* DTT (*see* **Note 8**). Using 12-kDa exclusion dialysis tubing, dialyze overnight against 4 L of 50 m*M* Tris-HCl, pH 7.2, containing 1 m*M* DTT.
5. The next morning adjust the pH to 7.2 as necessary. Remove any protein precipitate by centrifuging at 20,000*g* for 10 min. Flash-freeze the fraction II in dry ice–ethanol and store at –80°C.

3.2.2. Affinity Isolation of E1

Covalent affinity purification of E1 is adapted from earlier protocols for the isolation of E1 and E2 isoforms from rabbit reticulocyte extract ***(1,14)***. One-step affinity isolation of E1 is based on its ability to form a covalent thiol ester intermediate with column-bound ubiquitin. Because the E1 reaction is highly temperature dependent, all steps should be performed at room temperature and the erythrocyte fraction II should be warmed to 30°C as described prior to applying to the affinity column (*see* **Note 9**).

1. Equilibrate a 2.5 × 2 cm Sephadex G25 precolumn and the 10 cc of ubiquitin-Affi-Gel 10 affinity column prepared earlier with 50 m*M* Tris-HCl, pH 7.5, 20°C, containing 2 m*M* ATP and 10 m*M* $MgCl_2$ (*see* **Note 10**).
2. Flash thaw an aliquot of human erythrocyte fraction II equivalent to 1 U of packed red cells and then place on ice. Adjust the pH to 7.7 with 1 *M* Tris base as necessary and then adjust to 2 m*M* ATP, 10 m*M* $MgCl_2$, 10 m*M* creatine phosphate, and 1 U/mL of creatine phosphokinase (*see* **Note 11**). Warm the fraction II to 30°C in a water bath and then filter through the equilibrated Sephadex G25 precolumn. Save 200 µL as a starting fraction sample.
3. Slowly pass the filtered sample through the ubiquitin-Affi-Gel 10 affinity column at a flow rate of approx 0.5–1 mL/min. Save the unadsorbed flow through fraction as the postcolumn fraction for later determining recovery of E1.
4. Wash the column successively with two bed volumes of 50 m*M* Tris-HCl, pH 7.5, three bed volumes of 50 m*M* Tris-HCl, pH 7.5, containing 0.2 *M* KCl to remove weakly adsorbed proteins, and two bed volumes of 50 m*M* Tris-HCl, pH 7.5.
5. Elute the bound E1 with three bed volumes of 50 m*M* Tris-HCl, pH 7.5, containing 2 m*M* AMP and 2 m*M* PP_i. Immediately adjust the eluate to 1 m*M* DTT and place on ice for subsequent FPLC resolution (*see* **Subheading 3.2.3.**).
6. Elute the remaining E1 and E2 isoforms with three bed volumes of 0.1 *M* Tris-HCl, pH 9.0, containing 10 m*M* DTT and then immediately adjust to pH 7.5 with 1 *N* HCl and place on ice for FPLC resolution (*see* **Subheading 3.2.3.**).

3.2.3. Fast Protein Liquid Chromatography Purification of E1

Although the E1 is substantially pure following covalent affinity chromatography, the AMP-PP_i and pH 9-DTT eluates contain variable amounts of E2 isoforms, ubiquitin C-terminal hydrolase, and other ubiquitin-interacting proteins that can interfere with subsequent studies. Fast protein liquid chromatography (FPLC) is used to resolve E1 from these other components ***(14)***. The FPLC steps should be performed at 4°C.

1. Equilibrate a Mono Q HR5/5 anion-exchange column (Pharmacia) with 50 m*M* Tris-HCl, pH 7.5, containing 1 m*M* DTT. Load the entire AMP-PP_i eluate from **step 5** in **Subheading 3.2.2.** onto the column at a flow rate of 1 mL/min and monitor the column eluate at 280 nm with the chart recorder set to 1 absorbance unit full scale. After the unadsorbed fraction has passed through the column (monitored by the marked absorption peak due to AMP), start a linear 0–0.5 *M* NaCl gradient at 12.5 m*M*/mL (0–0.5 *M* in 40 mL) and immediately begin to collect 1-mL fractions. The E1 typically elutes as a sharp peak at 0.23 *M* NaCl (*see* **Fig. 2**). Pool the peak fractions for subsequent assay of E1 activity.
2. Reequilibrate the Mono Q column with 50 m*M* Tris-HCl, pH 7.5, containing 1 m*M* DTT and apply the pH 9 DTT eluate from **step 6** in **Subheading 3.2.2..** Repeat the NaCl gradient and collect into fresh tubes as before.
3. The Mono Q eluates are sufficiently homogeneous and of sufficient concentration that the NaCl present usually does not interfere because it is later diluted. If necessary, the Mono Q eluates can be resolved further by directly applying as 0.5-mL samples per run to a Superose 12 HR 10/30 gel filtration column (1 mL/min) equilibrated with 50 m*M* Tris-HCl, pH 7.5, containing 50 m*M* NaCl and 1 m*M* DTT. The E1 elutes as a symmetric peak at approx 110 kDa ***(14)***.
4. Because E1 is unstable to repeated freeze–thaw cycles, the enzyme should be divided into appropriate aliquots and flash frozen in liquid nitrogen then stored at –80°C ***(1,14)***.

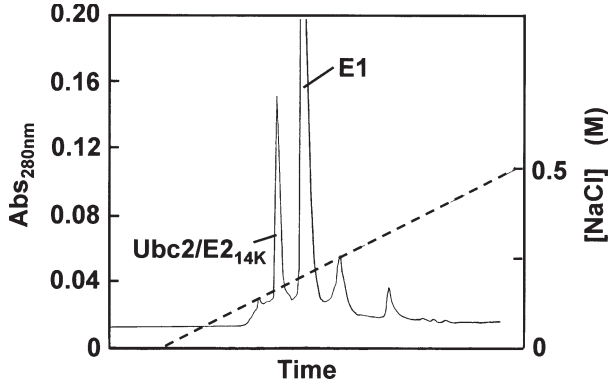

Fig. 2. Representative Mono Q FPLC trace of the AMP-PP_i elution from the ubiquitin affinity column. Eluate recovered from one unit equivalent of human erythrocytes was resolved as described in the text (*see* **Subheading 3.2.2.**). The *dashed line* plots the NaCl gradient in which E1 elutes at 0.23 *M*.

Samples are stable for at least 8 mo at –80°C. Aliquots should be flashed thawed briefly at 37°C and then placed on ice for use. The enzyme typically loses approx 25% of its activity with each thaw. If desired, stability can be enhanced by adding BSA as a carrier protein to a final concentration of 1 mg/mL. The E1 is less stable in 30% glycerol, a typical alternative means of stabilizing proteins.

3.3. Stoichiometric Assay of Active E1

The ability of E1 and its paralogs to form a stoichiometric ternary complex containing ubiquitin adenylate and ubiquitin thiol ester serves as the basis for two types of E1 activity assays ***(1,16)***. The *thiol ester assay* measures the amount of E1–^{125}I-ubiquitin thiol ester within the ternary complex by direct quantitation of associated radioactivity following nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolution from free radiolabeled polypeptide ***(1,2)***. Because the E1–ubiquitin thiol ester represents the proximal donor of activated ubiquitin in E2–ubiquitin thiol ester formation (*see* **Fig. 1**), this assay best estimates the amount of active E1 present. The *adenylate assay* measures the stoichiometric formation of ubiquitin [^{3}H]adenylate within the E1 ternary complex following fractionation from the [2,8-^{3}H]ATP substrate by TCA precipitation of the enzyme-bound intermediate ***(1,2)***. Analogous assays can be employed to quantitate the AppBp1-Uba3 heterodimeric Nedd8 activating enzyme ***(16)***.

3.3.1. Preparation of ^{125}I-Ubiquitin

1. Transfer 100 µL of 10 mg/mL of FPLC purified bovine ubiquitin (Sigma) to a 1.5-mL Eppendorf tube (*see* **Note 12**). Add 13 µL of 1 *M* Tris-HCl, pH 7.5. Warm the tube and contents to 37°C.
2. Add 10 µL (1 mCi) of carrier-free Na^{125}I (Amersham).
3. Begin the reaction by adding 25 µL of 10 mg/mL of chloramine-T. Incubate at room temperature for 1 min.

4. Quench the reaction by addition of 30 µL of 10 mg/mL of sodium bisulfite. Incubate for 1 min at room temperature.
5. Add 25 µL of 10 m*M* KI as a carrier to dilute the specific activity of the free ^{125}I.
6. Immediately load the sample onto a 1 × 45 cm column of Sephadex G25 equilibrated with 50 m*M* Tris-HCl, pH 7.5, at room temperature. Resolve ^{125}I-ubiquitin from free radioiodide at a flow rate of 1 mL/min. Collect 1-mL fractions.
7. To avoid the problem of coincident counts caused by the large amount of radiolabel, determine the ^{125}I radioactivity present in 5 µL of each fraction by γ-counting. Free ^{125}I-ubiquitin will elute in the void volume of the column (~fractions 10–12). To avoid excessive dilution of the radiolabeled protein, pool the peak 2 or 3 fractions only.
8. Accurately measure the absorbance of the pooled sample at 280 nm and then subtract the absorbance of a blank determined on a fraction appearing before the void volume (usually fraction 5). Calculate the absolute ubiquitin concentration of the pooled sample using an empirical extinction coefficient of 0.16 $(mg/mL)^{-1}$ ***(21)***.
9. To determine the fixed radioactivity, carefully dilute 10 µL of the pooled sample into 190 µL of 5 mg/mL of BSA. Transfer 10 µL of the latter dilution into 190 µL of 5 mg/mL of BSA and then add 200 µL of 20% (w/v) TCA. Allow the sample to stand on ice for 10 min and then centrifuge 14,000*g* for 10 min. Remove the supernatant by aspiration and determine radioactivity present in the pellet by γ-counting. Calculate the specific radioactivity of the ^{125}I-ubiquitin (typical values = 6000–12,000 cpm/pmol). The ^{125}I-ubiquitin should be divided into conveniently sized aliquots and flash frozen in liquid nitrogen and then stored at –20°C. Once thawed, aliquots can be kept at 4°C for several weeks without loss of activity.

3.3.2. E1–^{125}I-Ubiquitin Thiolester Assay

1. Prepare a standard 10% (w/v) SDS-PAGE gel and running buffer (equilibrated to 4°C).
2. Incubations of 50 µL final volume should contain 50 m*M* Tris-HCl, pH 7.5, 2 m*M* ATP, 10 m*M* $MgCl_2$, 1 m*M* DTT, 1 mg/mL of carrier BSA, 5 µ*M* ^{125}I-ubiquitin, and E1 sample. Because formation of the E1 ternary complex is rapid ***(2)***, the incubations should be equilibrated for several minutes at 37°C before initiating the assay by the addition of ^{125}I-ubiquitin (*see* **Note 13**).
3. After 1 min at 37°C, the reaction is quenched by adding 50 µL of standard SDS sample buffer from which 2-mercaptoethanol has been omitted. The sample is allowed to stand on ice for 5 min to allow proteins to unfold. Do not boil the samples because this will destroy the thiol ester linkage.
4. Immediately load the SDS-PAGE gel and resolve under standard conditions at 4°C. To prevent heating of the gel and hydrolysis of the thiol esters during the run, completely immerse the gel in ice cold running buffer for good heat transfer.
5. When the SDS-PAGE is completed, float the gel onto a piece of Whatman filter paper, overlay with Saran Wrap and dry the gel using a standard vacuum gel drier. Mark the filter paper with ^{125}I-labeled India ink (made by adding 25 µL of ^{125}I-ubiquitin to 1 mL of India ink) or glow-in-the-dark paint so that the gel can be overlaid on the resulting autoradiogram later. Autoradiograph overnight at –80°C using Kodak X-Omat film and an appropriate intensifying screen. The next day overlay the developed autoradiogram over the dried gel and cut out the corresponding E1 thiol ester bands for quantitation of associated radioactivity by γ-counting. Determine the absolute amount of E1 thiol ester by using the specific radioactivity of the ^{125}I-ubiquitin.

3.3.3. E1 Ubiquitin [^{3}H]Adenylate Assay

1. Incubations of 50 µL final volume should contain 50 m*M* Tris-HCl, pH 7.5, 1 µ*M* [2,8-^{3}H]ATP, 10 m*M* $MgCl_2$, 1 m*M* DTT, 1 mg/mL carrier BSA, 5 µ*M* ubiquitin, and E1 sample. Because formation of the E1 ternary complex is rapid ***(2)*** , the incubations should be equilibrated for several minutes at 37°C before initiating the assay by the addition of ubiquitin.
2. Incubate for 1 min at 37°C then quench the reaction by addition of 150 µL of 10% (w/v) TCA. Set on ice for 10 min and then centrifuge for 10 min at 14,000*g*.
3. Aspirate the supernatant and discard. Gently rinse the surface of the pellet with ice-cold 10% TCA and then aspirate.
4. Dissolve the pellet in 200 µL of 0.2 *M* triethylamine-HCl, pH 9.0, and then quantitatively transfer to a vial containing a suitable scintillation cocktail. Determine the radiolabel present in the solubilized TCA pellet and calculate the absolute E1 content from the specific radioactivity of the [2,8-^{3}H]ATP.

4. Notes

1. Commercial bovine ubiquitin is used to prepare the affinity column because it is identical in sequence to human ubiquitin ***(6)***.
2. Because the *N*-hydroxysuccinimide released during coupling absorbs at 280 nm and interferes with Lowry protein assays, the ligand concentration on the affinity column can be determined from the difference in unbound ligand protein between the starting and final coupling solutions by the Bradford dye binding assay ***(22)*** or by using quantitative SDS-PAGE followed by Coomassie staining. Alternatively, a small amount of ^{125}I-ubiquitin (*see* **Subheading 3.3.1.**) can be added directly to the initial coupling solution to allow one to calculate bound ligand from the radioactivity remaining in the postcoupling solution.
3. The *N*-hydroxysuccinimide ester activated support is sensitive to hydrolysis, which results in a diminished coupling efficiency for the protein ligand. Work quickly while washing the affinity support to avoid excessive hydrolysis. Gentle vacuum filtration is preferably to gravity filtration because the support can be washed more rapidly. Because coupling is through lysyl ε-amino groups on the ligand, never use buffers containing primary amines because they will react with the activated support and diminish coupling efficiency. Care should be taken to avoid introducing water into the unused support, which is stored in anhydrous ethanol; the bottle containing unused support should be carefully resealed with Parafilm and returned to –80°C.
4. The use of leukocyte-free human red blood cells will obviate having to remove the buffy coat by aspiration while washing the cells. Leukocytes do not pose a problem to the protocol but can introduce unwanted enzyme contaminants. Although modern blood bank screening procedures largely eliminate the risk of infectious agents, one should follow normal safety precautions for blood-borne pathogens through the DEAE step: (a) wear a laboratory coat and approved surgical gloves (while cutting open the blood bank bags one should also wear a face shield and surgical mask; (b) dispose of blood bank bags, gloves, and so forth in a biohazard bag and immediately autoclave; (c) decontaminate all glassware with dilute bleach; and (d) collect all disposable supernatants by aspiration into a side-arm flask for subsequent decontamination with bleach before disposal.
5. The ATP depletion step is essential to allow endogenous isopeptidases to disassemble residual ubiquitin conjugates ***(23)***. Otherwise, the isopeptidases will disassemble the con-

jugated ubiquitin in later steps and contaminate the erythrocyte fraction II with free ubiquitin. Free ubiquitin efficiently competes with column-bound polypeptide, even though the latter is present at a much higher concentration, and significantly reduces the yield of E1 recovered in the affinity purification step. Depletion of ATP depends on 2,4-dinitrophenol to decouple any remaining mitochondria present in the red cells and endogenous hexokinase to form 2-deoxyglucose-6 phosphate.

6. If fraction II equivalent to one unit of packed erythrocytes is being processed, the bed volume of DEAE will equal 10% of the total $10^5 g$ lysate. Our experience has been that a column of DE-52 is much more efficient at resolving free ubiquitin than bulk adsorption. The conditions described here have been optimized for lysate binding capacity by DE-52 and the resolution of free ubiquitin. Although it is generally bad practice to use an anionic buffer with an anion-exchange matrix, in the present application phosphate buffer aids in blocking ubiquitin adsorption to the DE-52.
7. Free ubiquitin is contained in the unadsorbed fraction of the DE-52 column under the conditions described even though the polypeptide has a pI of 6.7. Equilibrating the column to higher pH than specified results in increased contamination of fraction II with free ubiquitin.
8. Most of the protein will not initially dissolve because of the high concentration of residual ammonium sulfate contained in the pellet; however, protein will resolubilize during dialysis.
9. Covalent affinity purification of E1 requires that the ubiquitin be linked to the activated support so that it is not sterically hindered from proceeding through the catalytic cycle. Although the ubiquitin is probably preferentially linked through Lys^6, owing to the inherently greater reactivity of this group ***(24,25)***, there will exist a statistical distribution of linkages to all seven lysines present on ubiquitin. We have observed a general increased recovery of E1 with successive uses of the affinity column as sterically preferred sites are ubiquitinated and blocked from further reaction with E1 ***(14)***. No more than two bed volumes of fraction II should be processed through the column at one time because later steps in the conjugation reaction tend to elute the E1 from the column-bound thiolester, reducing the overall recovery. Poor recovery of E1 from fraction II usually results from inadequate removal of free ubiquitin in the DEAE step.
10. Protein solutions invariably contain microparticulate denatured proteins that are too small to detect by light scattering when viewed directly. However, these contaminants can collect at the top of affinity columns and, in part, elute with the affinity-bound protein, leading to contamination of the affinity-purified fraction. To prevent this, we use a small Sephadex G25 column as a prefilter to collect the microparticulates. This step proves to be a useful precaution in all affinity methods.
11. Free ATP is a competitive inhibitor of the true substrate, ATP/Mg^{2+} ***(2,26)***. This ratio of $ATP/MgCl_2$ guarantees that the ATP is quantitatively present as its Mg^{2+} chelate. The creatine phosphate and creatine phosphokinase are present as an ATP regenerating system. A stock solution of creatine phosphokinase at 10^3 IU/mL can be made in 50 m*M* Tris-HCl, pH 7.5, containing 1 m*M* DTT. Aliquots should be flash frozen and stored at –20°C. Aliquots should be thawed by hand only once and stored on ice for immediate use.
12. Commercial ubiquitin preparations that are not sufficiently pure for radioiodination can be purified to apparent homogeneity by FPLC ***(27)***.
13. Ubiquitin-activating enzyme shows a nonlinear stoichiometry at high concentrations, resulting in an underestimation of the true concentration of active enzyme. Therefore,

one should assay a series of four to five doubling dilutions of the enzyme in 50 m*M* Tris-HCl, pH 7.5, containing 1 m*M* DTT and 1 mg/mL of BSA as a carrier protein. Because the affinity-purified E1 is present at a low concentration, the enzyme should always be diluted in Tris–BSA to prevent loss by nonspecific adsorption to the sides of tubes. The Tris–BSA alone should be used as a negative control. The E1 sample volume within the thiol ester assay should be at least 10 µL for pipetting accuracy.

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