A specific endpoint assay for ubiquitin
(ubiquitin-activating enzyme)

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ABSTRACT Simple endpoint assays for free ubiquitin (Ub) and for the Ub-activating enzyme are described. The method for measuring Ub makes use of the reaction of iodoacetamide-treated Ub-activating enzyme (E): [3H]ATP + Ub + E \rightarrow [3H]AMP-Ub + PP, and PP \rightarrow 2P, (in the presence of pyrophosphatase). The Ub is then measured by determining the acid-insoluble radioactivity. The reaction is accompanied by a slow enzyme-catalyzed hydrolysis of the complex to AMP plus Ub. The presence of ubiquitin-activating enzyme in excess of Ub by \( \sim 0.1 \, \mu M \) assures that the steady state will be close to the endpoint for total Ub. A preparation of the activating enzyme from human erythrocytes that does not depend on affinity chromatography is described. Several applications of the assay are presented.

Since its discovery as a highly conserved protein of eukaryotic cells, ubiquitin (Ub) has been found in a variety of cell loci involved in key biological phenomena. In the nucleus, Ub is linked to histones H2A and H2B (1-3). Its presence may be important in gene expression (4, 5). The amount of Ub linked to these histones changes in a synchronous manner during the stages of the cell cycle (2, 6, 7). In the cytoplasm, Ub is required in a multienzyme pathway of energy-dependent protein breakdown, again acting by way of a C-terminal isopeptide linkage (8). Also, the presence in cells of diverse Ub-protein conjugates (9) may indicate that the attachment of Ub plays a role in regulating normal protein function. As a component of cell surface glycoproteins, Ub has a role in cell-cell recognition required for the homing of lymphocytes (10). Additional specific functions for Ub are suggested by the observation of a number of Ub-carrier proteins (11) and protein-conjugating proteins (12) of unknown function.

In spite of its importance, there is not a convenient assay for Ub. The methods now available require that a sample of Ub be used as a standard, even though Ub is not readily available in pure form. A suitable method should be able to assay Ub itself in the presence of a variety of derivative forms that may be present in a sample. Therefore, for example, an assay based on stimulation of protein breakdown by the reticulocyte system (13) is not generally appropriate since many of the conjugate forms of Ub would be included. An assay method that avoids this problem is based on stimulation of PP-ATP isotope exchange by highly purified Ub-activating enzyme (14). This method, being catalytic, is quite sensitive and will not respond to conjugated forms. However, not being an endpoint assay, it requires careful standardization and use of an internal standard to correct for rate effects caused by the sample. Furthermore, the exchange rate is inhibited by Ub at concentrations \( \geq 1 \, \mu M \) (15), which might lead to confusion. More complicated methods that depend on variously raised antibodies to Ub (16) or the combination of gel electrophoresis, transfer to nitrocellulose, and immunoblot analysis (9) are valuable in showing the distribution of Ub among different molecular weight species inasmuch as the antibody in use has low specificity, but they do not serve the need for a quantitative assay for Ub per se.

The simple endpoint assay for Ub at the subpicomolar level that we describe here is based on a nearly complete conversion of Ub to AMP-Ub by the Ub-activating enzyme (E). This enzyme, discovered by Hershko and colleagues (14), carries out the three-step sequence, Eq. 1:

\[
\begin{align*}
E_{SH} & + \text{ATP} + \text{Ub} \rightarrow E_{AMP-Ub} + \text{PP} \\
E_{AMP-Ub} & + \text{Ub} \rightarrow E_{S-Ub} + \text{AMP} \\
E_{S-Ub} & + \text{PP} \rightarrow E_{AMP-Ub} + \text{S-Ub}
\end{align*}
\]

It is the Ub that is in the thiolester linkage that is transferred to acceptor nucleophiles both enzymatically and nonenzymatically. To avoid loss of Ub by this route and to double the sensitivity of the assay, the enzyme is treated with iodoacetamide so that, with pyrophosphatase present to make the first step irreversible, the endproduct E-AMP-Ub is produced. The assay, based on conversion of \([3H]ATP\) to acid-insoluble \([3H]AMP-Ub\), depends on use of ATP of known specific activity (usually about 15,000 cpn/pmol) and an excess of iodoacetamide-treated Ub-activating enzyme. A crude preparation of Ub can easily be made for use in locating the enzyme in column fractions and for checking the reproducibility of assays.

MATERIALS AND METHODS

Ub and Ub-Activating Enzyme. Preparations of highly purified Ub from human erythrocytes (13) and Ub-activating enzyme from reticulocytes (17) have been reported and are quite reproducible (18, 19). Purification of the enzyme to homogeneity makes use of its affinity for Ub on a Ub-Sepharose 4B column in the presence of ATP and its specific elution by a solution of AMP and PPi. To obtain good yields of enzyme by this method, it is necessary to decrease the Ub/enzyme ratio from \( \sim 100:1 \) in the lysate to below 1:1 prior to application to the affinity column. This is readily done when rabbit reticulocytes are used, but it has been a problem when starting with human erythrocytes, possibly because of a continuous release of Ub from conjugates during the preparation. A procedure for preparing enzyme for assay purposes has been worked out that replaces the affinity chromatography step with salt-gradient elution on a DE-52 cellulose anion-exchange column followed by separation based on size by chromatography on Sephacryl S-200.

Abbreviations: Ub, ubiquitin; E, ubiquitin-activating enzyme.
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About 30 pmol of enzyme is usually obtained per milliliter of packed erythrocytes, which is enough for 10 assays. It is convenient to begin with 50 ml of cells, although the high stability of the enzyme allows larger size preparations to be of value. The initial steps of the procedure of Ciechanover et al. (17) for reticulocytes are used with human erythrocytes obtained from a hospital clinical laboratory as whole-blood samples that have been stored overnight in citrate or EDTA. Several of the red blood cells are removed from the centrifuged samples by aspiration, and the cells are washed twice by suspension in 4 volumes of 0.154 M saline. Cells are lysed by the addition of 4 volumes of cold 5 mM 2-mercaptoethanol. After 15 min in the cold, the lysate is centrifuged at 37,000 x g for 60 min to remove stroma. The optically clear supernatant obtained from 50 ml of cells is carefully taken off and added to a column (1.5 x 14 cm) of DE-52 (Whatman) that has been equilibrated with 3 mM Pi, pH 7.0/2 mM 2-mercaptoethanol. The unadsorbed hemoglobin-containing fraction has most of the Ub and is treated as follows to obtain a crude Ub preparation: The fraction is heated in a 90°C water bath with stirring for an additional 5 min after denaturation of the hemoglobin becomes apparent. The sample is filtered on a Buchner funnel, and the filtrate is cooled. Bovine serum albumin is added followed by trichloroacetic acid such that their final concentrations are 0.2 mg/ml and 12% (vol/vol), respectively. After 10 min on ice, the suspension is centrifuged at 12,000 x g. The pellet and tube are carefully rinsed with cold water; and the pellet, which contains ~4 nmol of Ub per ml of cells, is dissolved in 100 mM Tris-HCl (pH 7.2) given solution with ~15 nmol of Ub per ml at pH 7-7.5. Subdivided samples are stored at -70°C. They have been used interchangeably with pure Ub in the studies that follow.

To obtain the Ub-activating enzyme, the DE-52 column is washed with 5 column volumes of 3 mM Pi, pH 7.0/20 mM KCl/2 mM 2-mercaptoethanol and eluted with 3 column volumes of 0.5 M KCl/20 mM Tris-HCl, pH 7.2/2 mM 2-mercaptoethanol. Protein is precipitated with ammonium sulfate (to 80% saturation), centrifuged, suspended in a minimal volume of 20 mM Tris-HCl, pH 7.2/2 mM 2-mercaptoethanol, and dialyzed against the same solution. Precipitation and dialysis are important for removal of any residual ATP that would dilute the labeled ATP used in the assay.

The dialyzed solution is applied to a DE-52 column the same size as used before, equilibrated with 20 mM Tris-HCl, pH 7.2/50 mM KCl/2 mM 2-mercaptoethanol. The column is washed with 5 column volumes of the same mixture followed by a linear gradient prepared from 8 column volumes each of 50 and 350 mM KCl in the same buffer. The total effluent is collected in 80 fractions. The enzyme is found in fractions 20-40 as determined by assaying 5-ml samples that have been treated with 5 mM iodoacetamide (10 min, 37°C) and then with 4 mM dithiothreitol. For the assay, 50 pmol of Ub from the above preparation is added to a mixture containing 10 pmol of [3H]ATP, 0.1 μmol of MgCl2, 1 μmol of Tris-HCl (pH 7.6), and 0.04 μm of pyrophosphatase (Sigma) giving a total volume of 20 μl. After a 10-min incubation at 37°C, 1 ml of 12% (vol/vol) trichloroacetic acid is added following by 0.1 M NaOH (0.1 ml), and its radioactivity is determined. Under the conditions of the assay with excess Ub, the response is linearly related to the amount of Ub-activating enzyme added up to at least 2 pmol. The fractions within the peak of activity, appearing at ~150 mM Cl- (20) of the gradient, are pooled and precipitated with 80% ammonium sulfate. The pellet is transferred in a minimal volume of 20 mM Tris-HCl, pH 7.6/1 mM EDTA/5 mM 2-mercaptoethanol to dialysis tubing and dialyzed against 2 liters of the same buffer overnight.

The dialyzed enzyme from a 50-ml cell preparation is concentrated by lyophilization, if necessary, and is applied in 0.3 ml to a Sephacryl S-200 column (1 x 81 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.6/0.2 mM dithiothreitol/0.1 mM EDTA containing ovalbumin (0.05 mg/ml). One-milliliter fractions are collected, and those in the region expected for 100 kDa proteins are assayed for Ub-activating enzyme activity above and below the enzyme alcohols with iodoacetamide. The fractions of high activity are combined and subdivided, and they may be stored at -70°C indefinitely. Recovery of activity in the two column steps is greater than 50%.

Whether the enzyme is prepared by the affinity method or the alternative procedure presented here, it will contain enzymes that liberate Ub from conjugates with simple nucleophiles: thiols, esters, amides, and even from AMP-Ub itself (21, 22) and high molecular weight conjugates. More extreme treatment with iodoacetamide beyond that required to block the sulphydryl group of Ub-activating enzyme (19), 10 mM for 20 min at 37°C, inactivates those enzymes without altering the assayable activating enzyme. Enzyme treated in this way may be kept at -70°C for several freezing and thawing cycles.

Evidence that the assay of Ub is essentially an endpoint assay is provided in Fig. 1. The formation of the complex E-AMP-Ub is shown to be rapid under conditions recommended for the assay. A second addition of Ub would give almost the same increment of acid-insoluble cpm as the first. If ATP is removed from the system by means of added hexokinase, and 2-deoxyglucose, the labeled complex disappears with t1/2 = 10 min at 37°C (Fig. 1 Inset; k = 1.1 x 10^-3 sec^-1). This phenomenon and rate are also observed with affinity-purified activating enzyme. The loss of the E-AMP-Ub complex does not arise from a failure to block the active thiol site in some of the molecules since a single first-order rate constant accounts for decay of the whole population. That Ub is the product of the decay is evident since the assay value obtained does not decrease upon prolonged incubation as long as ATP is present. The breakdown is enzymatic; it is inhibited by EDTA and occurs maximally with as little as 0.1 mM MgCl2. It does not, therefore, represent an abortive transfer of Ub to water, which is a process that does not require Mg2+ (23), in lieu of the normal sulphydryl group of the enzyme. It is more likely to be the simple reverse of the first step of Eq. 1 using H2O instead of Pi, which is a Mg2+-dependent step already believed to be of unusually broad specificity as shown by its use of Pi when present at high concentration (23).

The instability of E-AMP-Ub suggests that its time-independent value in Fig. 1 may be the result of a steady state rather than an endpoint. To determine the extent to which an endpoint has been approached, the data of Fig. 1 were analyzed by the equation: d[E-AMP-Ub]/dt = k[E][Ub] - k2[E-AMP-Ub] using 1.1 x 10^-3 sec^-1 for k2 and [E] + [E-AMP-Ub] = [ET] = 1.5 pmol/20 μl (where ET = total enzyme). The best least-squares fit to the data, as shown by the continuous lines, comes from the following values: Ub0 = 8.75 x 10^-9 M, k1 = 4 x 10^5 M^-1sec^-1, and Ub (steady state) = 0.05 Ub0 (where Ub0 = Ub at time zero), all within 95% confidence bounds. Therefore, the assay is virtually quantitative and complete within 2 min using Ub-activating enzyme only 1 pmol/20 μl greater than Ub.

In spite of the irreversibility of step 1 (Eq. 1) of the activating enzyme in the presence of pyrophosphatase, the steady-state analysis of the assay requires that the total concentration of Ub-activating enzyme be greater than the concentration of Ub, since free enzyme or rather E-ATP determines the rate of this step. This is shown in Fig. 2 in
which E-AMP-Ub was measured at constant Ub and increasing amounts of Ub-activating enzyme in a 10 min assay. The assay appears to be \(\approx 75\%\) complete when the total amount of Ub-activating enzyme is in excess of free Ub by \(\approx 0.4\) pmol in the 20-\(\mu\)l assay. This is expected from the rate constants derived above.

Further evidence for a proportional response of the assay is shown by the linearity of the plot of acid-insoluble radioactive material versus the volume of an unknown Ub sample added (Fig. 3). The lower limit range of the assay is determined almost entirely by the presence of Ub in the activating enzyme. Radioactivity that is physically occluded in the precipitate is \(<0.2\%\) of the total cpm present in the incubation as can be determined by adding an excess of carrier ATP. This represents \(\approx 0.02\) pmol of a Ub-equivalent and can be decreased further by reprecipitation. The Ub present with the enzyme after the two-column procedure is 2–10\% of an enzyme equivalent. With an additional purification step to resolve the enzyme from the source of this Ub, the sensitivity of the assay can be increased to 0.02–0.1 pmol, giving 300–1500 cpm above background. This may be done by applying the enzyme to a small DE-cellulose column, washing the column with dilute buffer containing 20 mM KCl, and eluting with 0.5 M KCl as in the first step of the purification.

Routine our procedure is to make a stock solution, which for 10 assays contains (in 50 \(\mu\)l) 2 \(\mu\)mol of Tris-HCl (pH 7.6), 1 \(\mu\)mol of MgCl\(_2\), 0.03 \(\mu\)mol of EDTA, 100 pmol of [\(2,8\)-

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Rate of formation of E-[\(3^\text{H}\)]AMP-Ub and its hydrolysis under standard conditions. Two hundred fifty-microliter samples of 50 mM Tris-HCl, pH 7.6/5 mM MgCl\(_2\)/0.5 \(\mu\)M [\(3^\text{H}\)]ATP (15,000 cpm/pmol) containing 0.5 unit of pyrophosphatase (Sigma) was brought to 37°C. Ub-activating enzyme treated with iodoacetamide (10 mM, 20 min, and 37°C) was accurately assayed as 1.5 pmol per 20 \(\mu\)l using Ub in \(\approx 30\)-fold excess. The unknown was \(\approx 0.5\) pmol/20 \(\mu\)l using the Ub prepared as described. Samples of 20 \(\mu\)l were taken until 10 min at which time yeast hexokinase (Boehringer) and deoxyglucose were added to remove ATP, and sampling was continued. One milliliter of 12% (vol/vol) cold trichloroacetic acid and 100 \(\mu\)g bovine serum albumin (Sigma) were added to each sample. After 5 min on ice, the sample was centrifuged in an Eppendorf microfuge, and the supernatant sample was removed by aspiration. One milliliter of 2% trichloroacetic acid was used as a rinse without mixing. The pellet was dissolved in 0.1 ml of 1 M NaOH and was assayed for radioactivity. The value at 10 min, which was before hexokinase addition, agreed with the 300-sec point.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Effect of enzyme concentration on the Ub assay. Incubations of 20 \(\mu\)l containing \(\approx 0.7\) pmol of Ub, 0.5 \(\mu\)M [\(3^\text{H}\)]ATP, and varying amounts of Ub-activating enzyme, \(E_T\), were incubated for 10 min at 37°C. Correction was made for 0.02 equivalent of Ub that was added with the enzyme. ●, E-AMP-Ub; ×, \(E_T - (\text{E-AMP-Ub})\).
RESULTS AND DISCUSSION

An apparently well-behaved enzymatic method for the assay of subpicomole amounts of Ub is a practical product of simple laboratory procedures applied to readily available blood samples as a source for the Ub-activating enzyme and Ub. In the purification of the enzyme it seems important to maintain its active thiol group in the reduced state until it is alkylated. Therefore, dithiothreitol or 2-mercaptoethanol is present in all the steps. If this group was protected, possibly in the Ub-thiolester form, and deprotected during the assay, it might provide a route for Ub transfer to thiol such as dithiothreitol and amines during the assay. That such a process is not occurring is shown by the failure of high levels of dithiothreitol to stimulate the breakdown of E-AMP-Ub using iodoacetamide-treated enzyme (19) and the observation that a single first-order rate constant for hydrolysis applies to the whole population of acid-insoluble labeled complexes (Fig. 1). This instability contrasts with the long half-life (10 hr in the cold) of the same complex obtained from a Sepharose S-200 column as reported earlier (19), the difference being due to the activating effect of Mg++. We have made use of this column method to establish the true specific activity of preparations of 125I-labeled Ub using 3H]ATP of known specific activity to generate the complex.

A number of precautions concerning the assay should be kept in mind: (i) To repeat, samples known to contain ATP should be precipitated with cold trichloroacetic acid [10% (vol/vol)], dissolved in buffer, and reprecipitated to avoid dilution of the labeled ATP used in the assay. Recovery of Ub from such precipitations is found to be \(\approx 90-95\%\) when 0.2 mg of bovine serum albumin is present. (ii) It is desirable to initiate all assays by the addition of enzyme since the enzyme preparation described here has a small amount of ATPase activity. The concentration of ATP used in the assay (0.5 \(\mu\)M) is somewhat below that required for the half-maximal rate, and it was chosen for enzyme and assay reasons. The blank is low enough to allow 5 times this concentration of ATP to be used in assays where the amount of Ub is in the range of 0.5 pmol. This accomplishes only a small net increase in precipitable cpm when the amount of enzyme is 1 pmol/20 \(\mu\)l greater than the total amount of Ub. Increasing the concentration of ATP, however, does allow conservation of enzyme, which may be useful when the supply is low or if decreasing the Ub introduced by enzyme is important. The \(K_m\) of ATP is not altered by replacing the chloride of the buffer with acetate or by the presence of 5 mM sulfate. However, 2 mM P_i decreases the yield of E-AMP-Ub by about 50% under standard assay conditions. Therefore P_i, as well as ATP should be removed from Ub-containing samples by precipitation with trichloroacetic acid before assays are conducted. (iii) Although the enzyme has been freed of Ub-C-terminal ligand hydrolase to the extent of \(>95\%\) by the iodoacetamide treatment (20 min at 37°C in 10 mM iodoacetamide), the presence of that enzyme in the Ub sample can complicate the specificity of the assay since the hydrolase will be partially reactivated upon neutralization of the acid-precipitated sample (20) and be active during the assay. When such a possibility is suspected, the sample should be treated to denature the enzyme before the assay. This may be done without loss of Ub by heating the pellet formed in 10% (vol/vol) trichloroacetic acid at 60°C for 10 min before taking it up in assay buffer. (iv) The sensitivity of Ub to cleavage by trypsin at the Arg-74 residue (24) makes it desirable to include a suitable protease inhibitor, such as phenylmethanesulfonyl fluoride (0.1 mM), when neutral extracts that may be rich in catheptic enzymes are assayed (25). An internal standard of Ub should be added to test for recovery in such cases. The presence of isopeptidases may give false high values for free Ub. We have tried to avoid this problem by homogenizing cells in dilute acid or by using 80°C water for extraction. (v) Because Ub is readily absorbed on glass and plastic, it is important to maintain a concentration of protein \((\approx 2\ mg\ of\ bovine\ serum\ albumin\ or\ ovalbumin\ per\ ml)\) in solutions used for dilution of samples, in addition to the protein that is added to obtain a satisfactory acid precipitation. It has sometimes been found necessary to preequilibrate pipettes with solutions of Ub to be transferred.

As an alternative to centrifugation from acid to determine 3H]AMP-Ub, one may use the adsorption of isotope onto nitrocellulose equally well. The stability of AMP-Ub in acid is such that multiple samples can be kept on ice for at least 1 hr without significant loss of activity, thus allowing flexibility in doing multiple determinations.

The assay has been used to examine a number of practical questions: (i) By raising the concentration of Ub-activating enzyme in the assay, the conversion of Ub to E-AMP-Ub can be made proportionately faster than that shown in Fig. 1, which allows renaturation rates to be measured. (ii) When the complete assay mixture is used with a conjugate form of Ub such as the C-terminal amide, AMP-Ub is formed unless a suitable hydrolase is present. With low activities of added hydrolase, a coupled assay for the hydrolase, linear with time and proportional to the hydrolase, is obtained. (iii) The antibody titer to Ub has been measured by determining the decrease in assayable Ub. (iv) The assay has been used to measure the response of Ub concentration to an altered physiological state. Thus, when the temperature of a cell culture was raised from 37°C to 44°C, the free Ub level was
found to fall rapidly ($t_{1/2} \approx 3$ min) to about 25% of its level in control cells.

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