Ubiquitin-Dependent Proteolytic Pathway in Wheat Germ: Isolation of Multiple Forms of Ubiquitin-Activating Enzyme, E1†

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ABSTRACT: Ubiquitin is a highly conserved protein involved in several important regulatory processes through its ATP-dependent, covalent ligation to a variety of eukaryotic target proteins. We describe here the characterization of ubiquitin conjugation in wheat germ extracts and the subsequent isolation of enzymes involved in conjugation. With ^{125}I-ubiquitin as a substrate, wheat germ extracts form conjugates with either endogenous or added proteins. Conjugation requires ATP and has a pH optimum of ~8, and the conjugating activity is relatively stable over time. In addition, activities responsible for the ATP-dependent degradation and disassembly of ubiquitin conjugates have been detected in vitro. Ubiquitin-activating enzyme (E1) was purified from wheat germ extracts by using a modification of the covalent affinity chromatography procedure of Ciechanover et al. [(1982) *J. Biol. Chem. 267*, 2537–2542]. E1 from wheat germ, like that from rabbit reticulocytes, formed thiol ester intermediates with ubiquitin in the presence of ATP. Purified E1 preparations contained three polypeptides of apparent molecular masses of 117, 123, and 126 kDa after NaDodSO$_4$–PAGE. Under nondenaturing conditions, these proteins have native molecular masses of ~115 kDa, indicating that they exist as monomers. We concluded that all three species were E1 on the basis of their coelution with E1 activity, by immunorecognition by anti-human E1 antibodies, and by the similarity of their peptide maps. Furthermore, antibodies prepared against wheat germ E1's recognized E1 from rabbit reticulocytes. All three wheat germ E1's were detected in crude extracts prepared under conditions that minimized proteolysis, suggesting that the heterogeneity of the purified E1 preparations was not the result of posthomogenization breakdown. The immunological similarity of animal and plant E1's indicates that this conjugation enzyme, like ubiquitin, has been conserved through evolution.

The highly conserved, 76 amino acid protein ubiquitin is present in eukaryotic cells in a free form and covalently bound to various intracellular proteins (Finley & Varshavsky, 1985; Hershko & Ciechanover, 1986). As a consequence of ligation, ubiquitin is involved in several regulatory processes. Best characterized of these is its role in selective protein degradation, where conjugation serves to commit proteins to degradation (Hershko & Ciechanover, 1986; Hough & Rechsteiner, 1986). Less characterized functions include the possible regulation of chromatin structure (Goldknopf & Busch, 1977), DNA repair (Jentsch et al., 1987), and cell surface recognition (Siegelman et al., 1986).

Ligation of ubiquitin to cellular proteins requires ATP and is catalyzed by a multienzyme system initially observed in rabbit reticulocyte lysates (Hershko et al., 1983). In this series of reactions, an unusual peptide bond is formed between the C-terminal glycine carboxyl group of ubiquitin and primary amino groups on the target protein. In the initial step of ligation, ubiquitin-activating enzyme (E1) adenylates the carboxy terminus of ubiquitin using ATP. The activated molecule is then attached via a thiol ester linkage to another site on E1 with the concomitant release of AMP (Haas et al., 1982). The ubiquitin moiety is next transferred by a transesterification reaction to ubiquitin carrier proteins (E2's), which comprise a family of related proteins (Pickart & Rose, 1985). Finally, ubiquitin is covalently attached to target proteins with or without the participation of a family of ubiquitin protein ligases (E3's) (Hershko et al., 1983; Lee et al., 1986). Target proteins modified with one or more ubiquitins are rapidly degraded by an ATP-dependent protease(s) specific for ubiquitin–protein conjugates, with the release of functional ubiquitin (Hough & Rechsteiner, 1986). Alternatively, ubiquitin–protein conjugates are disassembled by ubiquitin–protein lyase(s) (isopeptidases), which cleave only the isopeptide bond between ubiquitin and the target protein, leaving both proteins intact (Andersen et al., 1981; Matsui et al., 1982; Hershko et al., 1984).

To date, the enzyme reactions required for ubiquitin conjugation have been thoroughly characterized only in one specific cell type, the rabbit reticulocyte (Hershko & Ciechanover, 1986). To determine if such a reaction scheme occurs in other eukaryotic cells, we have begun to characterize the mechanisms responsible for ubiquitin conjugation in plants. ATP-dependent ubiquitin conjugation has been demonstrated in vitro for several plant species (Vierstra, 1987). However, major limitations to further dissection of these systems include (1) the extreme instability of the conjugation systems [$t_{1/2}$ = 30 min at 30 °C for oats (Vierstra, 1987)], (2) the high rates of conjugate disassembly and degradation which interfere with conjugation assays (Vierstra, 1987; Vierstra & Sullivan, 1988), and (3) the presence of proteolytic activities that rapidly inactivate ubiquitin by removing C-terminal amino acids (Vierstra et al., 1985). The presence of these proteases is especially problematic because it precludes the use of ubiquitin affinity chromatography for purification of conjugation enzymes (Ciechanover et al., 1982). We describe here a highly active in vitro conjugation system from wheat germ that lacks these detrimental characteristics. Moreover, we have purified ubiquitin-activating enzyme, E1, from wheat germ extracts,

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and we have identified multiple forms of this enzyme.

Materials and Methods

Reagents. Human ubiquitin was purified according to the method of Haas and Wilkinson (1985). Human ubiquitin and egg white lysozyme were radiolabeled with ^{125}I by the chloramine T method (Ciechanover et al., 1980). Carrier-free $Na^{125}I$ (5.6×10^8 Bq/μg) was from Amersham. The initial specific radioactivity of ^{125}I-ubiquitin was $(3.0–14) \times 10^3$ cpm/pmol and $(1.6–3.1) \times 10^4$ cpm/pmol for ^{125}I-lysozyme. Bovine ubiquitin, leupeptin, hemin, hexokinase, phosphocreatine kinase, and inorganic pyrophosphatase were purchased from Sigma.

Biological Materials. Untoasted wheat germ (*Triticum vulgare*) was a gift of General Mills (Minneapolis, MN) and was stored at 4 °C. Wheat germ was ground to a fine powder at liquid N_2 temperatures and mixed with 50 mM Tris-HCl, 1 mM Na_2EDTA, 300 mM sucrose, and 14 mM 2-mercaptoethanol (pH 8.1, 4 °C) (5 mL/g of wheat germ) for 1 min. The extract was centrifuged at 35000*g* for 10 min, and the resulting supernatant was filtered through four layers of cheesecloth and centrifuged again to yield a clarified crude extract. Oat crude extracts were prepared from 4–5-day-old etiolated seedlings (*Avena sativa* [L.] cv. "Garry") according to Vierstra et al. (1985), using the buffer described above. Fraction II from ATP-depleted rabbit reticulocytes was prepared as described by Hershko et al. (1983).

Characterization of Wheat Germ Extracts. Proteolytic inactivation of ubiquitin was detected by the loss of antigenic recognition of the protein subjected to immunoblot analysis with anti-human ubiquitin antibodies specific for the C terminus (Haas et al., 1985; Vierstra et al., 1985). Human ubiquitin (final concentration 17 mg/mL) was added to either trypsin (8 μg/mL), wheat germ extracts, or oat extracts. The reaction mixtures were incubated with or without 200 μM leupeptin, and at various times, aliquots were boiled in an equal volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE)[1] sample buffer (Vierstra et al., 1985). Samples were subjected to $NaDodSO_4$–PAGE (Laemmli, 1970), and either gels were stained with Coomassie Blue or protein was transferred onto nitrocellulose and immunoblotted with anti-human ubiquitin antibodies.

Ubiquitin conjugation activity was assayed in 30-μL reactions containing 20 μL of clarified crude extract and 6 μL of ^{125}I-ubiquitin (0.5 μg) (Vierstra, 1987). For ATP-dependent activity, 3 μL of 2 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM creatine phosphate, and 50 mM Tris (pH 7.6, 25 °C) and 1 μL (1 unit/μL) of phosphocreatine kinase (ATP and ATP-regenerating system) were added. For ATP-independent activity, 3 μL of 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM deoxyglucose, and 50 mM Tris (pH 7.6, 25 °C) and 1 μL (1 unit/μL) of hexokinase (ATP-depleting system) were added. Reaction mixtures were incubated at 30 °C, and at designated intervals, reactions were terminated by boiling in an equal volume of $NaDodSO_4$–PAGE sample buffer and subjected to $NaDodSO_4$–PAGE. The gels were subsequently stained with Coomassie Blue, dried between two sheets of cellophane, and used for autoradiography. Quantitation of ubiquitin conjugates was accomplished by liquid scintillation counting slices of individual gel lanes after removing the lower portion of the gel containing free ubiquitin (as determined by autoradiography) (Vierstra, 1987).

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; $LiDodSO_4$, lithium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

For conjugate-specific protease and lyase experiments, crude wheat germ extracts were prepared as above except the buffer to tissue ratio was 7.5 mL/g of wheat germ. Ubiquitin–^{125}I-lysozyme conjugates were synthesized by using a modification of the method of Hough and Rechsteiner (1986) as described by Vierstra and Sullivan (1988). Reaction mixtures contained 10 μL of clarified crude extract, 3 μL of either ATP and ATP-regenerating system or ATP-depleting system, 10 μL of ubiquitin–^{125}I-lysozyme conjugates, and 6 μL of either 50 mM Tris (pH 7.6, 25 °C) or 200 μM hemin (in 50 mM Tris). Reactions were incubated at 30 °C, and at various times, two aliquots were removed. One aliquot was boiled in an equal volume of $NaDodSO_4$–PAGE sample buffer and subjected to $NaDodSO_4$–PAGE. The gels were prepared for autoradiography as described above. The other aliquot was mixed with 10 volumes of 10% (w/v) trichloroacetic acid, incubated on ice for 20 min, and centrifuged. The resulting pellet was resuspended in 1 N NaOH, and the radioactivity in both the pellet and supernatant was measured by liquid scintillation counting.

Enzyme Purification. E1 was purified from wheat germ by a modification of the covalent affinity procedure of Ciechanover et al. (1982). Twenty grams of powdered wheat germ was extracted (7.5 mL/g) in 50 mM Tris, 1 mM Na_2EDTA, 300 mM sucrose, 50 mM $(NH_4)_2SO_4$, and 14 mM 2-mercaptoethanol (pH 8.0, 4 °C). The extract was made 0.05% (v/v) poly(ethylenimine) (10% stock, pH 7.8, 25 °C) and clarified twice at 35000*g* for 10 min. Protein that precipitated between the additions of 0.2 g/mL and 0.3 g of $(NH_4)_2SO_4$/mL was collected by centrifugation at 29000*g*. The precipitate was resuspended in 6 mL of 10 mM Tris and 1 mM dithioerythritol (pH 8.0, 4 °C) and desalted on a 2.5 cm × 50 cm column of Sephadex G-25 equilibrated with the resuspension buffer. The excluded volume (pH 8.0, 4 °C) was subsequently applied to a 3.5 cm × 4.5 cm column of DE-52 (Whatman) equilibrated with the resuspension buffer. The column was washed with 2–3 column volumes of the resuspension buffer, and protein was eluted with 3 column volumes of 10 mM Tris, 1 mM dithioerythritol, and 300 mM KCl (pH 7.4, 4 °C). The concentration of Tris in the eluate was made to 100 mM and the pH adjusted to 8.0 (4 °C). Protein was precipitated with 0.56 g of $(NH_4)_2SO_4$/mL, collected at 29000*g*, then resuspended in 8 mL of 50 mM Tris and 0.5 mM dithioerythritol (pH 8.0, 25 °C), and dialyzed (4 °C) against the same buffer. The resultant dialysate was adjusted to 2 mM ATP, 5 mM $MgCl_2$, and 10 mM creatine phosphate and clarified, and the pH was adjusted to 8.0 (25 °C). Phosphocreatine kinase (1 unit/mL) was added to the dialysate and applied at room temperature to a 20-mL column of bovine ubiquitin coupled to Affigel 10 (2–5 mg/mL beads) (Haas et al., 1985) equilibrated with 50 mM Tris, 2 mM ATP, 5 mM $MgCl_2$, and 0.1 mM dithioerythritol (pH 8.0, 25 °C). The column was washed with 1 column volume of the equilibration buffer followed by 2–3 volumes of 50 mM Tris and 500 mM KCl (pH 8.0, 25 °C). Protein was eluted with 1 column volume of 50 mM Tris and 10 mM dithioerythritol (pH 9.0, 25 °C). Fractions containing E1 were pooled, concentrated with Centricon-10 microconcentrators (Amicon), and applied to a Bio-Gel A-1.5m column (2.5 cm × 96 cm) equilibrated with 50 mM Tris and 0.5 mM dithioerythritol (pH 8.0, 4 °C). Fractions containing E1 were pooled and concentrated as above and stored at −80 °C. Rabbit reticulocyte E1 was purified from fraction II by the covalent affinity chromatography procedure of Ciechanover et al. (1982), as modified by Haas et al. (1985).

Assay for Ubiquitin–E1 Thiol Ester Linkage. Reaction mixtures (20-μL total volume) containing E1 (5–13 μL), 0.5 μg (5 μL) of ^{125}I-ubiquitin, 2 μL of 50 mM Tris, 100 mM $MgCl_2$, 10 mM ATP, and 1 mM dithioerythritol (pH 7.6, 25 °C), and 1 unit (1 unit/μL) of inorganic pyrophosphatase were incubated at 30 °C for 1 min (Haas et al., 1982). The reactions were terminated either by boiling the samples for 10 min in 25 mM Tris, 5% (v/v) glycerol, 4% (w/v) $LiDodSO_4$, and 4% (v/v) 2-mercaptoethanol or by incubating the samples at 30 °C for 15 min in the above buffer containing 4 M urea instead of 2-mercaptoethanol. Samples were subjected to $NaDodSO_4$–PAGE at 4 °C, stained with Coomassie Blue, and used for autoradiography as described above.

Immunological Methods. Antisera were prepared in BALB/c mice against wheat germ E1 purified through the Bio-Gel A-1.5m column. The sample (150 μg of protein) was boiled in 1% (w/v) $NaDodSO_4$, mixed 1:1 with complete Freund's adjuvant, and injected into the interperitoneal cavity. A second immunization (100 μg of denatured protein 1:1 with incomplete Freund's adjuvant) was given after 2 weeks, and mice were bled 1 week later. Mouse serum was diluted 1:1000 before use. Rabbit anti-ubiquitin antibodies were prepared against either human or oat ubiquitin cross-linked to bovine γ-globulin (Hershko et al., 1982; Haas et al., 1985). Cross-linked antigen was boiled in 1% (w/v) $NaDodSO_4$ before injection. Rabbit anti-human E1 antibodies were prepared against native E1 purified from human erythrocytes. Immunoblot analysis was performed according to the procedure of Vierstra et al. (1985) using alkaline phosphatase linked, goat anti-mouse, or goat anti-rabbit immunoglobulins (Kirkegaard & Perry) in conjunction with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Membranes were autoclaved for 25 min immediately after transfer and before blocking (Swerdlow et al., 1986).

Peptide Mapping of Wheat Germ E1. Purified wheat germ E1's were subjected to peptide mapping as described by Cleveland (1983). Wheat germ E1's were purified through the Bio-Gel A-1.5m column and then subjected to $NaDodSO_4$–PAGE. The gel was stained with Coomassie Blue, and the regions of the gel containing the 123- and 126-kDa species, and the 117-kDa species, were excised and equilibrated in 125 mM Tris, pH 6.8, 0.1% (w/v) $NaDodSO_4$, 0.3% (v/v) 2-mercaptoethanol, 1 mM Na_4EDTA, and 10% (v/v) glycerol. The gel slices were loaded into sample wells of a 1-cm 4.5% acrylamide stacking gel with a 15% $NaDodSO_4$–acrylamide resolving gel. The slices were overlayed with 10 μL of buffer [125 mM Tris, pH 6.8, 0.1% (w/v) $NaDodSO_4$, 0.3% (v/v) 2-mercaptoethanol, 1 mM Na_4EDTA, and 20% (v/v) glycerol] and 10 μL (200 ng) of *Staphylococcus aureus* V8 protease. The samples were electrophoresed at 25 mA/0.15-cm gel until completely stacked; the voltage was turned off for 30 min, and the voltage was then reapplied at 35 mA/0.15–cm gel until resolved. The gel was prepared for immunoblot analysis as described above.

Results and Discussion

ATP-dependent conjugation of ubiquitin to a variety of proteins has been demonstrated in vitro for several plant species (Vierstra, 1987). Whereas extracts from etiolated wheat seedlings displayed little conjugation activity (Vierstra, 1987), extracts from wheat germ contained a highly active ubiquitin conjugation system. The wheat germ system effectively utilized both endogenous substrates (Figure 1, lane C) and added lysozyme (Figure 1, lanes D and E). Up to 50% of the ^{125}I-labeled human ubiquitin became conjugated in the presence of ATP. Regardless of the substrate, an array of conjugates

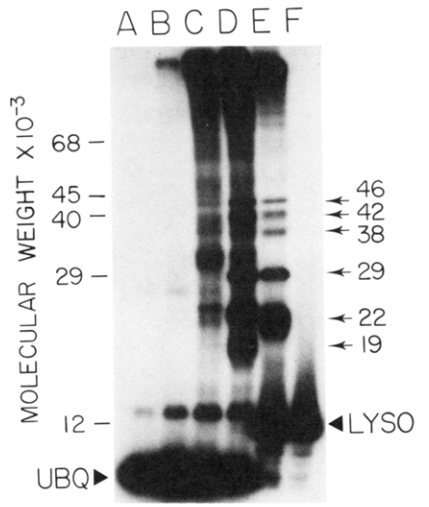

FIGURE 1: Detection of ATP-dependent ubiquitin conjugation in a crude extract from wheat germ. A crude wheat germ extract was added to reaction mixtures containing either (lane B) ^{125}I-ubiquitin and ATP-depleting system, (lane C) ^{125}I-ubiquitin, ATP, and ATP-regenerating system, (lane D) ^{125}I-ubiquitin, ATP, and ATP-regenerating system and unlabeled lysozyme, or (lane E) unlabeled ubiquitin, ATP, ATP-regenerating system, and ^{125}I-lysozyme. The reaction mixtures were incubated for 90 min at 30 °C and terminated by boiling in an equal volume of $NaDodSO_4$–PAGE sample buffer. The samples were subjected to $NaDodSO_4$–PAGE in a 13.5% acrylamide gel, followed by autoradiography. Lanes A and F contain ^{125}I-ubiquitin and ^{125}I-lysozyme alone, respectively (large arrowheads). Small arrows to the right indicate the apparent molecular masses of several ubiquitin–lysozyme conjugates.

was formed, some having high molecular mass (>100 kDa). With lysozyme as the substrate, six low molecular mass conjugates were synthesized, ranging in size from 19 to 46 kDa. These conjugates appear similar to those formed in rabbit reticulocyte lysates, where they represent the conjugation of individual ubiquitins to each of the six lysine residues of the lysozyme (Hershko & Heller, 1985; Hough & Rechsteiner, 1986). Conjugates with apparent molecular mass greater than 65 kDa probably represent the ligation of ubiquitin to ubiquitin moieties already coupled to lysozyme (Hershko & Heller, 1985).

Conjugation activity in wheat germ extracts was relatively stable. Wheat germ extracts preincubated at 30 °C for 1 h before addition of ATP and ubiquitin completely retained the ability to ligate ubiquitin (data not shown). As a consequence of this stability, conjugates accumulated for at least 2 h at 30 °C in the presence of ATP (Figure 2). The wheat germ in vitro system represents one of the best such systems in plants (Vierstra, 1987; data not shown), though the kinetics of conjugate accumulation in wheat germ extracts are several times slower than those observed in rabbit reticulocyte lysates (Ciechanover et al., 1980). The addition of leupeptin or hemin [both necessary for maximal conjugate accumulation in oat extracts (Vierstra, 1987; Vierstra & Sullivan, 1988)] did not substantially alter the kinetics or level of conjugate accumulation (Figure 2) in wheat germ extracts. The failure of hemin, a potent inhibitor of conjugate degradation and disassembly (Haas & Rose, 1981; Vierstra & Sullivan, 1988), to affect conjugate levels suggested that degradation and (or) disassembly proceeded more slowly relative to conjugation. In vitro conjugation exhibited a slightly alkaline pH optimum (~8), decreasing to 50% at pH 7 on the acidic side of the optimum and to 50% at pH 10 on the basic side (Figure 3).

To detect disassembly and (or) degradation of ubiquitin conjugates in wheat germ extracts, ubiquitin–^{125}I-lysozyme conjugates were synthesized in vitro (Vierstra & Sullivan,

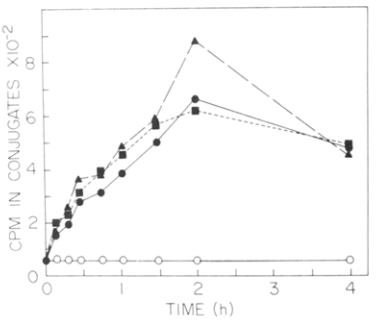

FIGURE 2: Kinetics of ubiquitin conjugation in a crude extract from wheat germ. A crude extract was prepared from wheat germ with (○, ●, ▲) or without (■) leupeptin. The extract was added to ^{125}I-ubiquitin and either (○) ATP-depleting system, (●, ■) ATP and ATP-regenerating system, or (▲) ATP, ATP-regenerating system, and 200 μM hemin. The reaction mixtures were incubated at 30 °C, and at various times, aliquots were boiled in an equal volume of NaDodSO$_4$–PAGE sample buffer. The samples were subjected to NaDodSO$_4$–PAGE using a 13.5% acrylamide gel, and the amount of free ubiquitin converted into the conjugated form was determined by scintillation counting of slices of individual gel lanes above free ubiquitin (see Materials and Methods).

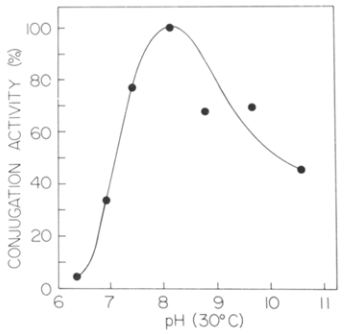

FIGURE 3: pH optimum for the ATP-stimulated synthesis of ubiquitin–protein conjugates in crude extracts from wheat germ. The extract was prepared in 50 mM Tris, 50 mM PIPES, 300 mM sucrose, 1 mM Na$_2$EDTA, and 14 mM 2-mercaptoethanol (pH 8.0, 4 °C). Crude extracts were adjusted to the desired pH at 0 °C. Aliquots were added to a mixture containing ^{125}I-ubiquitin, ATP, and ATP-regenerating system. The reaction mixtures were incubated at 30 °C for 30 min and terminated by boiling in NaDodSO$_4$–PAGE sample buffer. The amount of ubiquitin converted into the conjugated form at the various pHs was determined as in Figure 3 and expressed relative to the value obtained with the extract at pH 8.0.

1988) and then added to the extracts. Disassembly (ubiquitin–protein lyase activity) was measured by the conversion of conjugates into free ^{125}I-lysozyme, and degradation (ubiquitin–conjugate protease activity) was measured by conversion of conjugates into TCA-soluble ^{125}I. Both activities were observed in wheat germ extracts (Figure 4). Conjugate disassembly did not require ATP, was inhibited by 200 μM hemin, and accounted for ~90% of the loss of ubiquitin–^{125}I-lysozyme conjugates (Figure 4). The rate of disassembly was apparently faster without ATP than with ATP. This likely resulted from high rates of lysozyme reconjugation occurring in the presence of ATP. The remaining ~10% of conjugates were degraded by a conjugate-specific protease(s). The protease activity was also inhibited by hemin but required ATP, as does a similar protease characterized from rabbits (Hough & Rechsteiner, 1987) (Figure 4). Acid-soluble products were generated by the protease for only a short time (30 min) using this assay (Figure 4, lower panel).

The accumulation of ubiquitin conjugates in vitro may also be affected by the action of a protease(s) which rapidly inactivate(s) ubiquitin by proteolytic cleavage of the C-terminal glycine residues of ubiquitin (Vierstra et al., 1985). Inactivation is detected by using antibodies that preferentially recognize the C-terminus of ubiquitin (Haas et al., 1985; Vierstra et al., 1985). Loss of the C-terminus significantly decreases immunorecognition by these antibodies, as is demonstrated following the removal of the C-terminus of ubiquitin with either trypsin or a leupeptin-sensitive, naturally occurring oat protease(s) (Figure 5). In these experiments, the detection of ubiquitin by immunoblot analysis is diminished substantially within 1 h, even though no change is observed in the size or concentration of the protein. Conversely, ubiquitin is stable in wheat germ extracts with no loss of immunorecognition following incubation for up to 2 h (Figure 5).

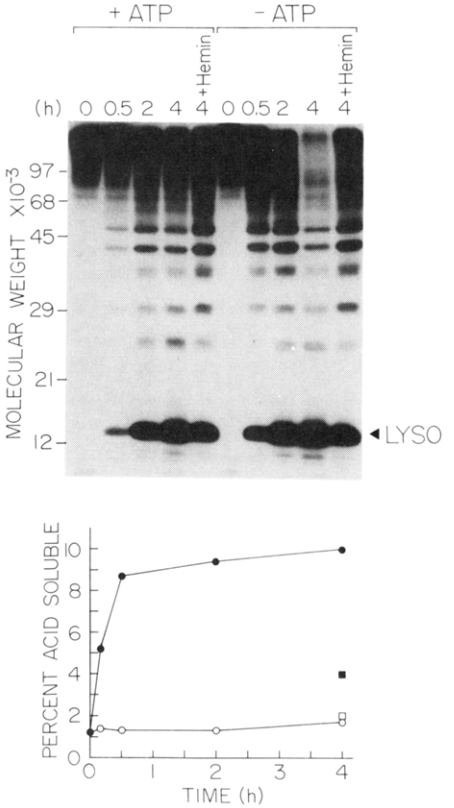

FIGURE 4: Detection of activities responsible for the disassembly and ATP-dependent degradation of ubiquitin–protein conjugates in crude extracts from wheat germ. Crude wheat germ extracts with (■, □) or without (●, ○) 200 μM hemin were added to a reaction mixture containing purified ubiquitin–^{125}I-lysozyme conjugates and either ATP and ATP-regenerating system (closed symbols) or ATP-depleting system (open symbols). The reactions were incubated at 30 °C, and at various times, aliquots were subjected to either TCA precipitation or NaDodSO$_4$–PAGE in a 13.5% acrylamide gel followed by autoradiography. Disassembly was detected electrophoretically by the conversion of ubiquitin–^{125}I-lysozyme conjugates into lower apparent molecular mass species (<69 kDa, >14 kDa) and, finally, to free ^{125}I-lysozyme (LYSO) (autoradiogram, upper panel). Degradation of ubiquitin–^{125}I-lysozyme conjugates to free ^{125}I was quantitated by the generation of acid-soluble radioactivity (graph, lower panel).

It was possible to isolate ubiquitin-activating enzyme (E1) from wheat germ as a consequence of both the stability and activity of the conjugation system, and the lack of protease(s) that inactivate(s) ubiquitin. The absence of proteolytic activity enabled us to use the ubiquitin covalent affinity chromatography procedure of Ciechanover et al. (1982) as a major purification step (Figure 6). By use of the procedure outlined here, approximately 3 mg of purified E1 could be recovered from 100 g of wheat germ. An estimation of the overall yield of the enzyme was not possible because we were unable to quantitate directly the amount of E1 in crude extracts.

Three major proteins of apparent molecular masses 117, 123, and 126 kDa (on NaDodSO$_4$–PAGE) were purified by

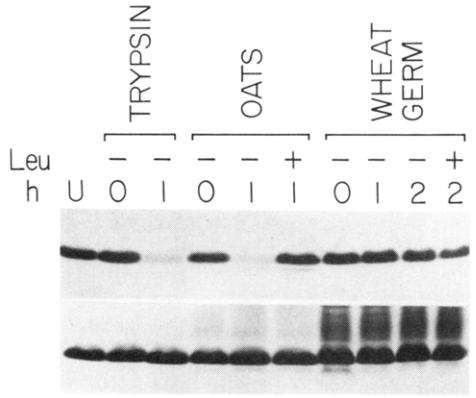

FIGURE 5: Proteolytic stability of ubiquitin in crude extracts from wheat germ. The extent of proteolysis was measured after various incubations by the loss of antigenic reactivity of ubiquitin as visualized by immunoblotting with anti-ubiquitin antibodies. These antibodies were specific for the C-terminus, such that proteolytic removal of this domain resulted in substantially less immunorecognition. Human ubiquitin was added either to trypsin or to a freshly prepared crude extract from either etiolated oat seedlings or wheat germ. The reaction mixtures were incubated at 30 °C with or without the addition of 200 μM leupeptin (Leu). At various time intervals, aliquots were boiled in an equal volume of $NaDodSO_4$–PAGE sample buffer. The samples were subjected to $NaDodSO_4$–PAGE in a 15% acrylamide gel, and protein was either stained with Coomassie Blue (lower panel) or transferred to nitrocellulose and immunoblotted with anti-human ubiquitin antibodies (upper panel). Equal volumes of reaction mixtures were applied to each lane, 10 μL for the Coomassie-stained gel and 5 μL for the immunoblot. U = human ubiquitin.

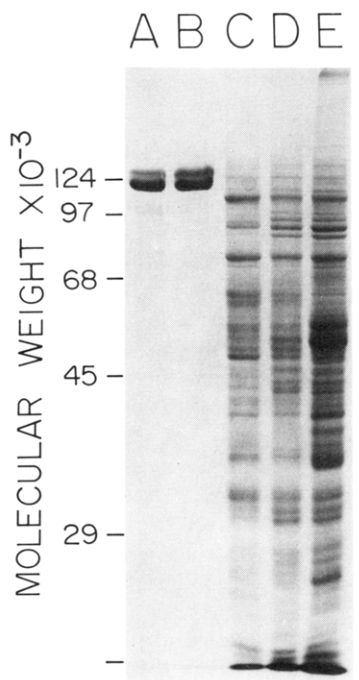

FIGURE 6: Electrophoretic analysis of various fractions during the purification of E1 from wheat germ: (lane A) E1-containing pool eluted from the Bio-Gel A-1.5m column; (lane B) proteins eluted from the ubiquitin covalent affinity column; (lane C) DE-52 eluate; (lane D) proteins precipitated from the clarified crude extract between additions of 0.2 g/mL and 0.3 g of $(NH_4)_2SO_4$/mL; (lane E) the clarified crude extract following poly(ethylenimine) precipitation. $NaDodSO_4$–PAGE was accomplished in a 10% acrylamide gel.

using ubiquitin covalent affinity chromatography (Figure 6). These were similar in mass to reticulocyte E1 (105–110 kDa) (Ciechanover et al., 1982). The three proteins coeluted during nondenaturing gel filtration with an estimated molecular mass of 115 kDa, suggesting they existed as monomers (data not shown). All three proteins also coeluted with an activity

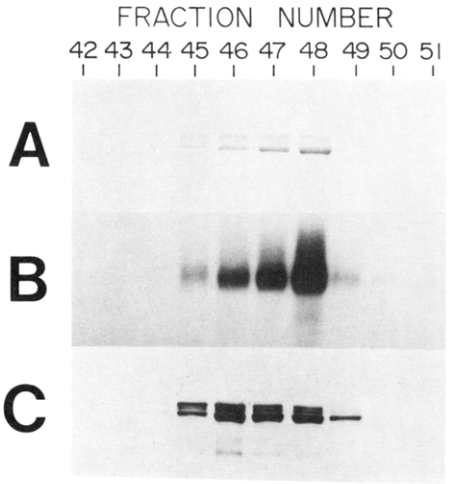

FIGURE 7: Size-exclusion chromatography of E1 purified from wheat germ. E1 was purified through the ubiquitin covalent affinity step and was applied to a 2.5 cm × 96 cm Bio-Gel A-1.5m column. Five-milliliter fractions were collected. An aliquot of each fraction indicated was subjected to $NaDodSO_4$–PAGE and stained with Coomassie (panel A) and tested for its ability to form a thiol ester linkage with ubiquitin in the presence of ATP (as described in Figure 8, panel B). An additional aliquot of each fraction indicated was subjected to $NaDodSO_4$–PAGE at 25 °C, and immunoblot analysis was performed with antisera against wheat germ E1 (panel C) as described under Materials and Methods. $NaDodSO_4$–PAGE was carried out using 8% acrylamide gels.

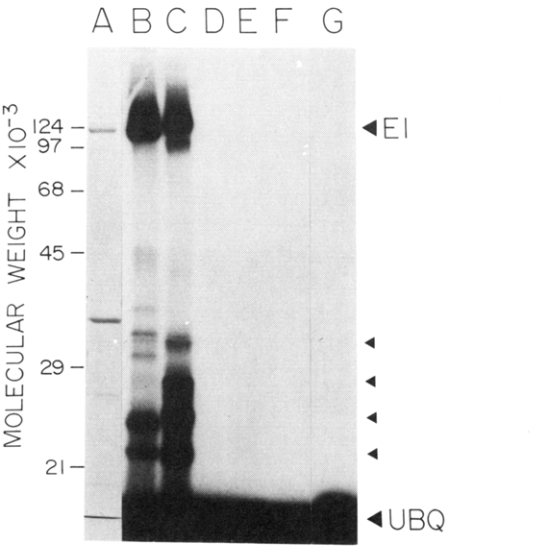

FIGURE 8: Electrophoretic detection of ubiquitin bound to purified wheat germ E1. E1 was purified from wheat germ and rabbit reticulocytes through the ubiquitin covalent affinity column step (see Materials and Methods). Reaction mixtures contained either wheat germ E1 (lanes C and E–G), reticulocyte E1 (lane B), or no E1 (lane D). ^{125}I-Ubiquitin was added to each with (lanes B–D, F, and G) or without (lane E) ATP and inorganic pyrophosphatase. Reactions were incubated at 30 °C for 1 min. They were stopped either by adding an equal volume of $LiDodSO_4$–PAGE sample buffer containing 4 M urea and then incubating at 30 °C for 15 min to maintain the thiol ester linkage (lanes B–E) or by boiling for 10 min in sample buffer containing 4% (v/v) 2-mercaptoethanol to disrupt the thiol ester linkage (lane G). After 1 min, the reaction in lane F was made 1 M hydroxylamine (pH 6.1) and then processed with sample buffer containing 4 M urea. Samples were then subjected to $NaDodSO_4$–PAGE at 4 °C using a 10% acrylamide gel. The gel was stained with Coomassie Blue and used for autoradiography (lanes B–G). Lane A represents the Coomassie staining of lane G. Large arrowheads indicate the position of E1–ubiquitin adducts and small arrowheads the position of E2–ubiquitin adducts.

characteristic of E1 involving the ATP-dependent formation of a thiol ester linkage with ^{125}I-ubiquitin (Figure 7, panel B).

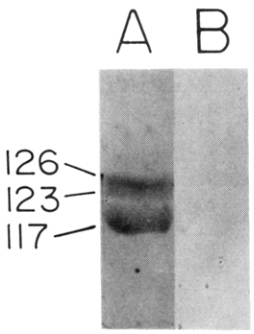

FIGURE 9: Immunological detection of wheat germ E1 by anti-human E1 antisera. Wheat germ E1 purified through the Bio-Gel A-1.5m column was subjected to $NaDodSO_4$–PAGE using a 6% acrylamide gel. Immunoblot analysis was performed using rabbit anti-human E1 antibodies as described under Materials and Methods, except nitrocellulose membranes were not autoclaved. Lane A = anti-human E1 antisera; lane B = nonimmune rabbit sera.

The attachment of ubiquitin to wheat germ E1 was sensitive to either the sulfhydryl reagent, 2-mercaptoethanol (Figure 8, lanes A and G), or 1 M hydroxylamine (pH 6.1, 25 °C) (Figure 8, lane F), demonstrating that this linkage was a thiol ester bond (Ciechanover et al., 1981). The three proteins were recognized by antibodies raised against purified human E1 (Figure 9). The relative intensity of each polypeptide on immunoblots was equivalent to that seen by protein staining, indicating that all three proteins had similar cross-reactivity with the antibodies.

The coelution of the three proteins with E1 activity and their cross-reaction with anti-human E1 antibodies suggested that these proteins were E1's. Peptide maps of the three proteins were generated by the method of Cleveland (1983) with *S. aureus* V8 protease to determine their relationship. A difference of >1% sequence divergence can be detected (Cleveland, 1983) by using this procedure. The three proteins were purified through the Bio-Gel A-1.5. column and separated into two fractions using $NaDodSO_4$–PAGE, the upper fraction containing the 123- and 126-kDa species and the lower fraction containing the 117-kDa species. The two higher molecular mass species could not be adequately resolved by electrophoresis and therefore were analyzed together. Subsequent reelectrophoresis of these fractions in the absence of protease indicated that the relative mobilities of the proteins were unaffected by the initial separation (data not shown). The partial digests of the lower and upper fractions yielded an array of peptide fragments (Figure 10). The peptide maps of the two fractions were indistinguishable, indicating that the proteins in the two fractions are closely related. Since the amount of the 123-kDa species was low relative to the 126-kDa species, it was possible that its peptide map could be unique from the 126- and the 117-kDa species but be below immunoblot detection. However, we were unable to detect any minor patterns even after increasing the amount of protein and overdeveloping the immunoblots.

On the basis of the results of these experiments, we have concluded that all three proteins represent structurally related forms of E1. In addition to E1, four lower molecular mass proteins were purified by using the ubiquitin affinity column, and these bound ^{125}I-ubiquitin only in the presence of E1 (Figure 8; Sullivan and Vierstra, unpublished results). These proteins likely represent E2's, which in reticulocyte lysates copurify with E1 during the ubiquitin covalent affinity step (Hershko et al., 1983).

Antibodies were raised in mice against the pool of Bio-Gel A-1.5m column fractions containing the three E1 proteins. Immunoblot analysis of the Bio-Gel A-1.5m fractions with the

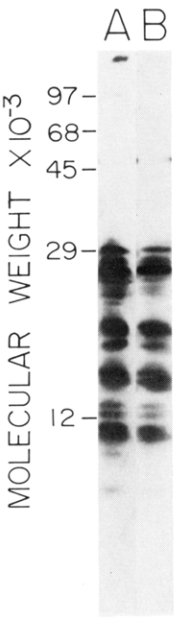

FIGURE 10: Immunoblot analysis of peptides generated by *S. aureus* V8 protease digestion of wheat germ E1. Wheat germ E1 was purified through the Bio-Gel A-1.5m column and subjected to $NaDodSO_4$–PAGE using a 4.5% acrylamide gel. The gel was stained with Coomassie Blue, and the regions of the gel containing the two, higher molecular mass E1 species (123 and 126 kDa) and the lower molecular mass E1 species (117 kDa) were excised. The proteins were digested with *S. aureus* V8 protease (200 ng) (Cleveland, 1983) and subjected to electrophoresis in a 15% $NaDodSO_4$–acrylamide gel. Peptides were visualized by immunoblot analysis with anti-wheat germ E1 antibodies. Lane A = 117-kDa E1 species; lane B = 123- and 126-kDa E1 species combined.

resultant antibodies indicated that all the immunoreactive species coeluted with fractions containing active E1 (Figure 7, panel C). In addition to recognition of wheat germ E1 by anti-human E1 antibodies, anti-wheat germ E1 antibodies recognized E1 from rabbit reticulocytes. Coomassie-stained gels of rabbit E1 purified by ubiquitin covalent affinity chromatography (as above) indicated a single, major species of 112 kDa (data not shown), whereas multiple species were observed by immunoblot analysis (Figure 11, lane C).

The three proteins observed in our E1 preparations could be the result of in vitro proteolytic modification of the native form into lower molecular mass species, or different isoforms of E1. To address the possibility of proteolytic modification, wheat germ anti-E1 antibodies were used to probe immunoblots of wheat germ extracts prepared under conditions that minimized postextraction proteolysis [i.e., extraction of tissue directly into boiling $NaDodSO_4$–PAGE sample buffer (Vierstra & Quail, 1982)]. Using this procedure, we detected three species with mobilities identical with those of our purified E1 preparations (Figure 11). Thus, the observed heterogeneity does not appear to be the result of limited proteolytic degradation during extraction.

The alternate forms of E1 may be a consequence of the amphidiploid nature of bread wheat, which evolved from the combination of three diploid progenitors (A, B, and D genomes) (Jones et al., 1982). Thus, each genome could contribute one species of E1 to modern wheat. However, multiple E1 species were also detected by immunoblot analysis of rapidly prepared extracts from green leaf tissue of *Triticum urartu*, *Aegilops speltoides*, and *Aegilops squarosa* [potential A, B, and D genome progenitors, respectively (Jones et al., 1982)] (data not shown). Multiple bands were observed also in other plant species by immunoblot analysis with anti-wheat germ E1 antibodies, including *Avena sativa*, *Zea mays*, and

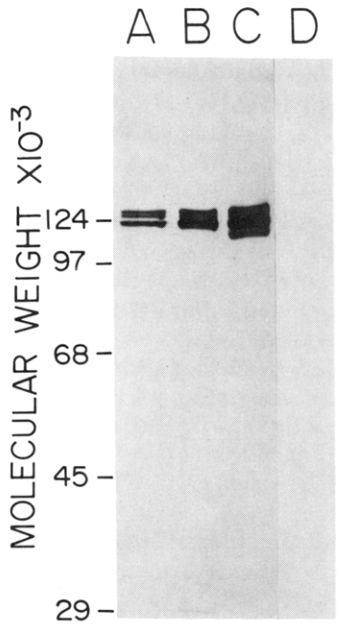

FIGURE 11: Immunological detection of E1 by anti-wheat germ E1 antisera. Samples were subjected to NaDodSO$_4$–PAGE using an 8% acrylamide gel and immunoblotted either with antisera prepared against E1 purified from wheat germ (lanes A–C) or with preimmune serum (lane D). Samples include (lanes A and D) a crude wheat germ extract rapidly prepared by boiling wheat germ directly in NaDodSO$_4$–PAGE sample buffer, (lane B) E1 purified from wheat germ, and (lane C) E1 purified from rabbit reticulocytes.

Arabidopsis thaliana (data not shown), indicating that multiple forms of E1 are common among higher plants. None of the wheat germ E1's were recognized by anti-oat ubiquitin antibodies, discounting the possibility that heterogeneity results from ubiquitination of a single E1 species (data not shown).

Conclusions

In this study, we show that wheat germ extracts contain a highly active ubiquitin conjugation system similar to the in vitro system derived from rabbit reticulocytes (Ciechanover et al., 1980; Hershko et al., 1983, 1984) and other higher plants (Vierstra, 1987). Wheat germ extracts can incorporate 40–50% of radiolabeled ubiquitin into higher molecular weight conjugates, approximately 6–10 times more activity than is observed with oat extracts (Vierstra, 1987). The high activity in wheat germ, as compared with oats, appears to result from several factors. First, wheat germ extracts do not possess proteolytic activities that inactivate ubiquitin. Second, the conjugation system in wheat germ is more stable, with full activity remaining after 1 h at 30 °C. In oats, conjugation activity is extremely unstable, with a $t_{1/2}$ of 30 min at 30 °C (Vierstra, 1987). Third, lyase activity in wheat germ extracts is significantly less than in oat extracts (Vierstra & Sullivan, 1988), resulting in less conjugate disassembly during conjugation assays. These attributes make wheat germ a convenient source for synthesizing ubiquitin conjugates in vitro, and for isolating the enzymes involved.

Conjugate accumulation exhibits an alkaline pH optimum in wheat germ extracts, with little accumulation under more acidic conditions. Several major proteolytic activities are associated with the acidic vacuole of higher plants, leading to the suggestion that the vacuole is a major site of protein degradation (Matile, 1982). The lack of conjugation activity at a low pH corresponding to that of the vacuole in vivo (Roberts et al., 1980) diminishes the possibility that the ubiquitin conjugation system is localized in this compartment.

The purification protocol initially outlined for rabbit reticulocytes (Ciechanover et al., 1982) is suitable for the purification of E1 from wheat germ with minor modifications. Three major protein species were purified. The data are consistent with the interpretation that all three are isoforms of E1, especially the observations that they are recognized by anti-human E1 antibodies and they have indistinguishable peptide maps. The nature of this heterogeneity is unknown but is likely not the result of posthomogenization proteolysis, the polyploid nature of wheat, or ubiquitin conjugation. Initial studies with rabbit E1 did not indicate the presence of multiple E1's. However, our data here (Figure 11), as well as more recent studies by Ciechanover (personal communication), demonstrate that multiple E1's may exist in rabbits as well. The two other components of the conjugation system, E2 and E3, have been shown to have multiple forms and (or) activities (Pickart & Rose, 1985; Jentsch et al., 1987; Lee et al., 1986). It is reasonable, therefore, that heterogeneity would also exist with E1. This heterogeneity may reflect different functions and (or) locations of the enzyme.

Amino acid sequence analysis of ubiquitin has shown that this protein is extremely conserved among several species. The observation that antibodies to animal E1's cross-react with wheat germ E1 and vice versa indicates that E1's from these species also share substantial homology. Future studies will determine whether this structural conservation through evolution applies as well to the other enzymes involved in ubiquitin conjugation.

Acknowledgments

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Registry No. Ubiquitin, 60267-61-0.

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Thermal Stability of RNA Hairpins Containing a Four-Membered Loop and a Bulge Nucleotide†

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ABSTRACT: Fourteen RNA hairpins containing a four-membered loop and a bulge nucleotide were synthesized and their thermal stabilities determined. The combined contribution of a four-membered loop and bulge A to the free energy of a hairpin is calculated to be 9.3 kcal/mol at 37 °C and successfully predicts the stability of an independent RNA hairpin. The introduction of a bulge nucleotide to the helical stem of an RNA hairpin destabilizes the molecule in a sequence-dependent manner. The individual thermodynamic contributions of a four-membered loop and bulge A, G, and U residues to the stability of an RNA hairpin loop are presented.

Since they were first published, the thermodynamic parameters for RNA secondary structure stability (Tinoco et al., 1971, 1973) have been improved and expanded. The contributions of RNA secondary structure features such as nearest-neighbor stacking interactions, nucleotide mismatches, and terminal unpaired nucleotides to the stability of RNA have now been well characterized (Hickey & Turner, 1985; Sugimoto et al., 1986; Freier et al., 1986) and can successfully predict the stability of a number of RNA duplexes.

Bulge nucleotides and hairpins are also common elements of RNA secondary structure. For example, bulge nucleotides appear in 12 locations in the current *Escherichia coli* 16S RNA secondary structure (Gutell et al., 1985). The same molecule contains 31 hairpins ranging in size from 3 to 11 nucleotides. Both of these structural features have been associated with the binding of proteins (Peattie et al., 1981; Mougel et al., 1986). It is quite likely that each individual hairpin and bulge has a defined conformation. Nucleotides in a loop can form defined structures as a result of stacking interactions and intraloop hydrogen bonds as seen in the anticodon loop of yeast $tRNA^{Phe}$ (Quigley & Rich, 1976). Bulge nucleotides can assume intercalated (Patel et al., 1982) and extrahelical (Morden et al., 1983) states or even an equilibrium between the two. Since the contribution of loops and bulges to RNA stability may be very sequence and neighbor dependent, it is important to determine if these structures can even be considered independent thermodynamic entities for the purpose of predicting RNA secondary structure stability.

Early attempts to characterize the stability of RNA hairpins were hampered by limitations in synthetic capabilities such that only a few simple sequences could be studied (Uhlenbeck et al., 1973; Gralla & Crothers, 1973). Although RNA hairpins have been purified from larger RNA molecules (Coutts, 1971; Gralla et al., 1974; Baan et al., 1977), their isolation depends upon the fortuitous cleavage properties of each RNA source and thus limits the range of sequences available for study. Thus, the current thermodynamic parameters for RNA hairpin loop and bulge nucleotides (Tinoco et al., 1973; Freier et al., 1986) do not reliably represent RNA sequences and structures that occur naturally.

Recent advances in the enzymatic synthesis of RNA make possible the synthesis of virtually any RNA for physical studies (Lowary et al., 1986; Milligan et al., 1987). In this work, we have synthesized 10 RNA hairpins that share the same 4-membered loop sequence and have a bulge A residue at the same nucleotide position. The helix–coil transition of these molecules was analyzed to determine the combined contribution of the four-membered loop and bulge A to the stability of a hairpin. Three additional hairpins were studied to de-

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