A Novel, Arsenite-Sensitive E2 of the Ubiquitin Pathway: Purification and Properties†

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ABSTRACT: In the multienzyme ubiquitin-dependent proteolytic pathway, conjugation of ubiquitin to target proteins serves as a signal for protein degradation. Rabbit reticulocytes possess a family of proteins, known as E2's, that form labile ubiquitin adducts by undergoing transthiolation with the ubiquitin thiol ester form of ubiquitin activating enzyme (E1). Only one E2 appears to function in ubiquitin-dependent protein degradation. The others have been postulated to function in regulatory ubiquitin conjugation. We have purified and characterized a previously undescribed E2 from rabbit reticulocytes. $E2_{230K}$ is an apparent monomer with a molecular mass of 230 kDa. The enzyme forms a labile ubiquitin adduct in the presence of E1, ubiquitin, and MgATP and catalyzes conjugation of ubiquitin to protein substrates. Exogenous protein substrates included yeast cytochrome *c* (K_m = 125 μM; $k_{cat} \sim 0.37$ min^{-1}) and histone H3 ($K_m < 1.3$ μM; $k_{cat} \sim 0.18$ min^{-1}) as well as lysozyme, α-lactalbumin, and α-casein. $E2_{230K}$ did not efficiently reconstitute Ub-dependent degradation of substrates that it conjugated, either in the absence or in the presence of the ubiquitin–protein ligase that is involved in degradation. $E2_{230K}$ may thus be an enzyme that functions in regulatory Ub conjugation. Relative to other E2's, which are very iodoacetamide sensitive, $E2_{230K}$ was more slowly inactivated by iodoacetamide (k_{obs} = 0.037 min^{-1} at 1.5 mM iodoacetamide; pH 7.0, 37 °C). $E2_{230K}$ was also unique among E2's in being subject to inactivation by inorganic arsenite (k_i^{max} = 0.12 min^{-1}; $K_{0.5}$ = 3.3 mM; pH 7.0, 37 °C). Arsenite is considered to be a reagent specific for vicinal sulfhydryl sites in proteins, and inhibition is usually rapidly reversed upon addition of competitive dithiol compounds. Inactivation of $E2_{230K}$ by arsenite was not reversed within 10 min after addition of dithiothreitol at a concentration that blocked inactivation if it was premixed with arsenite; inactivation is therefore irreversible or very slowly reversible. We postulate that a conformation change of $E2_{230K}$ may be rate-limiting for interaction of enzyme thiol groups with arsenite.

Covalent conjugation of the small, highly conserved (Goldstein et al., 1975) protein Ub[1] to cellular proteins is a posttranslational event that may serve a variety of metabolic purposes. The best understood of these is to render the target protein susceptible to degradation by a cytosolic MgATP-dependent protease [reviewed in Hershko and Ciechanover (1986)]. Three enzymes are required for the formation of degradatively competent Ub–protein conjugates: Ub activating enzyme (E1), a small Ub carrier protein (E2), and Ub–protein ligase (E3). They respectively catalyze the reactions (Hershko et al., 1983)

$$\text{E1-SH} + 2\text{ATP} + 2\text{UbC(=O)O}^- \xrightleftharpoons{Mg^{2+}} \text{E1-SC(=O)Ub·AMP-Ub} + \text{AMP} + 2\text{PP}_i \quad (1)$$

$$\text{E1-SC(=O)Ub} + \text{E2-SH} \rightleftharpoons \text{E2-SC(=O)Ub} + \text{E1-SH} \quad (2)$$

$$\text{E2-SC(=O)Ub} + \text{RNH}_2 \xrightleftharpoons{E3} \text{RN(H)C(=O)Ub} + \text{E2-SH} \quad (3)$$

Most conjugates have isopeptide structures in which carboxyl-terminal glycine residues of Ub molecules are linked to ϵ-amino groups of lysine residues of the substrate (Hershko et al., 1980) or of already conjugated Ub molecules (Hershko & Heller, 1985).

Conjugation of Ub to proteins may relate to processes other than protein degradation. Possible regulatory functions for Ub–protein conjugation are suggested by the locations, behaviors, and identities of certain Ub–protein conjugates. Thus, some cell surface proteins, which should be inaccessible to the Ub-specific protease, are Ub conjugates (Siegelman et al., 1986; Yarden et al., 1986). In addition, a subset of histone 2A and 2B molecules in the nucleus is ubiquitinated at specific lysine residues (Busch & Goldknopf, 1981; Thorne et al., 1987); the degree of ubiquitination of nucleosomes correlates with transcriptional activity of chromatin (Levinger & Varshavsky, 1982; Huang et al., 1986), with cell cycle (Matsui et al., 1979; Mueller et al., 1985), and with developmental state (Wunsch et al., 1987; Hensold et al., 1988). Finally, Ub is conjugated to at least one metabolically stable intracellular protein, actin (Ball et al., 1987).

Rabbit reticulocytes and other cells possess a number of low molecular weight E2's that can accept Ub in labile linkage from E1 (eq 2) (Hershko et al., 1983; Pickart & Rose, 1985; Jentsch et al., 1987). Only one of these, $E2_{14K}$, acts as Ub donor in E3-catalyzed conjugation (eq 3 above) (Pickart & Rose, 1985; Haas & Bright, 1988). Some of the others catalyze (E3-independent) ubiquitination of proteins such as histones (Pickart & Vella, 1988a,b; Haas et al., 1988) and cytochrome *c* (Pickart & Rose, 1985). Where metabolic roles for E2's (other than $E2_{14K}$) have been identified, they are not related to protein degradation in any obvious way: in yeast,

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[1] Abbreviations: Ub, ubiquitin; E1, Ub activating enzyme; E2, Ub carrier protein (subscript denotes subunit molecular mass in kilodaltons); E3, Ub–protein ligase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; rcmBSA, reduced and carboxymethylated bovine serum albumin.

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the DNA repair enzyme encoded by the RAD6 gene is a 20-kDa E2 (Jentsch et al., 1987), and the cell-cycle control protein encoded by the CDC34 gene is also an E2 (Goebl et al., 1988). Since the products of E2-catalyzed conjugation do not appear to be degradatively competent (Pickart & Vella, 1988a), it is possible that some E2's function in regulatory conjugation of Ub to specific intracellular substrates.

We recently presented indirect biochemical evidence for the existence of a high molecular weight E2 (approximately 200 kDa subunit), designated $E2_L$ (Pickart & Vella, 1988a). We describe the purification and characterization of this protein, now designated $E2_{230K}$. It is distinguished from other E2's by its size and relative insensitivity to iodoacetamide and in being subject to inactivation by the reagent arsenite.

Experimental Procedures

Ub was purified from outdated human blood (Haas & Wilkinson, 1985). Ub (8000 cpm/pmol) and human α-lactalbumin (Sigma) (approximately 10^6 cpm/μg) were radioiodinated by the chloramine T procedure (Hershko et al., 1980) using carrier-free iodine-125 from Amersham Corp. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Du Pont-New England Nuclear, and yeast cytochrome *c* from Sigma. The purifications of histones 2A, 3, and 4 have been described previously (Pickart & Vella, 1988b). rcmBSA was prepared as described by Evans and Wilkinson (1985).

Reticulocytosis was induced in male rabbits by phenylhydrazine treatment; fraction II was prepared as previously described (Hershko et al., 1983). Reticulocyte E1, $E2_{20K}$, and $E2_{14K}$ were purified to electrophoretic homogeneity as described previously (Haas & Bright, 1988; Pickart & Vella, 1988a). Concentrations were determined from the sizes of the [^{32}P]PP_i bursts produced in the presence of Ub and Mg[γ-^{32}P]ATP (Pickart & Rose, 1985).

Crude E3 was prepared by ammonium sulfate fractionation of fraction II. Proteins precipitating between 0 and 30% saturation (Haas & Bright, 1988) were dialyzed versus 20 mM Tris-HCl (5% base), 0.1 mM EDTA, and 0.2 mM DTT, at 5 °C. (This was the buffer used in all dialyses described below.) To inactivate Ub–protein isopeptidases present in the preparation, it was treated with iodoacetamide prior to assay (2 mM, 15 min, pH 7.2, 37 °C); iodoacetamide was quenched with 4 mM DTT (Hershko et al., 1983). Conditions for assay of E3 were the same as those for assay of $E2_{230K}$ (below), except that cytochrome *c* was replaced by β-lactoglobulin (1 mg/mL), 140 nM $E2_{14K}$ was added, and the concentration of (iodoacetamide-treated) E3 protein was 1.0 mg/mL. Assays were quenched with an equal volume of SDS–PAGE sample buffer (Pickart & Vella, 1988a) and electrophoresed on 12.5% gels prior to drying and autoradiography. Ub–protein conjugates (radiolabeled proteins migrating more slowly than free Ub) were excised and counted (LKB 1275 Minigamma counter). Conjugation occurred linearly for at least 10 min, at a rate of 0.015 μM Ub/min. Negligible conjugation occurred if E1, $E2_{14K}$, or E3 was omitted from the assay.

Reconstitution assays of Ub-dependent degradation of radioiodinated human α-lactalbumin were carried out as described previously (Pickart & Vella, 1988a), using the unadsorbed fraction from a Ub affinity column as the source of the Ub-specific protease (Hershko et al., 1984).

Purification of $E2_{230K}$. All manipulations were carried out at 5 °C. Fraction II (150–400 mg of protein) was step-fractionated on Q-Sepharose fast flow (Pharmacia LKB Biotechnology) as described previously (20 mg of protein/mL of resin; Pickart & Vella, 1988b). Proteins eluting between 0.2 and 0.3 M KCl (fraction C) were pooled, concentrated by ultrafiltration (Amicon cell), and dialyzed (above). Fraction C, and other fractions described below, could be stored at −60 °C for several weeks without loss of $E2_{230K}$ activity.

(*a*) *Gel Filtration.* Fraction C was further concentrated (Centricon 10, Amicon) to a volume of 1–2 mL. One-milliliter aliquots were run on a 1 × 50 cm column of Ultrogel AcA34 (IBF Biotechnics) that had been equilibrated with a buffer of 0.1 M Tris-HCl (10% base), 0.1 mM EDTA, 0.5 mM DTT, and 0.2 mg/mL ovalbumin. Fractions of 1 mL were collected; fractions 19–27 were assayed for $E2_{230K}$ activity (below). Peak fractions (usually fractions 20–25) were pooled and concentrated by ultrafiltration to a volume of 3–5 mL. The column (exclusion limit 350 kDa) was calibrated by using glucose oxidase (180 kDa, peak fraction 25), hemoglobin (66 kDa, peak fraction 30), and $E2_{14K}$ (25 kDa, peak fraction 36).

(*b*) *Ammonium Sulfate Fractionation.* Cold saturated ammonium sulfate solution was added slowly and with good mixing to the pooled gel filtration fractions to give 38% saturation. After 30 min, precipitated protein was collected by centrifugation at 8000*g* for 15 min. The supernatant was decanted, and 1–2 mg of ovalbumin was added to it, followed by sufficient saturated ammonium sulfate solution to bring saturation to 50%. After 30 min, precipitated protein was again collected by centrifugation; 1–2 mg of ovalbumin was added to the decanted supernatant. Each pellet (0–38% and 38–50%) was dissolved in 1–3 mL of dialysis buffer (above); dissolved pellets and 50% supernatant were dialyzed simultaneously against 1 L of buffer (two changes). Seventy to eighty percent of the $E2_{230K}$ activity was in the 38–50% fraction.

(*c*) *FPLC Anion Exchange.* The dialyzed 38–50% ammonium sulfate fraction was run on an FPLC Mono Q column (Pharmacia LKB Biotechnology) that had been equilibrated with a buffer of 50 mM Tris-HCl (5% base) and 0.5 mM DTT. The column was eluted at 1 mL/min with a 40-mL linear gradient of KCl (0–0.5 M) in the same buffer; 0.5-mL fractions were collected into plastic tubes containing carrier ovalbumin (0.2 mg/tube). Ten consecutive fractions bracketing the region where $E2_{230K}$ was expected to elute (0.35–0.37 M KCl) were individually diluted to 2.5 mL with dialysis buffer (above) and reconcentrated to 0.3–0.5 mL (Centricon 10) prior to assay. The three peak fractions were pooled and stored at −60 °C in small aliquots. $E2_{230K}$ activity decreased slowly during several months of storage under these conditions. Enzyme activity was also somewhat labile to freezing and thawing.

Standard Assay. Standard $E2_{230K}$ assays contained (10 μL, pH 7.2, 37 °C) 50 mM Tris-HCl (24% base), 5 mM $MgCl_2$, 0.6 unit/mL inorganic pyrophosphatase, 10 mM phosphocreatine, 2 mM ATP, 3 units/mL creatine kinase, 79 μM (1 mg/mL) yeast cytochrome *c*, 2 μM ^{125}I-labeled Ub, 0.1 μM E1, and 0.05–0.3 microunit of $E2_{230K}$. Assays were initiated by addition of E1 or $E2_{230K}$ and were quenched after 10 min by addition of an equal volume of SDS sample buffer. After heating to 100 °C (1 min), 15-μL aliquots were run on a 12.5% polyacrylamide gel. The gel was dried and autoradiographed; the portion of each lane corresponding to Ub–protein conjugates was excised and counted. All rates were corrected by subtraction of blanks obtained by omitting $E2_{230K}$ from the assay. Blank values were always <5% of experimental values. Appropriate controls showed that no conjugation occurred if E1 was omitted from the assay and that rates were independent of a 2-fold increase in [E1].

Assay of $E2_{230K}$–Ub Adduct Formation. Conditions were identical with those for the standard assay (above) except that

cytochrome *c* was omitted. Duplicate assays were quenched 1 min after E1 addition, in one case with 10 μL of thiolester SDS–PAGE sample buffer (lacking mercaptoethanol and containing urea; Pickart & Vella, 1988b) and in the other case with 10 μL of standard sample buffer (containing 0.43 M mercaptoethanol). Only the latter samples were heated to 100 °C. Aliquots of 15 μL were immediately electrophoresed at 5 °C (8% or 12.5% gel). The gel was dried and autoradiographed.

Kinetics of Inactivation by Arsenite. Primary incubations contained (25 μL, pH 7.0, 37 °C) 45 mM Tris-HCl (5% base), 0.1 mM EDTA, approximately 0.1 microunit/μL $E2_{230K}$, and concentrations of arsenite ranging from 0 to 3.93 mM (Figure 5A). Aliquots (4 μL) were withdrawn at timed intervals and diluted into a standard assay containing equimolar DTT (with respect to arsenite). Values of k_{obs} for loss of activity were calculated by using half-times taken from semilog plots of activity versus incubation time. To obtain k_i (specific inactivation rate constant), k_{obs} values (plus arsenite) were corrected by subtracting the value of k_{obs} in the control (lacking arsenite).

Kinetics of Inactivation by Iodoacetamide. Conditions in the primary incubation were identical with those for incubations with arsenite except that [$E2_{230K}$] and [$E2_{20K}$] were 0.05 microunit/μL and 0.5 μM, respectively, and arsenite was replaced with iodoacetamide (1.5 mM). Aliquots (4 μL) were withdrawn at timed intervals and diluted into assay mixtures containing 4 mM DTT and 6.4 μM H3 as substrate; after 5 min, assays were initiated with E1. Loss of activity in the primary incubations followed pseudo-first-order kinetics. Specific inactivation rate constants were obtained by subtracting the rate constant for loss of activity in the control lacking iodoacetamide.

Other Methods. Protein concentrations were determined with Bio-Rad dye reagent concentrate, using bovine serum albumin as standard. SDS–PAGE was carried out in mini gels (8%, 10%, or 12.5% acrylamide) by the discontinuous slab procedure of Laemmli (1970). Dried gels were autoradiographed at −60 °C by using Du Pont Cronex screens and Kodak XAR-5 film. Quantitative densitometry was carried out by using an LKB Ultroscan XL laser densitometer (Pickart & Vella, 1988b).

Results

In a study of changes in E2 levels during erythroid maturation, we observed a high molecular weight labile Ub adduct in rabbit reticulocyte fraction II, suggesting that a high molecular weight E2 might be present (Pickart & Vella, 1988a). Initial attempts to purify this protein by the covalent Ub affinity method (Hershko et al., 1983) were unsuccessful. Therefore, a conventional purification scheme was developed. As will be described below, the enzyme preparation catalyzes Ub transfer to specific protein substrates in an E3-independent fashion (in the presence of E1). In addition, a protein in the preparation forms a labile Ub adduct with a molecular mass of approximately 230 kDa. For convenience, the conjugating enzyme will be referred to as $E2_{230K}$; evidence that the adduct-forming protein is identical with the protein which catalyzes Ub conjugation will be presented below. A unit of enzyme activity was defined as that amount which conjugated 1 μmol of Ub/min in the presence of 79 μM (1 mg/mL) yeast cytochrome *c* and 0.1 μM E1.

Purification and Properties. Results of the four-step purification procedure are summarized in Table I and Figure 1. $E2_{230K}$ activity could not be assayed in fraction II or in the first step (fraction C) because of the presence of other E2's that also conjugate Ub to cytochrome *c* (Pickart & Rose, 1985). $E2_{230K}$ was completely separated from these other E2's in the gel filtration step; $E2_{230K}$ activity migrated on the Ultrogel AcA34 column with an apparent molecular mass of 200–270 kDa (not shown). The final three steps resulted in a purification of about 7-fold with 20% recovery of activity. These factors are approximate because the concentrations of endogenous protein substrates, which apparently competed with cytochrome *c*, decreased during purification. Thus, in the assays, conjugate products with high and intermediate molecular weights (presumably endogenous protein conjugates) became less abundant with increasing $E2_{230K}$ purification, while the intensity of the band corresponding to monoubiquitinated cytochrome *c* increased (not shown).

The enzyme preparation was not electrophoretically homogeneous (Figure 1, lane 4). On the basis of native and subunit (below) molecular masses of about 230 kDa, $E2_{230K}$ activity seemed most likely to reside in a 230-kDa band that was the major polypeptide in four separate enzyme preparations (arrowhead, Figure 1). The intense band at 43 kDa (lane 4, Figure 1) is ovalbumin, which was added to improve recovery of protein (Experimental Procedures). In most prep-

Table I: Purification[a]

step	total protein[b] (mg)	total milliunits[c]	spec act. (milliunits/mg)
(1) anion exchange 1 (Q–Sepharose fraction C)	35.3	ND[d]	ND[d]
(2) gel filtration	12.6	1.46	0.12
(3) ammonium sulfate (38–50%)	1.8	0.67	0.37
(4) anion exchange 2 (Mono Q)	0.31[e]	0.25	0.81

[a] Details of the method are described under Experimental Procedures. The starting material was rabbit reticulocyte fraction II (268 mg of protein). [b] Not including carrier ovalbumin. [c] One unit conjugates 1 μmol of Ub/min in the presence of 1 mg/mL yeast cytochrome *c* (Experimental Procedures). [d] Not determined. [e] Estimated by laser densitometric scanning of Coomassie Blue stained gel (by comparison to peak from known quantity of ovalbumin in the same lane).

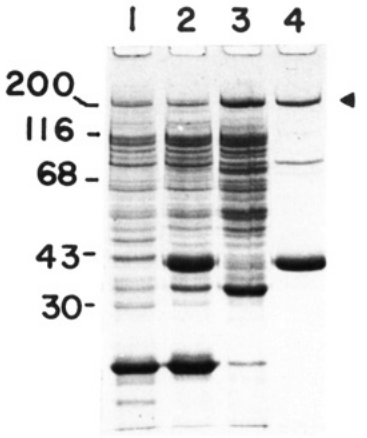

FIGURE 1: Purification of $E2_{230K}$ (10% SDS–polyacrylamide gel stained with Coomassie Blue). (Lane 1) Q–Sepharose fraction C (14 μg of protein loaded); (lane 2) concentrated peak of Ultrogel AcA34 column (14 μg, 1.7 microunits); (lane 3) 38–50% ammonium sulfate cut (9.4 μg, 3.4 microunits); (lane 4) concentrated peak of FPLC Mono Q column (2.3 μg, 1.9 microunits). These amounts of protein do not include the amount of carrier ovalbumin. (Total protein concentration was measured and then corrected by subtraction of the known concentration of ovalbumin.) Molecular masses (kDa) and mobilities of marker proteins run on the same gel are shown to the left: myosin (200); β-galactosidase (116); bovine serum albumin (68); ovalbumin (43); carbonic anhydrase (30). The shift in mobility of ovalbumin as a result of treatment with ammonium sulfate (38–50%) occurred reproducibly; the basis of this effect (lane 3 versus lanes 2 and 4) is not known.

Table II: $E2_{230K}$ Does Not Effectively Reconstitute Ub-Dependent Protein Degradation[a]

expt	$E2_{14K}$	E3	$E2_{230K}$	% cpm acid-sol
1	−	−	−	13.6
2	+	+	−	35.4
3	+	+	−	11.7[b]
4	+	−	−	17.0
5	+	−	+	16.2
6	−	+	−	19.8
7	−	+	+	23.5
8	+	+	+	35.7
9	−	−	+	14.4

[a] Reconstitution assays of degradation of radioiodinated human α-lactalbumin were carried out for 60 min, essentially as described previously (Pickart & Vella, 1988a). All assays contained (20 μL, pH 7.2, 37 °C) 50 mM Tris-HCl (24% base), 5 mM $MgCl_2$, 2 mM ATP plus regenerating system (Experimental Procedures), 0.6 unit/mL pyrophosphatase, 18.5 μg of protein from affinity-unadsorbed fraction (Experimental Procedures; source of protease), and 52 nM E1. Where indicated, assays were supplemented with the following: $E2_{14K}$ (112 nM), crude E3 (12.4 μg of protein, not treated with iodoacetamide), 0.36 microunits of $E2_{230K}$. All assays contained 11.7 μM Ub unless otherwise indicated. [b] Ub was omitted from the assay.

arations, the 230-kDa protein was about 50% of the total (non-ovalbumin) protein present after the Mono Q column, as evaluated by densitometric scanning of Coomassie-stained gels (not shown). The success of the purification scheme reflected primarily a change, during purification, in the behavior of the enzyme on anion-exchange resins: $E2_{230K}$ eluted between 0.2 and 0.3 M KCl on Q–Sepharose, but later on Mono Q (approximately 0.36 M; Experimental Procedures).

The $E2_{230K}$ preparation appeared to be devoid of activities of other Ub pathway enzymes, as indicated by the following. (1) Other E2's were removed in the first anion-exchange (Pickart & Vella, 1988b) and gel filtration steps (Pickart & Rose, 1985). The absence of low molecular weight E2's from the $E2_{230K}$ preparation was confirmed by the results of labile Ub adduct assays (Figure 2 below). The specific absence of $E2_{14K}$ was also shown by the finding that $E2_{230K}$ was not an effective substitute for $E2_{14K}$ in protein breakdown reconstitution assays (Table II, experiments 2, 6, and 7). (2) E3 precipitates between 0 and 30% ammonium sulfate (Haas & Bright, 1988) and should be removed in step 3. Its absence was confirmed by failure of $E2_{230K}$ to substitute effectively for E3 in protein breakdown reconstitution assays (Table II, experiments 2, 4, and 5). (3) E1 remaining after the gel filtration step was removed in the second anion-exchange step (Pickart & Vella, 1988a; Haas & Bright, 1988). Its absence was confirmed by the absolute requirement for added E1 in $E2_{230K}$ assays (Experimental Procedures). (4) While the $E2_{230K}$ preparation was not assayed directly for isopeptidase activity, the linearity of the assays (Figures 3A and 5B below) argues against extensive isopeptidase contamination of the preparation.

$E2_{230K}$ catalyzed Ub transfer to cytochrome *c*, independent of E3. This result suggested that it was an enzyme of the E2 class (Pickart & Rose, 1985). E2's also form labile Ub adducts that are intermediates in catalytic Ub conjugation (Hershko et al., 1983; Pickart & Rose, 1985). In the presence of E1, a protein in the $E2_{230K}$ preparation formed a labile Ub adduct with a molecular mass of about 230 kDa (lane 1 versus lane 2, Figure 2). Ub migrating at the position of the $E2_{230K}$–Ub adduct after mercaptoethanol treatment (lane 2) was presumably conjugated to one or more endogenous proteins. Since the gel (Figure 2) was run in the presence of 0.1% SDS, it is extremely unlikely that the 230-kDa labile adduct represents a noncovalent complex between a low molecular weight E2

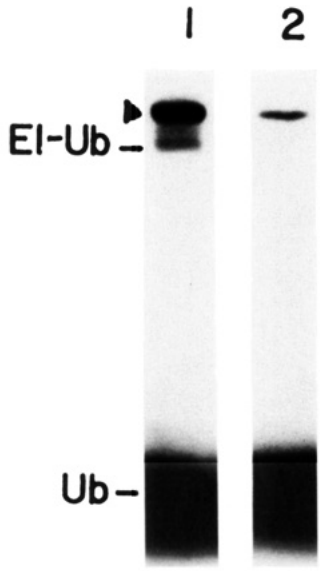

FIGURE 2: Formation of labile Ub adduct of $E2_{230K}$ (autoradiograph). Aliquots (4 μL) of the peak fraction of the FPLC Mono Q column were assayed with radioiodinated Ub, E1, and MgATP, as described under Experimental Procedures. (Lane 1) Assay quenched with sample buffer lacking mercaptoethanol; (lane 2) assay quenched with sample buffer containing mercaptoethanol and heated to 100 °C. The samples were run on a 12.5% gel. The arrowhead indicates the putative $E2_{230K}$–Ub adduct, E1–Ub the labile adduct of Ub and E1, and Ub free Ub. For estimation of the size of the adduct (text), samples were run on an 8% gel together with appropriate marker proteins. The gel was stained prior to drying and autoradiography (C. Pickart and Y. Cho, unpublished experiments).

and some other protein. The size of the adduct is thus consistent with $E2_{230K}$ activity residing in the major 230-kDa polypeptide (Figure 1), provided the stoichiometry of Ub adduct formation close to 1 mol of Ub/mol of $E2_{230K}$. This is the stoichiometry seen with nearly all low molecular weight E2's (Haas & Bright, 1988; Pickart & Vella, 1988a).

Conjugative Substrate Specificity. The best $E2_{230K}$ preparations were still contaminated by reticulocyte proteins that were good substrates for conjugation, as shown in Figure 3A (lanes 1–3) and Figure 4 (lane 4). However, conjugation of added exogenous proteins was easily detected against this background. Thus, addition of yeast cytochrome *c* resulted in formation of a prominent conjugate of the size expected for monoubiquitinated cytochrome (Figure 3A, lanes 4–6, arrowhead). Several minor products, presumably representing multiply ubiquitinated cytochrome, were also formed (see also Figure 4, lane 5). Total conjugation as well as formation of monoubiquitinated cytochrome was linear with time (Figure 3A; Figure 5B below), and rates were directly proportional to $E2_{230K}$ concentration (not shown).

The dependence of the rate of cytochrome monoubiquitination on cytochrome *c* concentration is shown in Figure 4B. The line was calculated by assuming values of 125 μM and 0.12 μM/min for K_m and V_{max}, respectively. Assuming that $E2_{230K}$ activity resides in the 230-kDa polypeptide, its concentration in the assay leads to a k_{cat} value of 0.37 min^{-1} (at V_{max}). These values may be compared to K_m = 274 μM and k_{cat} = 0.07 min^{-1} for monoubiquitination of the same substrate catalyzed by reticulocyte $E2_{20K}$ [calculated for 0.1 ionic strength from Figure 4B of Pickart and Rose (1985)].

$E2_{230K}$ also catalyzed Ub transfer to histones. At low histone concentrations (<5 μM), these reactions were distinguished from cytochrome conjugation by the extensive polyubiquitination seen with histones (Figure 4, lanes 1 and 2, versus Figure 3A, lanes 4–6). At higher H3 concentration, multiple

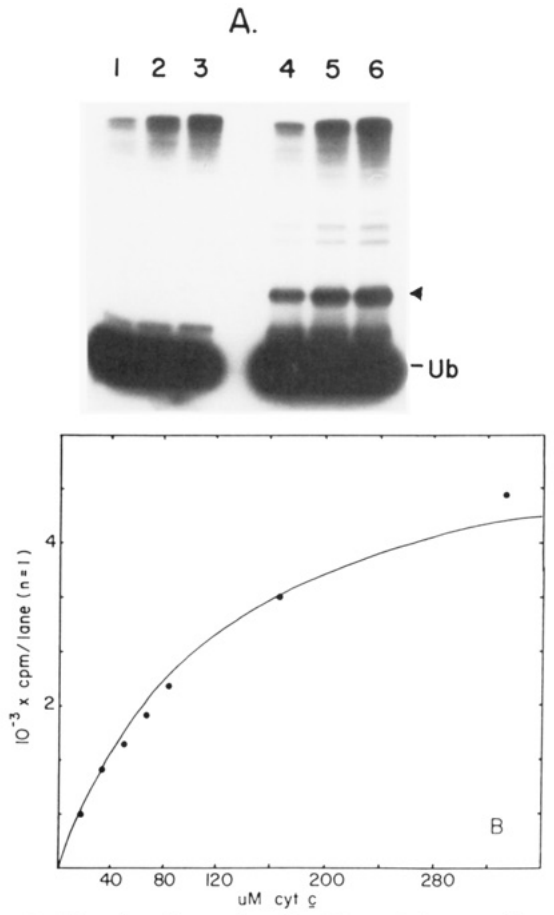

FIGURE 3: Ub conjugation catalyzed by $E2_{230K}$. (A) Autoradiograph. Assays were carried out with 0.26 microunits of $E2_{230K}$ as described under Experimental Procedures (standard assay), except as noted. (Lanes 1–3) No substrate protein added; (lanes 4–6) yeast cytochrome *c* (79 μM) added. Incubations were for 5 min (lanes 1 and 4), 10 min (lanes 2 and 5), or 15 min (lanes 3 and 6). The arrowhead denotes monoubiquitinated cytochrome (20 kDa) and Ub free Ub. (B) Concentration dependence of rate of cytochrome monoubiquitination. Assays were carried out as described under Experimental Procedures with 0.15 microunits of $E2_{230K}$, except that cytochrome concentration was varied. For each concentration, the band corresponding to monoubiquitinated cytochrome was excised and counted. Calculation of the line is described in the text.

ubiquitination was inhibited (Figure 4, lane 3). This behavior resembles that of $E2_{20K}$ with the same substrate (Pickart & Vella, 1988b). K_m for H3 in $E2_{230K}$-catalyzed monoubiquitination was <1.3 μM (Figure 4, lane 1 versus lane 2); V_{max} for the same reaction with H3 was about 50% of V_{max} for monoubiquitination of yeast cytochrome *c* (i.e., $k_{cat} \sim 0.18$ min^{-1}). These values may be compared to $k_{cat} = 0.1$ min^{-1} and $K_m = 1.8$ μM for monoubiquitination of H3 catalyzed by $E2_{20K}$ (Pickart & Vella, 1988b). Less quantitative experiments showed that H2A and H4 (approximately 15 μM) were (mono)ubiquitinated by $E2_{230K}$ with rates comparable to the rate at 15 μM H3 (not shown).

A number of other exogenous proteins were tested as substrates for $E2_{230K}$ (concentration of substrate protein in assay, 2 mg/mL). As shown in Figure 4 (lanes 6–9), $E2_{230K}$ conjugated Ub to lysozyme (5%), human (12%), and bovine (15%) α-lactalbumin and α-casein (42%) (numbers in parentheses indicate the rate of formation of the apparent monoubiquitinated product relative to that seen with 79 μM cytochrome *c*). Two other proteins, β-lactoglobulin and rcmBSA, were not detectably conjugated by $E2_{230K}$ (not shown).

The kinetics of these reactions were not investigated directly. However, lysozyme and casein both inhibited cytochrome

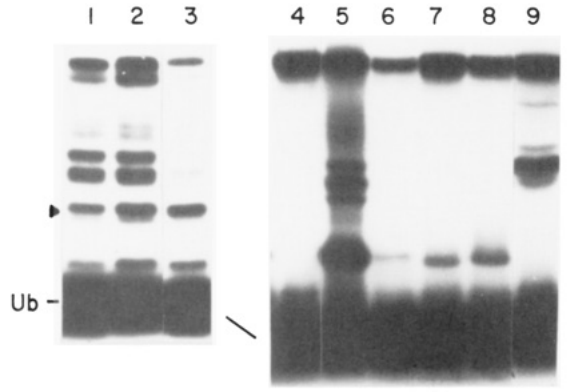

FIGURE 4: Substrate specificity (autoradiograph). Assays of Ub conjugation were carried out with 0.15 microunits of $E2_{230K}$ under standard conditions (Experimental Procedures), except that substrate protein identity was varied: (lane 1) 1.3 μM H3; (lane 2) 1.9 μM H3, (lane 3) 6.4 μM H3. The arrowhead denotes monoubiquitinated H3 (there were 1600 cpm in this band in lane 1 and 2500 cpm in lane 2). In lanes 5–9, concentrations of substrate proteins were 2 mg/mL. (Lane 4) No added substrate; (lane 5) yeast cytochrome *c*; (lane 6) lysozyme; (lane 7) bovine α-lactalbumin; (lane 8) human α-lactalbumin; (lane 9) α-casein.

conjugation: 139 μM lysozyme inhibited by 90%; 71 μM casein inhibited by about 50% (assays with 79 μM cytochrome). Assuming a model of competitive inhibition, these results lead to K_m values of 45 μM and about 10 μM for casein and lysozyme, respectively. Inhibition by lysozyme of Ub conjugation to contaminating endogenous protein substrates, apparent in Figure 4 (lane 4 versus lane 6), is also consistent with binding of lysozyme to $E2_{230K}$. Presumably, lysozyme has a low k_{cat} value. The other substrates shown in Figure 4 (lanes 4–9) were without significant effect on cytochrome conjugation (Klemperer and Pickart, unpublished experiments), indicating that binding of these proteins to $E2_{230K}$ is very weak.

The same proteins were tested as substrates for $E2_{20K}$. The results were qualitatively similar to those shown in Figure 4, although levels of conjugates other than that of cytochrome were too low to be quantitated (C. Pickart, unpublished experiments). These results, and those obtained with histones and cytochrome *c* (above), suggest that $E2_{230K}$ and $E2_{20K}$ have similar substrate specificities.

Role of $E2_{230K}$ in Ub-Dependent Protein Degradation. If the conjugate products of $E2_{230K}$ catalysis are degradatively competent, $E2_{230K}$ should substitute for the combination of $E2_{14K}$ and E3 in reconstituting Ub-dependent protein degradation. Data summarized in Table II show that $E2_{230K}$ did not reconstitute Ub-dependent degradation of human α-lactalbumin in the presence of E1 and a fraction containing the Ub conjugate protease (experiments 1–3 versus experiment 9), even though it conjugated Ub to this protein (Figure 4, lane 8). In addition, $E2_{230K}$ did not effectively substitute for $E2_{14K}$ in the presence of E3 (experiments 2, 6, and 7). This result suggests that $E2_{230K}$ cannot act as a Ub donor in E3-catalyzed conjugation that leads to degradation. While the possibility that $E2_{14K}$, E3, and $E2_{230K}$ are *all* required for degradation cannot be rigorously excluded, it would require that the small amount of $E2_{230K}$ activity contaminating the E3 and protease preparations (0.1 microunit total) be sufficient to support a maximal rate of breakdown in these assays. Results qualitatively similar to those shown in Table II were obtained in assays of rcmBSA and β-lactoglobulin degradation (N. Klemperer, unpublished experiments). These findings regarding $E2_{230K}$ are consistent with previous conclusions that E2's cannot support protein degradation in the absence of E3

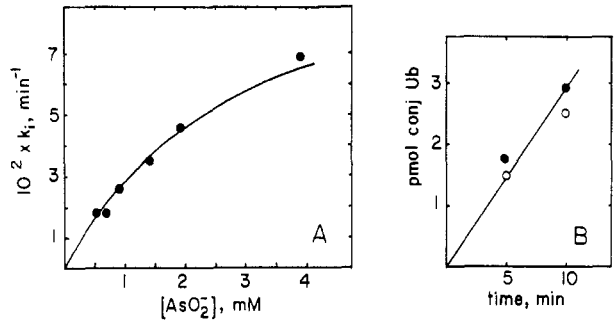

FIGURE 5: Inactivation of $E2_{230K}$ by arsenite. (A) Concentration dependence of specific inactivation rate constant (k_i). Values of k_i were measured as a function of arsenite concentration (Experimental Procedures). The calculation of the line is described in the text. (B) Protection against arsenite under turnover conditions. The ordinate shows the total amount of radioiodinated Ub conjugated to proteins as a function of time during the standard assay: no arsenite (○); 2 mM arsenite added approximately 10 s after the assay was initiated (●).

and that only $E2_{14K}$ interacts efficiently with E3 (Pickart & Rose, 1985; Pickart & Vella, 1988a; Haas & Bright, 1988).

Inactivation of $E2_{230K}$ by Arsenite. In other work, we found that arsenite, generally considered to be a reagent specific for vicinal sulfhydryl sites (Adamson & Stevenson, 1981), inhibits Ub-dependent protein degradation by at least two different mechanisms (Klemperer and Pickart, unpublished experiments). Arsenite was also tested as an inhibitor of $E2_{230K}$ and was found to inactivate in a time- and concentration-dependent fashion. In these experiments, incubations of $E2_{230K}$ with arsenite were terminated by diluting the enzyme into standard assays containing DTT equimolar with respect to arsenite, so that there was no free arsenite in the assay (Zahler & Cleland, 1968). Inactivation in the primary incubation followed first-order kinetics within experimental error (not shown); since inactivation persisted after DTT addition, it was irreversible on the time scale of the assays (10 min). It was shown in a control experiment that premixing arsenite with equimolar DTT abolished $E2_{230K}$ inactivation.

The dependence of the apparent inactivation rate constant (k_i) on arsenite concentration followed a saturating relationship. The line in Figure 5A was calculated according to eq 4, in which E and E_i are active and inactive forms (respectively) of $E2_{230K}$, assuming $K_{0.5}$ = 3.3 mM and k_i^{max} = 0.12 min^{-1}. The low value of k_i^{max} suggests that the rate-limiting step in the inactivation process may involve a slow enzyme conformation change. An alternative mechanism that is also consistent with the data involves a slow conformation change of free enzyme, followed by rapid second-order reaction of arsenite with the altered conformational form (not shown).

$$E + AsO_2^- \underset{K_{0.5}}{\overset{\text{fast}}{\rightleftharpoons}} E{\cdot}AsO_2^- \xrightarrow[\text{slow}]{k_i^{max}} E_i{\cdot}AsO_2^- \qquad (4)$$

The value of $K_{0.5}$ for arsenite was independent of the presence of 88 μM yeast cytochrome *c* in the inactivation reaction (not shown). However, during catalytic turnover $E2_{230K}$ was resistant to inactivation by arsenite. With free $E2_{230K}$, 35% inactivation occurred in 10 min in the presence of 1.93 mM arsenite (on the basis of data in Figure 5A); under turnover conditions (standard assay), no inactivation occurred in 10 min (Figure 5B). This result shows that an arsenite-insensitive form of the enzyme predominates during catalysis. Since cytochrome alone gave no protection (above), the labile Ub adduct of $E2_{230K}$ is most likely to be the arsenite-insensitive species. In the assay, this intermediate is formed rapidly (Figure 2) and irreversibly (due to the presence of pyrophosphatase to drive the E1 reaction; Experimental Procedures). These results suggest that the site at which arsenite interacts with $E2_{230K}$ is sensitive to active-site occupancy.

The sensitivity of $E2_{230K}$ to arsenite renders it unique among known E2's. We found that $E2_{14K}$ (and E3) were not affected by arsenite (Klemperer and Pickart, unpublished experiments). Arsenite (2–4 mM) also did not inhibit the histone-conjugating activity of $E2_{20K}$ or $E2_{35K}$ (C. Pickart, unpublished experiments).

Inactivation of $E2_{230K}$ by Iodoacetamide. The sensitivity of low molecular weight E2's, but not their Ub adducts, to iodoacetamide (Hershko et al., 1983) suggests that the Ub adducts are thiolesters, and this hypothesis is consistent with the presence of a single Cys residue in a conserved region in the sequence of two (yeast) E2's (Jentsch et al., 1987; Goebl et al., 1988). $E2_{230K}$ forms a labile Ub adduct and is sensitive to a putative vicinal sulfhydryl reagent, arsenite (above). We therefore tested its sensitivity to iodoacetamide: $E2_{230K}$ lost activity with k_{obs} = 0.037 min^{-1} at 1.5 mM iodoacetamide (pH 7.0, 37 °C). Under the same conditions $E2_{20K}$ was much more rapidly inactivated ($k_{obs} \geq 0.66$ min^{-1}). Inactivation of $E2_{14K}$ proceeded with $k_{obs} \sim 0.23$ min^{-1} at 5 mM iodoacetamide (Hershko et al., 1983) and with k_{obs} = 0.15 min^{-1} at 0.4 mM iodoacetamide (S. Dosch and C. Pickart, unpublished experiments). An apparent essential sulfhydryl group of $E2_{230K}$ thus reacts slowly with iodoacetamide; this property distinguishes $E2_{230K}$ from other E2's for which this reaction has been studied.

Discussion

We have described the partial purification of a high molecular weight Ub-conjugating enzyme. This protein appears to be a member of the E2 family on the basis of the following considerations. (1) Catalysis of Ub transfer to proteins is E1 dependent, but occurs without involvement of the E3 that participates in Ub-dependent proteolysis. Conjugative substrate specificity is in many respects similar to that of another well-characterized protein of the reticulocyte E2 family, $E2_{20K}$. (2) The preparation contains a protein that accepts Ub, in labile linkage, from the E1–Ub thiolester. The size of the labile Ub adduct of $E2_{230K}$ (approximately 230 kDa; Figure 2) is essentially identical with the size of the (native) conjugating activity (approximately 235 kDa) and with the size of the major polypeptide in the preparation (approximatey 230 kDa; Figure 1). Pending purification of $E2_{230K}$ to homogeneity, it is difficult to exclude rigorously the possibility that conjugation depends on a contaminating ligase activity (other than the E3 known to be involved in protein degradation). However, the simplest explanation of these data is that the major 230-kDa polypeptide is an E2 which can accept Ub from the E1–Ub thiolester and which can catalyze transfer of this Ub to proteins such as cytochrome *c* and histones.

Lee et al. (1986) described the partial purification and characterization of multiple Ub–protein "ligases" from human erythrocytes. Since $E2_{230K}$ is present in rabbit erythrocytes (Pickart & Vella, 1988a), we considered the possibility that it was one of these ligases. The previously described enzymes were insensitive to iodoacetamide and appeared to conjugate Ub to proteins independently of $E2_{14K}$; however, they did not appear to form labile Ub adducts (Lee et al., 1986). The former properties agree with those of $E2_{230K}$; the latter does not. Also, all of the previously described ligases conjugated Ub to rcmBSA; $E2_{230K}$ does not. We conclude that $E2_{230K}$ is unlikely to represent one of these previously described enzymes.

$E2_{230K}$ shares many features with other E2's (Pickart & Rose, 1985; Pickart & Vella, 1988a,b; Haas & Bright, 1988;

Haas et al., 1988). It conjugates Ub to small, basic proteins such as cytochrome *c* and free histones (Figures 3 and 4) and does not seem to function in Ub-dependent protein degradation. With regard to $E2_{230K}$, the latter conclusion is based on a direct test (Table II); it is also consistent with the features of arsenite inactivation of $E2_{230K}$ (Figure 5) versus arsenite inhibition of Ub-dependent protein degradation. Proteolytic inhibition by arsenite is rapid and reversible (Klemperer and Pickart, unpublished experiments); inactivation of $E2_{230K}$ is slow and apparently irreversible. In conjugative reactions and in failure to participate in protein degradation, $E2_{230K}$ resembles $E2_{20K}$ and $E2_{35K}$. However, $E2_{230K}$ also has several unusual features. These include large size, relative insensitivity to iodoacetamide, and sensitivity to arsenite.

Inhibition of monosubstituted trivalent arsenicals is often considered to be diagnostic for the presence of vicinal sulfhydryl groups on the affected enzyme. Substituted phenyl arsenoxides bind to and/or inhibit pyruvate dehydrogenase (via its lipoamide cofactor) (Adamson & Stevenson, 1981), thioredoxin (Brown et al., 1987), and lecithin–cholesterol acyltransferase (Jauhiainen et al., 1988). In the latter case, the sulfhydryl groups that complex with arsenicals are contributed by Cys residues involved in the formation of a catalytic acyl-enzyme intermediate. This case is of particular interest, since $E2_{230K}$ forms an apparent acyl(Ub)-enzyme adduct that is probably an intermediate in catalytic Ub transfer to proteins (Hershko et al., 1983; Pickart & Rose, 1985; Haas & Bright, 1988).

Inhibition of enzymes through binding of trivalent arsenicals to vicinal sulfhydryls is usually rapidly reversible upon addition of a competitive dithiol reagent such as DTT (Adamson & Stevenson, 1981). Inactivation of $E2_{230K}$ by arsenite was not reversed within 10 min after DTT addition. However, the slow kinetics of $E2_{230K}$ inactivation (maximum k_i, 0.12 min^{-1}) suggest that an enzyme conformation change could be rate-limiting for inactivation. If so, reversal of the conformation change could also be slow. This kind of slow step might also contribute to rate limitation in the reaction of $E2_{230K}$ with iodoacetamide. Since $E2_{230K}$ is resistant to arsenite under turnover conditions, studies on the mechanism of the inactivation reaction should provide information regarding the normal catalytic mechanism of the enzyme. Future studies may employ organic arsenicals, since preliminary experiments indicate that phenyl arsenoxide is a much more potent inactivator of $E2_{230K}$ than is inorganic arsenite (Y. Cho, E. Berleth, and C. Pickart, unpublished experiments).

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Registry No. Cytochrome *c*, 9007-43-6; lysozyme, 9001-63-2; arsenite, 15502-74-6; iodoacetamide, 144-48-9.

REFERENCES

Adamson, S. R., & Stevenson, K. J. (1981) *Biochemistry 20*, 3418–3424.

Ball, E., Karlik, C. C., Beall, C. J., Saville, D. L., Sparrow, J. C., Bullard, B., & Fyrberg, E. A. (1987) *Cell 51*, 221–228.

Brown, S. B., Turner, R. F., Roche, R. S., & Stevenson, K. J. (1987) *Biochemistry 26*, 863–871.

Busch, H., & Goldknopf, I. L. (1981) *Mol. Cell. Biochem. 40*, 173–187.

Evans, A. C., & Wilkinson, K. D. (1985) *Biochemistry 24*, 2915–2923.

Goebl, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., & Byers, B. (1988) *Science 241*, 1331–1335.

Goldstein, G., Scheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., & Niall, H. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 11–15.

Haas, A. L., & Wilkinson, K. D. (1985) *Prep. Biochem. 15*, 49–60.

Haas, A. L., & Bright, P. M. (1988) *J. Biol. Chem. 263*, 13258–13267.

Haas, A. L., Bright, P. M., & Jackson, V. E. (1988) *J. Biol. Chem. 263*, 13268–13275.

Hensold, J. O., Swerdlow, P. S., & Housman, D. E. (1988) *Blood 71*, 1153–1156.

Hershko, A., & Heller, H. (1985) *Biochem. Biophys. Res. Commun. 128*, 1079–1086.

Hershko, A., & Ciechanover, A. (1986) *Prog. Nucleic Acids Res. Mol. Biol. 33*, 19–56.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1783–1786.

Hershko, A., Heller, H., Elias, S., & Ciechanover, A. (1983) *J. Biol. Chem. 258*, 8206–8214.

Hershko, A., Leshinsky, E., Ganoth, D., & Heller, H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1619–1623.

Huang, S. U., Barnard, M. B., Xu, M., Matsui, S. I., Rose, S. M., & Garrard, W. T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3738–3742.

Jauhiainen, M., Stevenson, K., & Dolphin, P. J. (1988) *J. Biol. Chem. 263*, 6525–6533.

Jentsch, S., McGrath, J. P., & Varshavsky, A. (1987) *Nature 329*, 131–134.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lee, P. L., Midelfort, C. F., Murakami, K., & Hatcher, V. B. (1986) *Biochemistry 25*, 3134–3138.

Levinger, L., & Varshavsky, A. (1982) *Cell 28*, 375–385.

Matsui, S. I., Seon, B. K., & Sandberg, A. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6386–6390.

Mueller, R. D., Yasuda, H., Hatch, C. L., Bonner, W. M., & Bradbury, E. M. (1985) *J. Biol. Chem. 260*, 5147–5153.

Pickart, C. M., & Rose, I. A. (1985) *J. Biol. Chem. 260*, 1573–1581.

Pickart, C. M., & Vella, A. T. (1988a) *J. Biol. Chem. 263*, 12028–12035.

Pickart, C. M., & Vella, A. T. (1988b) *J. Biol. Chem. 263*, 15076–15082.

Siegelman, M., Bond, M. W., Gallatin, W. M., St. John, T., Smith, H. T., Fried, V. A., & Weissman, I. L. (1986) *Science 231*, 823–829.

Thorne, A. W., Sautiere, P., Briand, G., & Crane-Robinson, C. (1987) *EMBO J. 6*, 1005–1010.

Wunsch, A. M., Haas, A. L., & Lough, J. (1987) *Dev. Biol. 119*, 85–93.

Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. U., Ando, M. E., Harkins, R. N., Francke, Y., Fried, V. A., Ullrich, A., & Williams, L. T. (1986) *Nature 323*, 226–232.

Zahler, W. L., & Cleland, W. W. (1968) *J. Biol. Chem. 243*, 716–719.