Mechanism of Ubiquitin Conjugating Enzyme E2-230K: Catalysis Involving a Thiol Relay?†

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ABSTRACT: Covalent conjugation of ubiquitin to intracellular proteins is a signal for degradation by the 26S protease. Conjugation is usually accomplished by the sequential action of activating (E1), conjugating (E2), and ligase (E3) enzymes. Each of these enzymes forms a covalent thiol ester with ubiquitin as part of its catalytic cycle. In most cases, the apparent role of the ubiquitin conjugating enzyme (E2) is to transfer ubiquitin from the E1 active site to the E3 active site. Ubiquitin is then delivered from E3 to the substrate lysine residue. An unusually large, reticulocyte-specific enzyme, known as E2-230K, is unique among the large family of E2 enzymes is being susceptible to inhibition by inorganic arsenite [Klemperer et al. (1989) *Biochemistry 28*, 6035−6041]. We show that phenylarsenoxides potently inhibit E2-230K, apparently by binding to vicinal Cys residues of the enzyme: bound aminophenylarsenoxide partially protects the enzyme against inactivation by *N*-ethylmaleimide (NEM), and prior enzyme inactivation with NEM blocks enzyme binding to immobilized phenylarsenoxide. Studies on the mechanistic basis of inhibition showed that a concentration of (aminophenyl)arsenoxide that produced complete inhibition of steady-state turnover had no effect on the turnover of the preformed E2−ubiquitin adduct. However, when the enzyme was preincubated with this concentration of inhibitor prior to initiation of adduct formation, the level of E2-associated ubiquitin was reduced by 60%. These results are consistent with a model in which two Cys residues of the enzyme sequentially form thiol esters with ubiquitin and the second of these Cys residues is bound to arsenic in the enzyme-inhibitor complex. In this model, E2-230K functions as an E2−E3 hybrid.

The ubiquitin-mediated proteolytic pathway is the primary mechanism for selective protein turnover in eukaryotic cells. This pathway carries out turnover of most short-lived proteins (Ciechanover et al., 1984), and participates in intracellular regulation by degrading c-Jun, c-Mos, mitotic and G1 cyclins, and other important regulatory proteins (Ciechanover, 1994; Glotzer et al., 1991; Nishizawa et al., 1992; Treier et al., 1994; Deshaies et al., 1995; Yaglom et al., 1995). Covalent conjugation of C-termini of ubiquitin molecules to internal Lys residues of these and other substrates is a signal for degradation by the 26S protease [review in Ciechanover (1994)]. Substrates destined for rapid degradation are usually conjugated to many molecules of ubiquitin. Multiple ubiquitins linked in a homopolymeric chain interact strongly with a specific subunit of the protease (Chau et al., 1989; Deveraux et al., 1994).

Ubiquitination typically occurs through an intermolecular enzyme−ubiquitin thiol ester cascade (Ciechanover, 1994; Scheffner et al., 1995). The C-terminus of ubiquitin (Gly-76) is first activated through ATP-dependent formation of a thiol ester with a Cys residue at the active site of ubiquitin activating enzyme, or E1.[1] Ubiquitin is next transferred to a Cys residue at the active site of a ubiquitin conjugating enzyme, or E2 protein. Although some E2s transfer ubiquitin directly to certain model substrates (Pickart & Rose, 1985), the formation of multiubiquitinated conjugates usually requires a ubiquitin−protein ligase, or E3 (Hershko et al., 1983). An E3−ubiquitin thiol ester is an intermediate in the mechanisms of the two known types of E3s (Huibregste et al., 1995; Scheffner et al., 1995; V. Chau, personal communication).

Specificity in ubiquitin conjugation arises in part through the existence of multiple E2 and E3 proteins that cooperate in ubiquitinating specific substrates (Jentsch et al., 1991; Huibregste et al., 1995). The E2 protein family, and at least one E3 protein family, are characterized by distinctive conserved sequences that flank their respective active-site Cys residues (Jentsch et al., 1991; Huibregste et al., 1995). Most E2s are small proteins (14−35 kDa) that do not exhibit intrinsic affinity for physiological substrates. In contrast, known E3 enzymes are large proteins (100−200 kDa) that bind tightly to their substrates (Reiss & Hershko, 1990; Scheffner et al., 1993). Thus, a working model for the basis of conjugative specificity postulates that the E3 enzyme binds to the substrate, while the E2 enzyme binds to the E3 and charges it with ubiquitin. This model satisfactorily explains the ubiquitination of N-end rule substrates catalyzed by E3-α and E2-14K (Reiss et al., 1989; Berleth et al., 1992a) and the ubiquitination of p53 catalyzed by the E3 known as E6-AP and a 16-kDa E2 (Scheffner et al., 1993).

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[1] Abbreviations: E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin−protein ligase; BSA, bovine serum albumin; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NPAsO, *p*-aminophenyl arsenoxide; BrAcNPAsO, *p*-(bromoacetylamino)phenylarsenoxide; PAsO-Sepharose, phenylarsenoxide-Sepharose; BSA, bovine serum albumin; TEO buffer, 50 mM Tris-HCl (24% base), 0.1 mM ethylenediaminetetraacetic acid, and 1 mg/mL ovalbumin.

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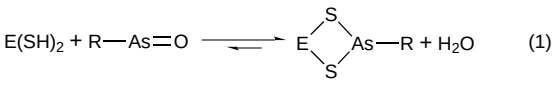
The unusually large mammalian enzyme known as E2-230K represents an apparent exception to the above-described model. Like other E2 enzymes, E2-230K ubiquitinates nonphysiological substrates such as denatured histones in an E3-independent fashion (Klemperer et al., 1989). But in marked contrast to other E2s, E2-230K also ubiquitinates endogenous proteins in erythroid cells, by a mechanism that does not depend upon any (known) E3.[2] High expression of E2-230K is restricted to reticulocytes, suggesting that this E2 enzyme may function in induced turnover of previously stable proteins at the reticulocyte stage of erythroid differentiation (Wefes et al., 1995).

E2-230K is further distinguished from other E2 enzymes by its sensitivity to arsenite (R = -OH in eq 1; Klemperer et al., 1989). Trivalent arsenoxides such as arsenite typically react with vicinal thiols according to the mechanism shown in eq 1 (Whitaker, 1947; Stevenson et al., 1978). Inhibition



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by this mechanism is usually reversed by DTT, which forms a competing cyclic dithioarsinite complex and shifts the equilibrium to the left. However, a treatment of arsenite-inhibited E2-230K with DTT did not restore enzyme activity (Klemperer et al., 1989).

Here we report that inhibition of E2-230K by aromatic (phenyl) arsenoxides, unlike inhibition by arsenite, is readily reversed by DTT. Several lines of evidence indicate that Cys residues of the enzyme mediate phenylarsenoxide binding and that arsenoxides inhibit conjugation by blocking covalent reaction of ubiquitin with an enzymic Cys residue. The results support a model in which E2-230K-catalyzed ubiquitination occurs through an *intra*molecular thiol relay mechanism and suggest that E2-230K may function as an E2−E3 hybrid.

EXPERIMENTAL PROCEDURES

Materials. Partially purified calf thymus histone 2B was from Worthington; α-casein was from Sigma; NEM and iodoacetamide were from Sigma or Aldrich. Ubiquitin (Sigma) was radioiodinated by the chloramine-T procedure, ∼8000 cpm/pmol (Hershko et al., 1983). Purified recombinant ubiquitin bearing a Lys-to-Arg substitution at residue 48 (K48R-ubiquitin) was generously provided by Robert Cohen (University of Iowa). Reductively methylated ubiquitin was prepared as described (Hershko & Heller, 1985). Syntheses and properties of NPAsO, BrAcNPAsO, and PAsO-Sepharose have been described (Berleth et al., 1992a). Protein concentrations were determined with Bio-Rad dye reagent concentrate, using BSA as standard, or by densitometric analysis of Coomassie blue-stained gels (relative to BSA standards run on the same gel).

Enzymes. Electrophoretically homogeneous E1s purified from rabbit reticulocytes or bovine erythrocytes (Pickart & Vella, 1988a) were used interchangeably. Homogeneous recombinant E2-25K was a gift of Margaret Haldeman (Pickart et al., 1992). The E2-25K used in our experiments bore a catalytically silent Cys-to-Ser mutation at residue 170.

[2] C. Pickart and E. Kasperek, unpublished experiments.

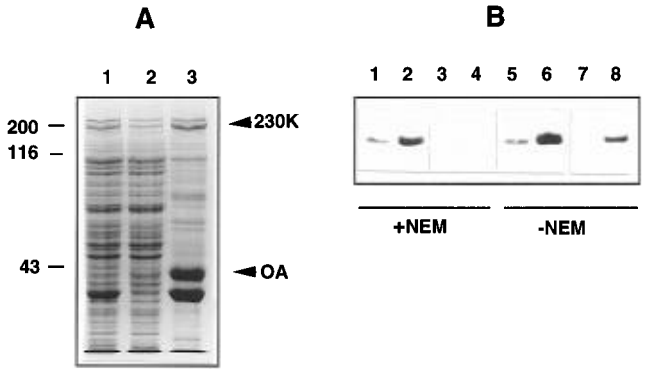


FIGURE 1: E2-230K binding to PAsO-Sepharose. (A) Purification (Coomassie-stained gel). About ∼1% of each fraction from the arsenoxide affinity step of the purification described in Experimental Procedures was run on an 8% gel. Lane 1, material loaded onto column; lane 2, unbound fraction; lane 3, DTT eluate. 230K, E2-230K; OA, ovalbumin (carrier). Molecular masses of marker proteins (in kilodaltons) are shown on the left. (B) NEM treatment prevents E2-230K binding to PAsO-Sepharose (immunoblot). Lanes 1−4, NEM-treated enzyme; lanes 5−8, control enzyme (see Experimental Procedures). Two aliquots of each fraction were run (3 and 10 μL; odd and even lanes, respectively). Lanes 1, 2, 5, and 6, material loaded onto resin; lanes 3, 4, 7, and 8, DTT eluates. As expected, the NEM-treated E2-230K was recovered in the unbound fraction (not shown).

For most purposes E2-230K was purified by a modification of the previously described procedure (Klemperer et al., 1989), with an added step of arsenoxide affinity chromatography. Briefly, proteins from rabbit reticulocyte fraction II (Hershko et al., 1983) that precipitated between 30% and 80% saturation with ammonium sulfate (or 30−50%) were collected, dialyzed, and applied to an anion-exchange column (Klemperer et al., 1989). Proteins eluting between 0.25 and 0.4 M (or 0.5 M) KCl were pooled, concentrated by ultrafiltration, and dialyzed. The order of the previous two steps was sometimes reversed, depending on whether other components were being purified from fraction II. Following these two steps, the E2-230K-containing fraction was applied to PAsO-Sepharose (Berleth et al., 1992a). Cysteine, 1 mM, was included during the loading and wash steps. E2-230K was eluted with buffer containing 5 mM DTT, then repreatedly diluted and concentrated to reduce [DTT] to 0.2 mM (Centricon-10, Amicon). The purification documented in Figure 1A provides an example. Here 650 mg of fraction II protein was fractionated on a 43-mL Q-Sepharose column. Proteins eluting between 0.2 and 0.3 M NaCl were concentrated and dialyzed. From this fraction (105 mg of protein), species precipitating between 30% and 50% saturation with ammonium sulfate were collected, dissolved, and dialyzed (44 mg). Half of this material (22 mg, 3.4 mL) was chromatographed on a 1-mL PAsO-Sepharose column. The final purification step was gradient elution from an FPLC Mono Q column (Pharmacia−LKB). The E2-230K preparations used in this work ranged in electrophoretic purity from ∼50% to ∼80%. Concentrations of E2-230K were estimated by densitometry (above).

Several lines of evidence indicate that the ∼20-kDa protein isolated by the above-described procedure was E2-230K and not the 200-KDa protein E3-α, which also binds to PAsO-Sepharose (Berleth et al., 1992a). First, our preparations of E2-230K conjugated ubiquitin to histones independently of E2-14K (Results), while the conjugating activity of E3-α is strictly dependent on the latter E2 (Pickart & Vella, 1988a).

Second, purified E2-230K (above) reacted with antibodies raised against a specific E2-230K peptide (Haldeman et al., 1995; Wefes et al., 1995). This antibody does not recognize E3-α, as shown by the finding that all of the immunoreactive protein precipitates between 30% and 50% saturation with ammonium sulfate, while E3-α precipitates at ≤30% saturation (Haas & Bright, 1988). Finally, the conjugating activity in our E2-230K preparations was *reversibly* inhibited by BrAcNPAsO (Results), while E3-α is *irreversibly* inactivated by this reagent (Berleth et al., 1992a).

Electrophoresis, Western Blotting, and Autoradiography. SDS−PAGE was carried out in a 0.75-mm-thick mini-gels (Laemmli, 1970). Gels (8% acrylamide) were blotted onto Immobilon-P (Millipore) at 70 V for 3 h as described previously (Haas & Bright, 1985). Immunostaining was carried out as described (Haas & Bright, 1985). Blots were developed with affinity-purified polyclonal antibodies (750-fold dilute) raised against a specific E2-230K peptide (Haldeman et al., 1995). Immune complexes were detected using ^{125}I-protein A at 1 μCi/mL (Bolton−Hunter-labeled, >30 μCi/μg; ICN).

Treatment of E2-230K with Iodoacetamide. This treatment was used to eliminate residual DTT in the enzyme storage buffer. E2-230K (0.5−5 μM) was treated with 2 mM iodoacetamide for 15 min at pH 7.3 and 37 °C, in TEO buffer. (Ovalbumin in this buffer served to counteract E2 adsorption.) The tube was kept on ice, and the enzyme was used within several hours. If the enzyme was to be used in assays involving E1, iodoacetamide was quenched with a 1.5−3-fold molar excess of DTT or cysteine (cysteine was used for experiments involving arsenoxides).

Effect of NEM Treatment on E2-230K Binding to PAsO-Sepharose. E2-230K (0.2 μM) was incubated with 30 μM NEM in TEO buffer (6 min, 37 °C; 68 μL). NEM was quenched by adding 7.5 μL of 10 mM β-mercaptoethylamine and incubating for 10 min more. For the control, E2 addition was delayed until completion of the mercaptoethylamine quench. At the end of the quench, a 20-μL aliquot was removed from each incubation and added to 20 μL of SDS sample buffer. A second aliquot (50 μL) of each incubation was added to 10 μL of PAsO-Sepharose (previously equilibrated with TEO buffer). The suspensions were rotated at 5 °C for 15 min and then microcentrifuged for 30 s. The supernatants (unbound fractions) were mixed with 50 μL of sample buffer. To each resin pellet was added 50 μL of TEO buffer containing 10 mM DTT. The suspensions were rotated and centrifuged (above), and each supernatant was mixed with 50 μL of sample buffer. After boiling, 3- and 10-μL aliquots of each fraction (load, unbound, and eluate) were electrophoresed, blotted, and immunostained for E2-230K (above).

Conjugation Assay. Standard assays contained (10 μL, pH 7.3, 37 °C) 50 mM Tris-HCl (24% base), 5 mM $MgCl_2$, 2 mM ATP, an ATP regenerating system, 0.6 unit/mL pyrophosphatase, 2 μM ^{125}I-ubiquitin, 0.1 μM E1, a conjugative substrate (2 mg/mL α-casein or 0.1 mg/mL H2B), and 20−200 nM E2-230K (Klemperer et al., 1989). Assays were quenched during the linear phase of conjugation (usually at 4 min) with SDS sample buffer. After boiling, an aliquot was electrophoresed (10% or 12.5% gel). The dried gel was autoradiographed, and the portion of the lane corresponding to ubiquitin−protein conjugates was excised and counted in a γ counter.

Assay of E2−Ubiquitin Adduct Formation. Assays (10 μL) were similar to those for conjugation, except that the substrate was omitted, and the assay was quenched 60 s after mixing. Reactions were quenched with 10 μL of thiol ester sample buffer (lacking mercaptoethanol). A 9-μL aliquot of the quenched reaction was mixed with 9 μL of normal sample buffer (with mercaptoethanol) and boiled. Aliquots of the unboiled (7 μL, lacking mercaptoethanol) and boiled (14 μL, containing mercaptoethanol) samples were electrophoresed (8% or 10% gels), followed by drying and autoradiography. The band corresponding to E2-230K was excised and counted. Radioactivity from the boiled assay was substracted from radioactivity in the unboiled assay to determine the amount of mercaptoethanol-labile adduct (thiol ester).

Kinetics of E2−Ubiquitin Adduct Formation and Effect of Arsenoxide. Iodoacetamide-treated E2 (above) was preincubated for 2 min with 80 or 200 μM NPAsO (15−30 μL, TEO buffer, 37 °C). The control contained either no NPAsO or 80 μM NPAsO together with 100 μM DTT. Adduct formation was initiated by adding approximately one-fourth volume of a cocktail that supplied the following components (final concentrations): E1 (0.1−0.3 μM), ^{125}I-ubiquitin (3 μM), $MgCl_2$ (5 mM), and ATP (2 mM). The final concentration of E2-230K ranged from 0.4 to 2 μM. Aliquots of 7 μL were quenched in 13 μL of thiol ester sample buffer. The concentration of E2−ubiquitin adduct was determined (above).

Kinetics of E2−Ubiquitin Adduct Turnover, and Effect of Arsenoxide. Iodoacetamide-treated E2-230K (∼2 μM) was preincubated for 3 min with 0.4 μM E1, 2 mM ATP, and 5 mM $MgCl_2$ (38 μL, TEO buffer, 37 °C). Thiol ester formation was initiated by adding 1.6 μL of 100 μM ^{125}I-ubiquitin. After 30 s, 8 μL was withdrawn and quenched with 12 μL of thiol ester sample buffer. Simultaneously, 6 μL of a chase mixture was added to the 30 μL of remaining pulse, providing 7 μM H2B and 88 μM unlabeled ubiquitin (final concentrations). After 30 and 180 s more, 10-μL aliquots were quenched with 10 μL of thiol ester sample buffer. At the end, 7 μL of each quenched time point was run on an 8% gel. Then a 9 μL aliquot of each quenched time point was mixed with 9 μL of standard sample buffer (containing mercaptoethanol) and boiled; aliquots of 14 μL were run on a 10% gel. The gels were dried and autoradiographed. The effect of NPAsO on E2−ubiquitin adduct turnover was determined similarly, except that 80 μM NPAsO was added ∼5 s before the chase mixture was added, and only a single time point was taken in the chase (at 90 s).

RESULTS

Reversible Inhibition of E2-230K by Phenylarsenoxides. The inhibitory interaction of inorganic arsenite with E2-230K is weak, develops slowly, and is not detectably reversed by DTT within 10 min (Klemperer et al., 1989). The first indication that phenylarsenoxides might exhibit different inhibitory properties came from studies on the interaction between E3-α and phenylarsenoxides (Berleth et al., 1992a). In this work we used a PAsO-Sepharose column to isolate E3. We found that the DTT eluates sometimes contained, besides E3, a low amount of a protein that appeared to be E2-230K (not shown). If this species was indeed E2-230K,

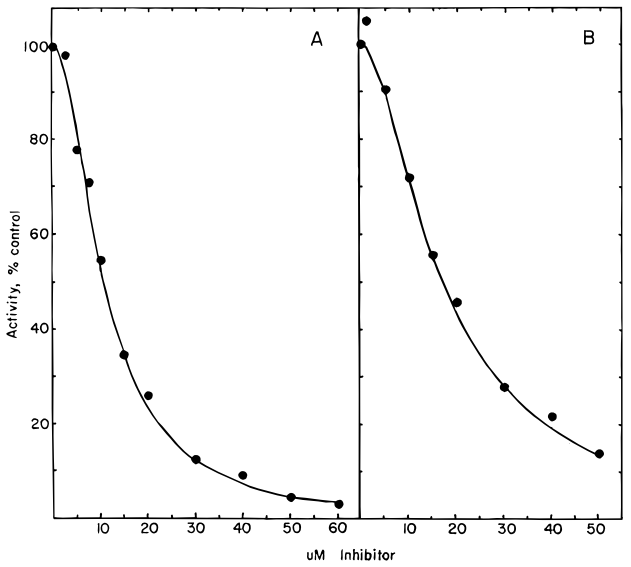


FIGURE 2: Inhibition of E2-230K activity by phenylarsenoxides. E2-230K (~0.4 uM) was preincubated with the indicated arsenoxide reagent for 30 s in TEO buffer at 37 °C. (Appropriate controls showed that a rapid phase of inhibition was complete within 30 s. A second phase of weaker inhibition occurred much more slowly and was not investigated further.) Assays were initiated by adding a cocktail containing the other assay components with casein as substrate. This caused a 2.5-fold dilution of the primary incubation. The final concentration of inhibitor in the assay is shown on the abscissa. Most points are the means of duplicate or triplicate determinations. A) NPAsO (monofunctional reagent). The line was calculated assuming $K_{0.5} = 17.2$ μM and $n_H = 1.7$. (B) BrAcNPAsO (potential bifunctional reagent). The line was calculated assuming $K_{0.5} = 10.7$ μM and $n_H = 1.9$. Addition of DTT (in excess over BrAcNPAsO) fully restored activity at all [BrAcNPAsO] (not shown). The basis of the cooperative concentration dependence is not known.

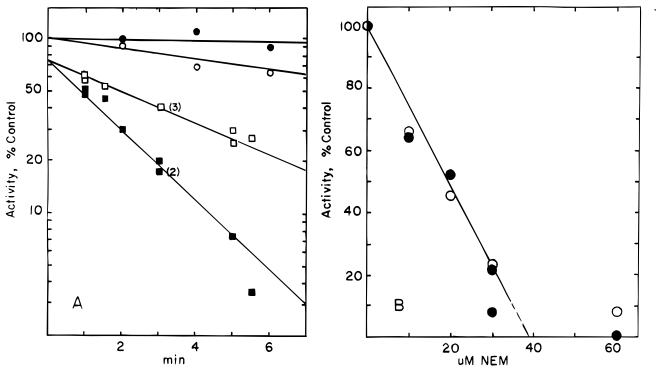


FIGURE 3: Inhibition of E2-230K by NEM and protective effect of phenylarsenoxide. (A) NPAsO protects against inactivation by NEM. E2-230K was first treated with iodoacetamide (Experimental Procedures) to quench DTT in the enzyme storage buffer. Aliquots from the iodoacetamide incubation were diluted into four secondary incubations (TEO buffer, ~10 μL, 37 °C) containing E2-230K (~0.4 μM) and no further addition (●), 80 μM NPAsO (○), 30−40 μM NEM (■), or 30−40 μM NEM together with 80 μM NPAsO (□). At the times indicated on the *abscissa*, 2-μL aliquots were withdrawn and added to assay tubes containing 4 μL of 1.5 mM DTT (in TEO buffer). After ~4 min at 37 °C, the tubes were transferred to ice until all aliquots had been quenched with DTT. Standard assays were then initiated by adding 4 μL of assay cocktail, with H2B used as substrate. Results of three experiments, each involving all four conditions, are combined. The activity observed at 2 min in the control (●) was defined as 100%. The corrected rate constants for inactivation by NEM are 0.43 min^{-1} (■) and 0.14 min^{-1} (□). (B) Inactivation of E2−ubiquitin adduct formation, and substrate ubiquitination, by NEM. Iodoacetamide-treated E2-230K (1 μM) was preincubated with the indicated concentration of NEM for 6 min in TEO buffer at 37 °C. NEM was then quenched by adding 0.21 mM DTT and incubating for 5 min more. One aliquot of the enzyme was then tested for E2−ubiquitin adduct formation (0.5 μM E2, ○), while a second aliquot was assayed for conjugation activity, with H2B as substrate (60 nM E2, ●).

it must have interacted reversibly with the immobilized arsenoxide. We investigated this unexpected phenomenon further.

When the purification scheme was optimized for recovery of E2-230K, the DTT eluate from the PAsO-Sepharose column reproducibly contained an abundant polypeptide of ~200 kDa, along with other, unidentified proteins (Figure 1A). The ~200-kDa protein was E2-230K and not E3-α, which also binds to PAsO-Sepharose (Berleth et al., 1992a), based on criteria that are described in Experimental Procedures. Incorporation of an arsenoxide affinity step provided a significant advantage of E2-230K purification: the yield of E2-230K obtained in the new procedure was 3−5-fold higher than the yield obtained in the original procedure (Klemperer et al., 1989). On the basis of quantitative Western analysis, we estimated that E2-230K represented 0.1−0.3% of the protein in rabbit retulocyte fraction II.[2]

Consistent with tight binding of E2-230K to PAsO-Sepharose, NPAsO was a potent inhibitor of the enzyme, with $K_{0.5} \sim 17$ μM (Figure 2A). Inhibition by NPAsO was manifested within 30 s (legend, Figure 2A) and was rapidly reversed upon addition of excess DTT (not shown), consistent with the ability of DTT to elute E2-230K from PAsO-Sepharose (Figure 1A). These properties contrast with the weak, slow, and irreversible inhibition seen with inorganic arsenite ($K_{0.5} \sim 3.3$ mM; Klemperer et al., 1989). E2-230K binds to PAsO-Sepharose in the presence of 1 mM cysteine (Experimental Procedures), suggesting that the enzyme−phenylarsenoxide complex is resistant to monothiols. This inference was confirmed by showing that a monothiol reagent, β-mercaptoethylamine, did not restore activity to the NPAsO-inhibited enzyme (not shown). These results are consistent with a mechanism in which phenylarsenoxides bind to vicinal thiols of the E2 (eq 1 above).

BrAcNPAsO is a bifunctional arsenoxide whose *p*-alkyl halide moiety may irreversibly alkylate a nucleophile proximal to the arsenoxide binding site (Stevenson et al., 1978). BrAcNPAsO acts in this fashion to inactivate at least two components of the ubiquitin pathway, Arg-tRNA protein transferase and E3-α (Berleth et al., 1992a,b; Li & Pickart, 1995). However, BrAcNPAsO was a strictly reversible inhibitor of E2-230K, with an affinity comparable to that of the monofunctional reagent NPAsO (Figure 2B).

Enzymic Thiols Mediate the Binding of Phenylarsenoxides to E2-230K. Although most E2s are rapidly inactivated by iodoacetamide due to alkylation of the active-site Cys thiol (Hershko et al., 1983), both the conjugative activity of E2-230K and its ability to form a labile ubiquitin adduct are resistant to millimolar concentrations of iodoacetamide [Klemperer et al. (1989) and data not shown]. However, E2-230K is rapidly inactivated by a different thiol-alkylating agent, NEM. At 30−40 μM NEM, 75% of the activity was lost in a first-order process, with a half-life of 1.6 min; this phase followed loss of 25% of the activity in a rapid burst whose origin is unclear (filled squares vs. filled circles, Figure 3A). This sensitivity to NEM indicates that E2-230K possesses one or more essential thiols. Presumably these thiol(s) are resistant to iodoacetamide due to specific

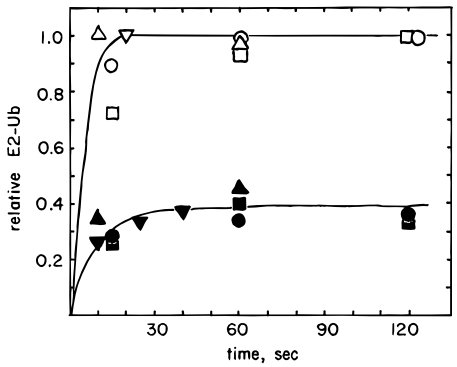


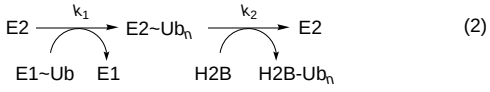
FIGURE 4: Kinetics of E2−ubiquitin adduct formation and inhibitory effect of phenylarsenoxide. Iodoacetamide-treated E2-230K was preincubated for 3 min without NPAsO (▽), in the presence of 80 μM NPAsO plus 100 μM DTT (○, □, △), in the presence of 80 μM NPAsO (●, ■, ▲), or in the presence of 200 μM NPAsO (▼). E2−ubiquitin adduct formation was initiated by adding a cocktail containing ^{125}I-ubiquitin, E1, and MgATP; aliquots were quenched at the indicated times (Experimental Procedures). Data from four independent experiments are combined. In each experiment, the level of E2−ubiquitin adduct was normalized to the maximum level in the corresponding control (open symbols).

environmental features.

Treatment of E2-230K with NEM abolished formation of the labile E2-ubiquitin adduct (open circles, Figure 3B). The similar concentration dependence of loss of activity in adduct formation and substrate ubiquitination (filled vs open circles, Figure 3B) is consistent with the expectation that the adduct is an obligatory intermediate in conjugation, as seen for other E2s (Jentsch et al., 1991). The data shown in Figure 3B moreover suggest that an NEM-reactive Cys residue is a site of ubiquitin thiol ester formation.

If phenylarsenoxides and NEM interact with a common Cys residue, then reversibly bound NPAsO might protect against irreversible alkylation by NEM. This was the case. A saturating concentration of NPAsO reduced the first-order rate constant for NEM inactivation by 3-fold, without affecting the burst (Figure 3A, open vs filled squares). The converse experiment is shown in Figure 1B: pretreatment with NEM completely prevented binding of E2-230K to PAsO-Sepharose (compare lanes 4 and 8). These results are consistent with a model in which phenylarsenoxides bind to vicinal Cys residues of E2-230K. Moreover, at least one of the vicinal thiols may also react with NEM and with ubiquitin (above).

A Nonchemical Step Is Rate-Limiting in E2-230K-Catalyzed Conjugation. Before addressing the mechanistic basis of inhibition by phenylarsenoxides, we characterized the kinetics of formation and turnover of the E2−ubiquitin adduct. The experiments shown in Figures 4−6 can be interpreted according to the following scheme (eq 2). The



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designation of the E2−ubiquitin adduct as E2∼Ub$_n$ acknowledges the likelihood, based on results to be described below, that the labile E2 adduct bears more than one ubiquitin.

As shown in Figure 4 (open symbols), E2−ubiquitin adduct formation was very fast, with a half-time of <10 s, indicating $k_1 > \sim 6$ min^{-1} (eq 2). The concentrations of E1 and E2-230K used in this experiment were 0.1−0.3 μM and 0.4−1 μM, respectively. At 3 μM E1 the reaction was even faster (complete by 10 s, not shown). Such rapid thiol ester formation is characteristic of E2 enzymes [*e.g.*, Pickart et al. (1994)].

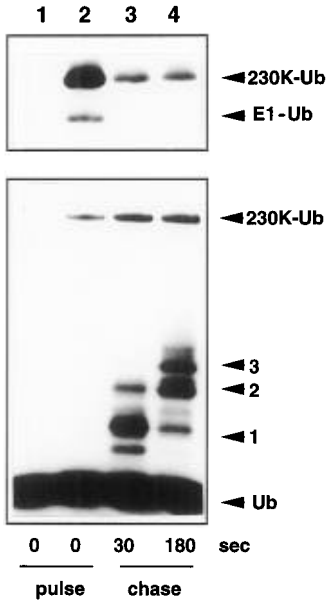

FIGURE 5: Turnover kinetics of E2−ubituitin adduct (autoradiographs): adduct (top) and conjugate products (bottom). The E2−ubiquitin adduct was formed in a 30-s preincubation (lane 2), followed by a chase with excess unlabeled ubiquitin and 7 μM H2B (Experimental Procedures). Aliquots were withdrawn at 30 s (lane 3) and 180 s (lane 4) in the chase. Lane 1 shows a 30-s control pulse in which unlabeled and labeled ubiquitin were mixed prior to E2 addition. Samples were analyzed both for labile E2−ubiquitin adduct (8% gel), and ubiquitin-protein conjugates (10% gel). In the top panel, only the region of the 8% gel containing the E1− and E2−ubiquitin adducts is shown. Both labile and (auto)-conjugated adducts are detected in this assay. The bottom panel shows the entire 10% gel. Only conjugated species are detected in this assay. The positions of labile (and/or conjugated) E2−ubiquitin adduct (230K-Ub), labile E1−ubiquitin adduct (E1-Ub), unconjugated ubiquitin (Ub), and mono-, di-, and triubiquitinated H2B (1, 2, and 3, respectively) are indicated.

Transfer of the E2-bound ubiquitin to the target protein was also very rapid. In the experiment shown in Figure 5, the thiol ester adduct was formed in a 30-s pulse with ^{125}I-ubiquitin (lane 2, top). Excess unlabeled ubiquitin was then added simultaneously with the substrate, histone H2B. All of the E2-bound ubiquitin was transferred to the histone within 30 s (lane 3, top and bottom). In another experiment, transfer was found to be complete within 20 s, indicating $k_2 > 8$ min^{-1} (eq 2). From parallel steady-state assays, the turnover number under these conditions was 0.8 min^{-1} (7 μM H2B; V_{max}). Thus the entire pool of E2−ubiquitin adduct is catalytically competent. Strikingly, neither the formation of this intermediate (Figure 4) nor its turnover (Figure 5) is rate-limiting for steady-state ubiquitination. Besides catalyzing histone conjugation, E2-230K also underwent an apparent autoconjugation reaction (lanes 3 and 4, top and bottom, figure 5). This reaction was time-dependent and was more prominent when substrate was omitted from the incubation (not shown).

At longer times in the chase, the sizes of the labeled conjugate products continued to increase due to conjugation of additional unlabeled ubiquitin(s) (lanes 3 vs 4, bottom, Figure 5). Thus the ubiquitinated histone product formed

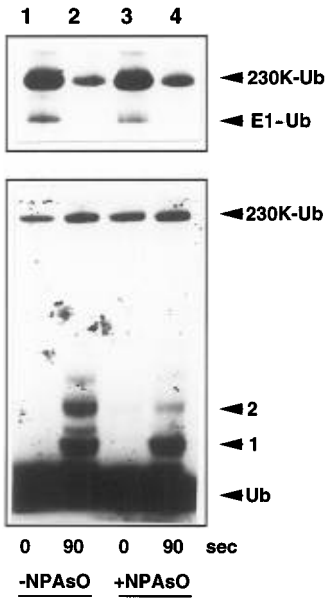

FIGURE 6: Phenylarsenoxide does not inhibit turnover of the E2−ubiquitin adduct (autoradiographs): adduct (top) and conjugates (bottom). The E2−ubiquitin adduct was formed in a 30-sec pulse incubation (lanes 1 and 3). The chase with unlabeled ubiquitin and H2B was carried out for 90 sec, without (lane 2) or with (lane 4) 80 μM NPAsO (see Experimental Procedures). As in Figure 5, only the region of the 8% gel containing the E1− and E2−ubiquitin adducts is shown (top panel), while the entire conjugate-containing 10% gel (bottom panel) is shown. Notations are the same as in Figure 5.

during the first turnover of the (labeled) E2 adduct (~1.9 μM) must have competed efficiently with the larger pool of unconjugated histone (~5.1 μM). The simplest explanation for this competition is kinetic; *i.e.*, dissociation of the conjugate product was slow relative to its further ubiquitination. If this explanation is correct, it is likely that product dissociation is rate-limiting for steady-state turnover.

In Figure 5 (bottom), and under steady-state conditions, histone conjugates bearing three or four ubiquitins were detected. These multiple ubiquitins were present as monoubiquitin moieties independently ligated to Lys residues of the histone, rather than as multiubiquitin chains, since the distribution of ubiquitinated histone species was unchanged when wild-type ubiquitin was replaced with K48R-ubiquitin or reductively methylated ubiquitin.[2] K48R-ubiquitin cannot be assembled into the most common type of multiubiquitin chain (Chau et al., 1989), while reductively methylated ubiquitin cannot form any type of isopeptide chain (Hershko & Heller, 1985).

Site of Inhibition by Phenylarsenoxides. Since the data shown in Figures 1 and 3 suggested that phenylarsenoxides might interact with active-site Cys residues, we examined the effect of NPAsO on formation and turnover of the E2−ubiquitin adduct. These experiments utilized NPAsO at a concentration, 80 μM, that essentially abolished steady-state activity (Figure 2A). Addition of NPAsO to the preformed adduct had no effect on its turnover (lanes 1 and 2 vs lanes 3 and 4; top, Figure 6). Since adduct turnover is so rapid, modest inhibition would not have been detected. On the other hand, since adduct turnover is at least 10 times faster than steady-state turnover (above), modest inhibition cannot account for the complete inhibition of overall activity seen at 80 μM NPAsO.

Although it did not inhibit turnover of the preformed E2−ubiquitin adduct, NPAsO blocked conjugation of unlabeled ubiquitins during the chase (lanes 2 vs 4; bottom, Figure 6), suggesting that the arsenoxide inhibited the next round of (unlabeled) adduct formation. In the experiment shown in Figure 4 (filled symbols), E2-230K was preincubated with NPAsO prior to initiating thiol ester formation with labeled ubiquitin. NPAsO decreased the *extent* of thiol ester formation by ~60% but had no detectable effect on the rate of this process. The effect of NPAsO on the extent of E2−ubiquitin adduct formation (Figure 4) was identical when the concentration of E1 was increased ~30-fold (to 3 μM, not shown). In addition, NPAsO (80 μM) did not inhibit charging of E2-25K with ubiquitin (not shown). These two observations confirm that the effect on charging seen in Figure 4 is due to interaction of NPAsO with E2-230K rather than with E1. The issue of whether the partial inhibition seen in Figure 4 can explain the virtually complete inhibition of turnover seen in Figure 2 is considered in the Discussion.

DISCUSSION

Mechanism of Phenylarsenoxide Binding to E2-230K. Reversible inhibition by trivalent arsenicals is usually considered to be diagnostic for the presence of vicinal thiol groups (Stevenson et al., 1978; Berleth et al., 1992a; Hoffman & Lane, 1992; Kalef et al., 1993), although another binding mode apparently exists for these reagents.[3] In the case of E2-230K, two additional observations strongly support a binding mechanism involving vicinal thiols (eq 1): pretreatment of the enzyme with the thiol-alkylating agent NEM blocked enzyme binding to immobilized phenylarsenoxide (Figure 1B), and the presence of bound inhibitor significantly slowed the rate of enzyme inactivation by NEM (Figure 3A). Bound arsenical protects enzymic thiols against alkylation in lecithin cholesterol acyltransferase (LCAT), an enzyme known to possess vicinal Cys residues (Jauhiainen et al., 1988). As with LCAT, the simplest explanation for our findings is that NEM and NPAsO react with at least one common, and functionally important, thiol group. However, we cannot exclude the possibiity that the two reagents react with distinct, but spatially proximal, residues, such that the presence of one reagent sterically hinders access of the other. As discussed below, we propose that at least one of the arsenoxide-binding Cys residues forms a thiol ester with ubiquitin. Characterization of the E2-230K-encoding cDNA, in progress, may allow us to use site-specific mutagenesis to address this hypothesis in future experiments. This hypothesis predicts that mutation of the relevant Cys residue, for example to Ala, should abolish both enzyme activity and enzyme binding to PAsO-Sepharose.

Catalytic Mechanism of E2-230K: *Insights from Microscopic Kinetic Analysis*. We orginally classified E2-230K as an E2 based in part on the observation that it formed a labile adduct with ubiquitin (Klemperer et al., 1989). We have now rigorously demonstrated the kinetic competence of this intermediate: it forms, and turns over productively, at rates that are about 10 times faster than steady-state turnover (Figures 4 and 5). As expected for a mechanism in which the adduct is an obligatory intermediate, blocking its formation prevented catalysis (Figure 3B). Our previous

[3] J. Li and C. Pickart, *Biochemistry*, in press.

observation that E2-230K is resistant to high concentrations of iodoacetamide raised the possibility that ubiquitin was bound to the E2 in a non-thiol ester linkage (Klemperer et al., 1989). However, the NEM sensitivity of the enzyme, the high specificity of this reagent for thiols, and the firmly established thiol ester mechanism of the E2 protein family (Jentsch et al., 1991) are all consistent in supporting a thiol ester structure for the E2−ubiquitin adduct.

The ubiquitinated histone formed in a single turnover of the labile E2 adduct competes successfully with excess unconjugated histone in subsequent rounds of turnover (Figure 5). This finding, and the observed rapidity of the chemical steps (Figures 4 and 5), suggest that dissociation of the conjugate product is rate-limiting for steady-state tunover, although further work will be necessary to substantiate this model. Slow dissociation of conjugate products, leading to processive conjugation, is seen with at least one E3 enzyme (Reiss et al., 1989) and in histone conjugation catalyzed by the low molecular weight enzyme E2-20K (Pickart & Vella, 1988b). However, E2-230K differs from E2-20K in being sensitive to arsenoxides and in autonomously ubiquitinating erythroid cell proteins.[2]

Mechanistic Basis of Inhibition by Phenylarsenoxides. Addition of NPAsO to the pre-formed adduct did not inhibit its turnover (Figure 6, top). However, the presence of bound inhibitor during adduct formation decreased the level of labile, E2-bound ubiquitin by 60% (Figure 4). The inability of this residual E2-associated ubiquitin to be transferred to substrate is shown by the results in Figure 6, bottom. In this experiment, the reduced level of adduct would pertain after the first round of adduct turnover in the presence of inhibitor (*cf.* Figure 4). Comparison of lane 4 to lane 2 shows that, in the presence of NPAsO, there was no ubiquitin transferred to substrate after this first turnover (Results).

The partial inhibition of adduct formation seen in Figure 4 can lead to the complete inhibition of substrate conjugation seen in Figure 2 if catalysis by E2-230K follows the model shown in Figure 7. This model postulates that there are at least three Cys residues in the active site, two of which form thiol esters with ubiquitin, in an obligatory sequence. Specifically, Cys_a is charged with ubiquitin by E1; ubiquitin is passed from Cys_a to Cys_b; and finally, ubiquitin is transferred from Cys_b to the substrate Lys residue. In this model, Cys_a and Cys_b can be simultaneously charged with ubiquitin. If the ligands of bound arsenic are Cys_b and a third Cys in the active site (Cys_x in Figure 7), then the presence of bound inhibitor is expected to decrease the total level of labile E2-associated ubiquitin by 50%, close to the decrease of ~60% observed in Figure 4.

Although we cannot exclude the possibility that phenylarsenoxide exerts an additional inhibitory effect beyond that documented in Figure 4, there is no positive evidence to support this idea. The model shown in Figure 7 is the simplest one that is consistent with the strikingly distinct effects of saturating concentrations of NPAsO on adduct level and steady-state turnover (Figure 2 vs Figure 4). This model is also consistent with several other features of our data. For example, it predicts that NPAsO will not bind to the fully charged enzyme, since one of the thiol ligands of arsenic is covalently bound to ubiquitin. Thus, there should be no effect on turnover of the fully charged enzyme, as seen in Figure 6 (top). Once the bound ubiquitin has been discharged to the substrate, however, NPAsO binding will

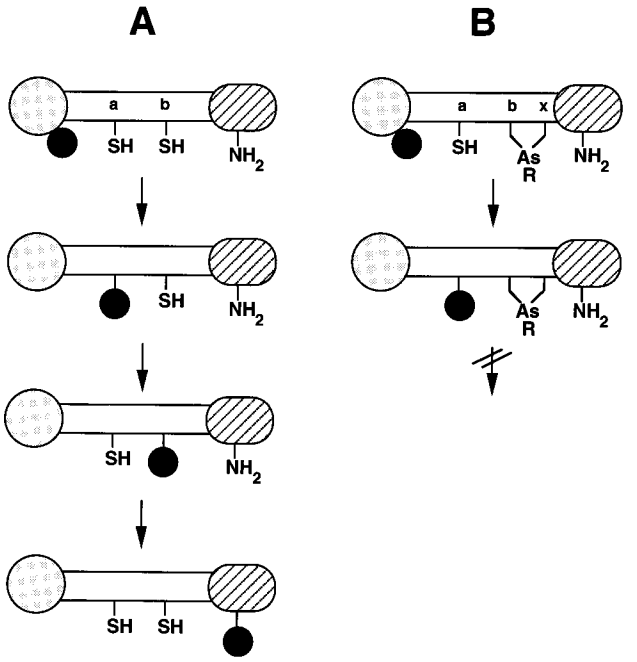

FIGURE 7: Proposed mechanism of E2-230K and mechanism of inhibition by phenylarsenoxides. The long box represents E2-230K; the gray circle is E1; the striped oval is a bound substrate. Panel A: Normal mechanism. Cys_a is charged with ubiquitin (black circle) by E1. Ubiquitin is passed from Cys_a, to Cys_b to a Lys residue of the bound substrate. Panel B: Effect of phenylarsenoxides. The arnseoxide binds to Cys_b and to a third Cys (Cys_x) near the active site. In the simplest case, Cys_x is not involved in the normal mechanism. Bound arsenoxide prevents charging of Cys_b, and thus substrate conjugation, but has no effect on charging of Cys_a.

compete successfully with the charging reaction in the "next" round of turnover (Figure 6, lanes 2 and 4, bottom). Our model is also consistent with the previous finding that the enzyme was protected against arsenite during steady-state turnover (Klemperer et al., 1989). Protection presumably reflected the presence of the charged form of the enzyme, since substrate alone afforded no protection (Klemperer et al., 1989). The failure of arsenite binding (unlike NPAsO binding) to compete with the ubiquitin charging reaction presumably reflects the extremely slow kinetics of inactivation seen with arsenite (Klemperer et al., 1989). We speculate that the marked kinetic differences between phenylarsenoxides and arsenite are due to the different polarities of the substituents in the two reagents, with the more hydrophobic phenyl group being better suited to occupy the active site. A relatively hydrophobic active site might also explain why the enzyme is very sensitive to NEM but resistant to the more polar reagent iodoacetamide. Finally, preliminary experiments suggest that $K_{0.5}$ for inhibition by NPAsO is identical at saturating and subsaturating concentrations of H2B.[2] This result argues against the unexpected possibility that phenylarsenoxides additionally compete for the substrate-binding site on E2-230K.

The model shown in Figure 7 predicts that the Ub/E2 stoichiometry in the labile adduct should be 2:1. We have not yet been able to verify this prediction. The large size of the enzyme makes it impossible to use size as a criterion of whether the adduct bears one or two ubiquitins. The enzymes's lability, and the low amounts of enzyme available to us, have so far prevented a reliable calculation of stoichiometry based on incorporation of ^{125}I-ubiquitin.

Implications for E2-230K Structure−Function. Ubiquitin conjugation catalyzed by E3 enzymes apparently involves an intermolecular relay of E1−, E2−, and E3−ubiquitin thiol esters (Scheffner et al., 1995). The model shown in Figure 7 postulates that E2-230K utilizes an *intra*molecular thiol relay in its catalysis. In this model, Cys_a functions to accept ubiquitin from E1 and transfers ubiquitin to a site that can access the substrate. This is analogous to the classical role of an independent E2 enzyme. Cys_b accepts ubiquitin from Cys_a and transfers it to the target protein. The proposed role of Cys_b is analogous to the mechanistic role of an E3 active-site Cys. Thus, in our model E2-230K functions as an E2−E3 hybrid. The unusually large size of E2-230K would then reflect the presence of these two types of catalytic sites, which are presumably combined with extensive regions that are dedicated to substrate specificity, as seen in the typical E3 (Huibregste et al., 1993). Testing the model shown in Figure 7 is the objective of ongoing work. These studies should be facilitated by the anticipated availability of the E2-230K cDNA.

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