Structure and Function of Ubiquitin Conjugating Enzyme E2-25K: The Tail Is a Core-Dependent Activity Element†

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ABSTRACT: Individual members of the conserved family of ubiquitin conjugating enzymes (E2s) mediate the ubiquitination and turnover of specific substrates of the ubiquitin-dependent degradation pathway. E2 proteins have a highly conserved core domain of ∼150 amino acids which contains the active-site Cys. Certain E2s have unique terminal extensions, which are thought to contribute to selective E2 function by interacting either with substrates or with *trans*-acting factors such as ubiquitin-protein ligases (E3s). We used the mammalian ubiquitin conjugating enzyme E2-25K in a biochemical test of this hypothesis. The properties of two truncated derivatives show that the 47-residue tail of E2-25K is necessary for three of the enzyme's characteristic properties: high activity in the synthesis of unanchored K48-linked polyubiquitin chains; resistance of the active-site Cys residue to alkylation; and an unusual discrimination against noncognate (nonmammalian) ubiquitin activating (E1) enzymes. However, the tail is not sufficient to generate these properties, as shown by the characteristics of a chimeric enzyme in which the tail of E2-25K was fused to the core domain of yeast UBC4. These and other results indicate that the specific biochemical function of the tail is strongly dependent upon unique features of the E2-25K core domain. Thus, divergent regions within the conserved core domains of E2 proteins may be highly significant for function. Expression of truncated E2-25K as a glutathione *S*-transferase (GST) fusion protein resulted in the apparent recovery of E2-25K-specific properties, including activity in chain synthesis. However, the catalytic mechanism utilized by the truncated fusion protein proved to be distinct from the mechanism utilized by the wild-type enzyme. The unexpected properties of the fusion protein were due to GST-induced dimerization. These results indicate the potential for self-association to modulate the polyubiquitin chain synthesis activities of E2 proteins, and indicate that caution should be applied in interpreting the activities of GST fusion proteins.

Ubiquitin-dependent proteolysis is the predominant mechanism for turnover of short-lived proteins in eukaryotic cells (*1*). The role of ubiquitin in proteolysis is that of a covalent signal: ubiquitination allows target proteins to be recognized by the 26S proteasome. The multienzyme ubiquitin pathway is responsible for the turnover of key regulatory proteins, including mitotic cyclins (*2*, *3*), transcription factors (*4*), and the tumor suppressor protein p53 (*5*). The pathway also degrades misfolded proteins (*6*).

Specificity in ubiquitin-mediated proteolysis appears to arise primarily at the level of ubiquitination. In this process (*1*), the carboxyl terminus of ubiquitin (G76) is first activated through ATP-dependent formation of a thiol ester with a Cys residue 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 certain E2 proteins can directly transfer ubiquitin to target proteins [*e.g.*, (*7*)], it appears that target protein ubiquitination is usually accomplished following ubiquitin transfer from the E2 to a Cys residue at the active site of a ubiquitin-protein ligase or E3 (*8*). The E3 then transfers this ubiquitin to an internal Lys residue of the substrate. Conjugation of additional ubiquitins, in the form of a K48-linked polyubiquitin chain, renders the target protein especially susceptible to degradation by the 26S proteasome (*9*).

A large family of E2 proteins is characterized by a core region consisting of ∼150 amino acids that is conserved at the level of both sequence and three-dimensional structure (*10*−*13*). Within this core region is a highly conserved active-site motif containing the Cys residue that forms a thiol ester with ubiquitin (*10*). Certain E2s have additional regions, which may be up to ∼125 residues in length, flanking the 15-kDa core region. These "extensions" are typically divergent relative to comparable regions in other E2 proteins within the same organism.

Molecular genetic studies in the yeast *Saccharomyces cerevisiae* have shown that certain functions of the ubiquitin pathway involve specific members of the E2 protein family

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GST, glutathione *S*-transferase; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin-protein ligase; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyllysyl chloromethyl ketone; Ub_{74}, des-GlyGly ubiquitin (lacking the two C-terminal residues); Ub_{76}, full-length ubiquitin.

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(see Discussion). Such specificity could arise in one (or both) of two ways: interaction of the E2 with the target protein, or interaction of the E2 with an E3 (or other *trans*-acting factor). In several cases, it is apparently the E3 protein that interacts with a specific primary sequence element in the target protein, while the E2 interacts with the E3 [*e.g.*, (*14*)]. However, there is evidence to suggest that certain E2s function in substrate recognition [*e.g.*, (*15*−*17*)]. Regardless of the mechanistic basis of E2 specificity, the interactions leading to such specificity will, in the simplest case, involve regions of the E2 protein that are divergent among the family members within a given organism. Thus, E2 terminal extensions may be specificity elements that direct the interactions of E2 proteins with E3 proteins and/or substrates (*10*, *18*). Several lines of evidence support this hypothesis (see Discussion).

Ubiquitin conjugating enzyme E2-25K is broadly expressed in mammalian tissues (*19*−*22*). *In vitro*, E2-25K is specific for ubiquitin as conjugation target, synthesizing unanchored polyubiquitin chains that are linked exclusively by K48−G76 isopeptide bonds (*19*). This activity, which is unique to E2-25K among known mammalian E2s, is suggestive of a role in protein degradation, as is the high homology between the core of E2-25K and the cores of the E2 proteins encoded by the yeast *UBC1*, *UBC4*, and *UBC5* genes (*20*). The latter three E2s form an essential subfamily that functions in the turnover of short-lived proteins; UBC4 and UBC5 are functionally redundant (*23*, *24*). The biological function of E2-25K remains unknown.

The 47-residue carboxyl-terminal tail of E2-25K is absent in UBC4 and UBC5, and is dissimilar to the corresponding region of UBC1. E2-25K, but not yeast UBC1, catalyzes the synthesis of unanchored polyubiquitin chains *in vitro* (*20*). Based on this finding, we proposed that the tail of E2-25K conferred activity toward ubiquitin as target protein. The studies presented here test this hypothesis through analyses of truncated and chimeric forms of E2-25K. The results support this hypothesis, but also show that specific features of the E2-25K core domain are critical for activity and specificity.

EXPERIMENTAL PROCEDURES

Materials, Enzyme Preparations, and General Methods. Reagents and proteins were from Sigma unless stated otherwise. Bovine ubiquitin or recombinant K48R-ubiquitin was radioiodinated to ∼8000 cpm/pmol with chloramine-T (*25*). E1 was purified to electrophoretic homogeneity from bovine erythrocytes (*26*) or wheat germ (*27*). E2-20K was purified from rabbit reticulocytes (*26*). Purified yeast E1 was a gift of R. Kulka (Hebrew University, Jerusalem). Purified recombinant K48R-ubiquitin was provided by R. Beal (SUNY-Buffalo). The plasmid pRSUbD, encoding a mutant ubiquitin with Asp as the 77th residue, was a gift of C. Larsen and K. Wilkinson (Emory University). Expression and purification of D77-ubiquitin were carried out by established procedures (*28*). Ub_{74} was prepared as described (*29*). SDS−PAGE was carried out by the discontinuous slab method of Laemmli (*30*).

Plasmid Preparations. Standard molecular biological methods were used throughout (*31*). Inserts prepared by PCR were amplified with the proofreading thermostable polymerases Vent (New England Biolabs) or pfu (Stratagene). All mutations were verified by DNA sequencing, either by PCR (fmol kit, Promega) or at the Hopkins Core facility.

(A) Plasmid *pET3d-25K* was generated by digesting pOTS-25K (*20*) with *Nco*I and *Bam*HI. The resulting insert, containing the coding sequence plus ∼130 bp of 3′ untranslated sequence, was ligated into *Nco*I/*Bam*HI-digested pET3d. We recently determined that a sequencing error had misidentified residue 23 of E2-25K as Thr instead of Ser, as confirmed by resequencing the original plasmid pUC19-25K[2] (*20*). Residue 23 is also Ser in human E2-25K (*15*).

(B) The plasmid *pET3d-C170S,F174L-25K* was generated by a two-step PCR method (*32*). Plasmid pUC19-25K (*20*) was used as the template in PCR with mutagenic 5′ primer D1 (5′-GAA AAC CTA TCT GCT ATG GGC TTG-3′) and 3′ flanking primer C2 which contained a *Bam*HI site (*20*). The purified double-stranded product was used as the 3′ primer in PCR with 5′ flanking primer C1 which contains an *Nco*I site (*20*). The final product was digested with *Nco*I and *Bam*HI, and ligated into pET3d.

(C) Plasmid *pET3d-25K*$_{151}$ was generated by PCR amplification using pUC19-25K as template with 5′ primer C1 and 3′ primer C3 (5′-TAG CGG ATC CGC CTA CAC ATG TGC CCA A-3′). The resulting PCR product had a stop codon after V151, followed by a *Bam*HI site. It was ligated into *Sma*I-digested pGEM3Zf(−), then excised with *Nco*I and *Bam*HI, and subcloned into pET3d.

(D) The insert for *pET3d-UBC4* was prepared from a coding insert derived from pPLUBC4 (provided by V. Chau, Wayne State University). A diluted aliquot of this insert was amplified using 5′ primer H1 (5′-GGC TCT AGA GTC GAC CCA TGG CTT CTT CTA AAC G-3′) and a 3′ primer (5′-CCG AGG AGG GAT CCG CAT GCT TAT AC-3′) that introduced sites for *Nco*I and *Bam*HI, respectively; these primers are mainly complementary to sequences flanking the UBC4 coding region. The appropriately digested insert was ligated into pET3d. Primer H1 introduced a Ser to Ala mutation at residue 2 of UBC4 (Results). Relative to the original *UBC4* gene (*23*), the gene in pPLUBC4 also carried an A to G mutation at base 115, which created a Met to Val mutation at residue 39 (Results). These mutations did not affect activity in ubiquitin thiol ester formation (Results); the M39V mutation is without effect on the growth of *S. cerevisiae* (V. Chau, personal communication). The *in vivo* effect of the S2A mutation has not been tested.

(E) The plasmid *pET3d-UBC4/25K* was prepared as follows. The UBC4 coding sequence was amplified as described above, using 5′ primer H1 (above) and a 3′ primer carrying a *Bss*HII site (5′-GGG TAC CGC ATG GCG CGC CCG CGT A-3′). The product was digested sequentially with *Nco*I and *Bss*HII. The E2-25K tail region was amplified using pUC19-25K as template, with 3′ flanking primer C2 (carrying a *Bam*HI site) and 5′ primer D2 carrying a *Bss*HII site (5′-GCT GGC GCG CCA GTT TCT AGT CCA GAG-3′). The product was digested sequentially with *Bam*HI and *Bss*HII. The core- and tail-encoding inserts were combined with *Nco*I/*Bam*HI-digested pET3d in a three-way ligation. We initially constructed a pET vector encoding an E2-14K/25K fusion by similar methods, but this protein was quantitatively proteolyzed to an E2-14K-sized fragment under all expression conditions tested.[3]

[2] L. Mastrandrea and C. Pickart, unpublished experiments.
[3] M. Haldeman and C. Pickart, unpublished experiments.

(F) To generate *pGEX-25K*, primers 25KN (5′-TCC ATG GGA GAC ATG GCC AAC-3′) and 25KC-200 (5′-TAA GGA TCC TTT CAG TTA CTC AGA AGC-3′) were used to amplify the complete E2-25K coding sequence (pUC19-25K as template). The product was cut with *Bam*HI, and then ligated into *Sma*I/*Bam*HI-digested pGEM-3Zf(−) to generate pGEM-25K. The insert was subcloned into *Nco*I/*Bam*HI-digested pGEX-2TK (Pharmacia) that had been modified to carry an *Nco*I site upstream of the *Bam*HI site. This vector also specifies a thrombin cleavage site between the GST and E2 moieties.

(G) To generate *pGEX-25K*$_{153}$, the insert was amplified using pGEM-25K as template, with an M13 reverse primer as the 5′ flanking primer and 25KC-153 (5′-TAA GGA TCC TAA GCA TAC ACA TGT GC-3′) as the 3′ primer. The appropriately digested insert was ligated into pGEX-2TK (above). The GST fusion protein encoded by this vector terminates at A153 of E2-25K.

Expression and Purification of Recombinant E2 Proteins: (A) Soluble pET-Encoded Proteins. Proteins encoded by pET vectors were expressed in *E. coli* strain BL21(DE3)-pLysS as described previously (*32*), except that the induction temperature was 37 °C. Cell pellets were lysed by resuspending in buffer containing 50 mM Tris-HCl (24% base, pH 7.6), 1 mM EDTA, ∼2 mM DTT, 0.1 mM TLCK, 1 mM PMSF, 10 μg/mL leupeptin (Boehringer), 10 μg/mL soybean trypsin inhibitor, and 0.4 mg/mL lysozyme (2 mL of buffer/g of cells). $MgCl_2$ and DNase I were added to 10 mM and 20 μg/mL, respectively, to digest DNA. The suspension was clarified by centrifugation at ∼9000*g* (20 min).

For wild-type E2-25K and C170S,F174L-25K, we collected proteins that precipitated from the 9000*g* supernatant between 40 and 80% saturation with ammonium sulfate, and then carried out gradient elution (*19*, *33*) from an FPLC monoQ column (Pharmacia−LKB Biotech). These E2-25K derivatives were 80−90% homogeneous following this two-step purification scheme, and were recovered at 10−50 mg of purified protein/L of cell suspension, with C170S,F174L-25K exhibiting severalfold higher expression.[3]

UBC4 and the UBC4/25K chimera were expressed at much lower levels, and were generally assayed in the 9000*g* supernatant (above). In some cases, these enzymes were partially purified by anion exchange chromatography. Crude lysate proteins were applied to a column of Q-Sepharose (8−15 mg of protein/mL of resin). For UBC4, we collected the unbound fraction. For E2-25K and UBC4/25K, the column was washed with several volumes of low-salt buffer, and then eluted with 3 volumes of buffer containing 0.17 M NaCl.

(B) Insoluble Protein: E2-25K$_{151}$. This derivative partitioned to inclusion bodies under all expression conditions tested. Inclusion bodies were purified by a modification of a published procedure (*34*). The cell pellet from a 2 L culture was resuspended in several volumes of 20 mM Tris (24% base) containing 20% w/v sucrose and 1 mM EDTA. After 10 min on ice, the cells were pelleted and frozen. One gram of cells was lysed by suspending in 10 mL of phosphate-buffered saline supplemented with 1 mM EDTA, 1 mM DTT, 0.1 mM TLCK, 0.1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL soybean trypsin inhibitor, and 1 μg/mL pepstatin. DNase I (0.8 mg) and RNase A (2.6 mg) were added. After 10 min at RT, the suspension was diluted with 40 mL more lysis buffer, and then centrifuged at 13000*g* (30 min). The pellet was suspended in 60 mL of phosphate-buffered saline supplemented with 25% w/v sucrose, 2.5 mM EDTA, and 1% v/v Triton X-100. Following 10 min on ice, inclusion bodies were pelleted by centrifugation at 25000*g* (10 min). This washing step was repeated to generate the final inclusion body preparation. Inclusion bodies were suspended in 20 mL of denaturation buffer containing 50 mM Tris-HCl (50% base, pH 8.0), 5 M urea, and 5 mM EDTA. After 1 h on ice, insoluble material was removed by centrifuging at 12000*g* (30 min). To renature the denatured protein, the supernatant was poured slowly into 200 mL of renaturing buffer containing 50 mM Tris-HCl (50% base), 1 mM DTT, 20% v/v glycerol, 0.1 mM PMSF, 10 mM TLCK, 1 μg/mL trypsin inhibitor, 1 μg/mL leupeptin, and 0.1 μg/mL pepstatin. The resulting suspension was stirred gently at 5 °C overnight. Insoluble material was removed by centrifugation (above); the concentration of urea was reduced from 0.5 to 0.1 M by concentration and dilution (Centricon-3, Amicon). In assays of E2-25K$_{151}$, the final concentration of urea ranged from 20 mM (Figures 3 and 4, Results) to 40 mM (Table 1, Results). Control studies showed that the presence of 0.1 M urea in the assay had no effect on diubiquitin synthesis catalyzed by E2-25K.[3] To provide a control for studies with E2-25K$_{151}$, wild-type enzyme was similarly denatured and refolded. Enzyme for this purpose was expressed from pOTS-25K in *E. coli* strain AR120 (*20*), since a higher yield of inclusion bodies was obtained in this system. Cell pellets were resuspended in lysis buffer (above) and lysed using a French press. The rest of the procedure was as described above, except that ovalbumin was added as carrier to enhance protein recovery during renaturation. For the experiment shown in Table 1 (Results), we diluted 2 mg of purified C170S,F174L-25K (below) into 1 mL of denaturation buffer. The rest of the protocol was as described above, except that the volumes were reduced 10-fold. The refolded E2 enzymes were 80−90% homogeneous based on SDS−PAGE and Coomassie or silver staining (data not shown).

(C) GST Fusion Proteins. These derivatives were expressed in *E. coli* strain BL21(DE3)pLysS (GST-25K$_{200}$) or UT5600 (GST-25K$_{153}$). Induction and lysis were carried out by procedures similar to those used for expression from the pET3d-25K vector (above), except that cells were grown at room temperature instead of 37 °C. Cell lysates were clarifed by centrifugation at 13000*g* for 10 min. The GST fusion proteins were purified using GSH−agarose according to a protocol from Pharmacia−LKB Biotech. Typically, 0.2 mL of resin allowed the recovery of ∼0.4 mg of fusion protein from lysate derived from a 100-mL culture. The GSH concentration in the eluate was reduced from 10 mM to <0.5 mM by repeated dilution and reconcentration in a Centricon-10 device (Amicon), using a buffer of 50 mM Tris-HCl (24% base), 0.1 mM EDTA, and 0.2 mM DTT. The two GST fusion proteins were 80−90% homogeneous based on SDS−PAGE and Coomassie staining (data not shown). To remove the GST moiety from purified GST-153, the protein (0.1 mg) was treated with 1.5 units of thrombin (U.S. Biochemicals) for 2 h at room temperature, in 0.2 mL of phosphate-buffered saline containing 1 mM DTT. Following this treatment, GST and uncleaved fusion protein were removed by passing the incubation through GSH−agarose. The cleaved E2 product was designated E2-25K$_{153}$. It bears 15 extra pGEX-encoded residues at its N-terminus (GSRRASVESHMPMGD).

Ubiquitin Thiol Ester Assay. Incubations (5−20 μL) contained 50 mM Tris-HCl (24% base), 5 mM $MgCl_2$, 2

mM ATP, a creatine phosphate/kinase-based ATP-regenerating system, 0.3 unit/mL inorganic pyrophosphatase, 20−100 nM E1, and 2 μM ^{125}I-ubiquitin (pH 7.3, 37 °C). Assays were initiated by adding E2 (∼0.5−2 μM), and quenched after 3−5 min with sample buffer lacking mercaptoethanol. E2−ubiquitin adducts were detected by electrophoresis on 12.5% SDS−PAGE gels, followed by autoradiography (*7*). In some cases, bands were exised and counted for quantitation. Controls showed that all of the E2-associated ^{125}I-ubiquitin was labile to treatment with β-mercaptoethanol, unless stated otherwise.

Diubiquitin Synthesis: Pulse−Chase Assay. Conditions in the pulse incubation (5 min) were as described for thiol ester formation (above). After removing an aliquot to monitor thiol ester formation, the chase was initiated by adding a cocktail providing 1 mg/mL ubiquitin and 2.5−10 mM EDTA, usually in the presence of 0.4 mg/mL ovalbumin as carrier. Aliquots were quenched at increasing times in sample buffer lacking mercaptoethanol, followed by electrophoresis. After autoradiography, E2−ubiquitin bands were excised from the dried gel and counted. Pseudo-first-order rate constants for diubiquitin synthesis were obtained from semi-log plots of thiol ester radioactivity *versus* chase incubation time (*19*).

Diubiquitin Synthesis: Continuous Assay. This assay monitors the ligation of G76 of ^{125}I-K48R-ubiquitin (2 μM) to K48 of D77-ubiquitin (0.25−5 mg/mL). Other conditions were essentially the same as in the pulse of the pulse−chase assay, except that the E2 concentration was usually 0.3 μM. Aliquots (5 μL) were quenched in an equal volume of sample buffer and boiled; 5 μL of the quenched sample was electrophoresed on a 13.5% SDS−PAGE gel. Following autoradiography, diubiquitin bands were excised and counted.

Iodoacetamide Inactivation. Wild-type or mutant E2-25K (∼1 μM) was preincubated for 10 min at 37 °C with iodoacetamide (1−3 mM) in a buffer containing 14 mM Tris-HCl (24% base), 0.1 mM EDTA, and ∼0.2 mg/mL carrier ovalbumin (7 μL volume). DTT (1 μL) was then added to a final concentration that was 60% of the iodoacetamide concentration. After 10 min more, thiol ester formation was assayed by adding 2 μL of a cocktail providing E1, MgATP, and ^{125}I-ubiquitin (above). For the control, iodoacetamide and DTT were premixed and incubated for 10 min; the E2 was then added; after 20 min more, thiol ester formation was assayed.

Gel Filtration. Analytical gel filtration analysis of E2 proteins was carried out on a 0.7 × 25 cm column of Sephacryl-200 (Pharmacia-LKB), in a buffer of 50 mM Tris-HCl (24% base), 0.1 mM EDTA, 0.4 mM DTT, and 0.1 mg/mL ovalbumin. In some runs, this buffer was supplemented with 0.2 M NaCl. Columns were run at 5 °C. Samples were applied in a volume of 0.2 mL; 0.27 mL fractions (9 drops each) were collected into plastic tubes. The void volume was determined with blue dextran. Standard proteins were E1 (116 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and RNase S (13.7 kDa). The peak of E1 was determined by thiol ester assay (above). The peaks of other standard proteins were determined by SDS−PAGE and Coomassie staining of fraction aliquots. E2 proteins were applied to the column at a concentration of 1−3 μM. Elution was monitored by assaying 8 μL aliquots of the fractions for E2-^{125}I-ubiquitin thiol ester formation (with 50 nM added E1, above). The E2-25K, UBC4, and UBC4/25K preparations analyzed in this fashion were partially purified, while GST-25K$_{200}$, GST-25K$_{153}$, and E2-25K$_{153}$ were purified (above).

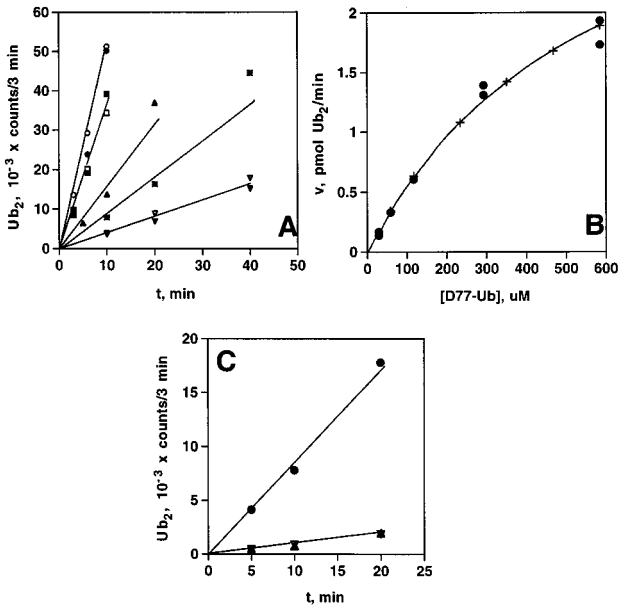

FIGURE 1: Diubiquitin synthesis: continuous assay. (A) Time and concentration dependence. Standard assays (Experimental Procedures) were carried out with C170S,F174L-25K at the following concentrations of D77-ubiquitin (mg/mL): 5 (circles); 2.5 (squares); 1.0 (upright triangles); 0.5 (asterisks); 0.25 (inverted triangles). For the filled symbols, the enzyme concentration was 0.3 μM. For the open symbols, the enzyme concentration was 0.15 μM (circles and squares) or 0.6 μM (inverted triangles), and the data were corrected to correspond to an enzyme concentration of 0.3 μM. Thus, agreement of the filled and open symbols indicates that the rate is linear in [enzyme]. (B) Replot. The filled circles correspond to the data from panel A. The crosses are calculated for K_m = 580 μM and k_{cat} = 0.56 min^{-1}. (C) Activities of truncated E2 derivatives. Assays were carried out at 2 mg/mL D77-ubiquitin, with C170S,-F174L-25K (circles), GST-25K$_{153}$ (inverted triangles), or E2-25K$_{153}$ (upright triangles). The results of simultaneous thiol ester assays were used to normalize the data to the same E2 concentration. The rates observed with both truncated E2 enzymes were 2-fold lower when [D77-ubiquitin] was reduced to 1 mg/mL (data not shown), confirming that the acceptor was free, *vs* E2-bound, ubiquitin (text).

RESULTS

Continuous Assay for Diubiquitin Synthesis. Some of the derivatives of E2-25K used in the present work had low activity in polyubiquitin chain synthesis. To facilitate the analysis of such derivatives, we developed a new assay, which monitors the conjugation of G76 of ^{125}I-K48R-ubiquitin to K48 of D77-ubiquitin. The resulting dimer is blocked at its proximal and distal termini, so labeled diubiquitin is formed as the terminal product. Since the background rate is negligible and product formation is linear with time and E2 concentration, the sensitivity of this assay is high. This contrasts with the pulse−chase assay used in certain experiments, which monitors diubiquitin formation during a single turnover of the labeled E2−ubiquitin thiol ester (*19*). The sensitivity of the pulse−chase assay is low when chain synthesis activity is low, because the E2−ubiquitin thiol ester, which is not regenerated during the assay, hydrolyzes at a significant rate (*33*).

Figure 1A and Figure 1B show the properties of the continuous assay as applied to a mutant form of E2-25K in which C170 and F174 have been changed to Ser and Leu, respectively. These mutations were made in an effort to improve the crystallization properties of E2-25K; the mutant

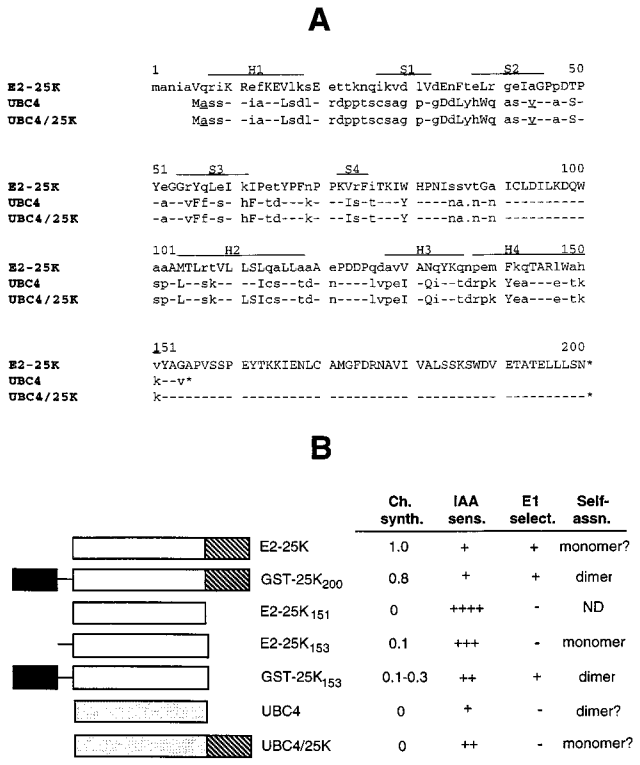

FIGURE 2: (A) Comparison of E2-25K, UBC4, and chimeric E2 protein sequences. Dashes, identical residues; upper case, conservative substitutions (in reference to the E2-25K sequence). The underlined residues in UBC4 (and UBC4/25K) were mutated during cloning (S2A and M39V; Experimental Procedures). The secondary structure elements seen in the UBC4 crystal structure are indicated (Cook et al. 1993). (B) Schematic representation of E2-25K derivatives and their properties. The GST moiety and linker are not to scale. Results: "Ch. synth.", relative k_{cat}/K_m in diubiquitin synthesis; "IAA sens.", relative degree of inactivation by 2 mM iodoacetamide (+, 50−60% inactivation; ++, 60−70%; +++, 70−80%; ++++, >80%; see Table 1 below); "E1 select.", relative selectivity for mammalian E1 (+, only functions with mammalian E1; −, functions with plant or mammalian E1); "Self assn.", self-explanatory (ND, not determined).

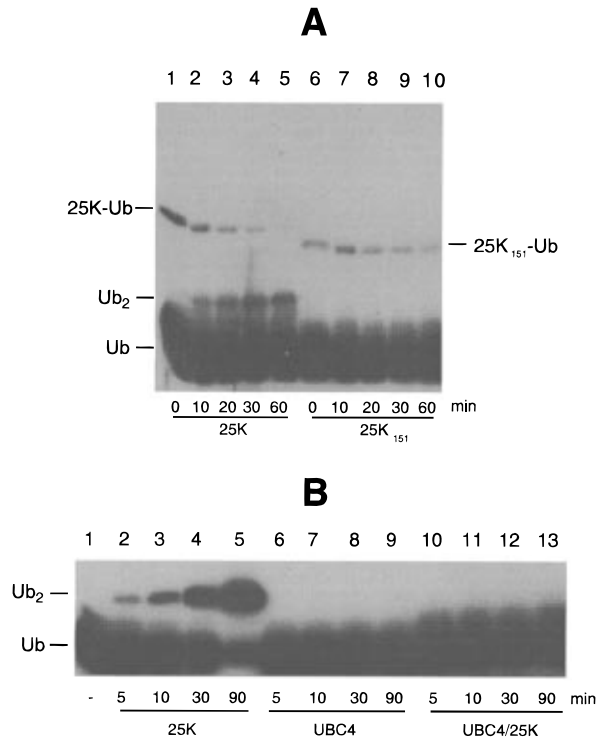

FIGURE 3: Diubiquitin synthesis activities of E2 derivatives (autoradiographs). (A) Purified refolded proteins. Activity was monitored by pulse−chase assay (Experimental Procedures). Chase incubations with E2-25K (lanes 1−5) or E2-25K$_{151}$ (lanes 6−10) were sampled at the indicated times. The full-length enzyme remained active when mixed with the truncated enzyme (not shown), excluding the presence of an inhibitor in the truncated enzyme preparation. (B) Chimeric E2 and controls. Activity was monitored by continuous assay in soluble *E. coli* lysates (Experimental Procedures). Lane 1 is a mock reaction lacking lysate (10 min incubation); lanes 2−5, E2-25K; lanes 6−9, UBC4; lanes 10−13, UBC4/25K.

protein was expressed in *E. coli* at a higher level than wild-type E2-25K (Experimental Procedures). As shown in Figure 1A, with the mutant enzyme product formation in the continuous assay was linear with time (Figure 1A). The rate at low concentrations of D77-ubiquitin ($\leq$100 μM) was directly proportional to the concentration of this acceptor (Figure 1B). At higher acceptor concentrations, there was weak saturation, consistent with K_m = 580 μM (line, Figure 1B). The calculated value of k_{cat}/K_m, 966 M^{-1} min^{-1} (k_{cat} = 0.56 min^{-1}), is similar to values of 500−1200 M^{-1} min^{-1} obtained for wild-type E2-25K (*19*, *33*) and C170S,F174L-25K (data not shown) in the pulse−chase assay. These results indicate that the C170S and F174L mutations are fully permissive for biochemical activity, and in some of the experiments described below, C170S,F174L-25K served as a positive control.

The Tail of E2-25K Is Necessary, but Not Sufficient, for Activity in Polyubiquitin Chain Synthesis. The 153-residue core of E2-25K is highly related to the cores of the UBC1, UBC4, and UBC5 proteins of yeast (Figure 2A) and higher organisms (not shown). However, besides apparent E2-25K homologs in *C. elegans* and *D. melanogaster*, identified in database searches, no other known E2s have tails that strongly resemble the tail of E2-25K. Neither yeast UBC1, which has an unrelated tail, nor yeast UBC4, which lacks a tail, catalyzes the synthesis of unanchored polyubiquitin chains *in vitro* (*20*; below). These observations suggest that the tail of E2-25K is necessary for activity in conjugation.

As a first test of this hypothesis, the 49 C-terminal residues of E2-25K were deleted by placing a stop codon after V151 (Figure 2A). Although E2-25K$_{151}$ was intractably insoluble when expressed in *E. coli*, a fraction of the (purified) truncated protein could be refolded following denaturation in urea (Experimental Procedures). Refolded E2-25K$_{151}$ formed a thiol ester with ubiquitin (Figure 3A, lane 6), but the truncated enzyme was inactive in chain synthesis as monitored by pulse−chase: the thiol ester hydrolyzed, but no diubiquitin was detected (Figure 3A, lanes 7−10). In contrast, full-length E2-25K was active in diubiquitin synthesis following refolding (Figure 3A, lanes 1−5). The simplest interpretation of these data is that residues 152−200 are necessary for activity in chain synthesis. The activity of the refolded full-length enzyme in chain synthesis and the ability of the refolded truncated enzyme to form a thiol ester both suggest that E2-25K$_{151}$ fails to synthesize diubiquitin due to the absence of the tail, as opposed to the presence of a strongly aberrant conformation in the refolded core (see also below).

We undertook additional studies on the function of the tail using GST-25K fusion proteins (Figure 2B). The full-length fusion protein GST-25K$_{200}$ formed a thiol ester with ubiquitin (see below), and retained nearly full activity in chain synthesis. Values of k_{cat}/K_m for GST-25K$_{200}$ were 78% and 75% of the values for C170S,F174L-25K determined in the pulse−chase and continuous assays, respectively (data

not shown). These results indicated that the GST moiety minimally perturbed the properties of full-length E2-25K. We thus expected that truncated E2-25K derivatives could be analyzed as intact GST fusion proteins.

Because GST-25K$_{151}$ was very poorly expressed,[4] we made a slightly shorter 47-residue deletion that also more accurately reflected the core−tail boundary, since Y152 and A153 are strictly conserved among E2-25K, UBC1, UBC4, and UBC5 (Figure 2A). GST-25K$_{153}$ was soluble, and formed a thiol ester with ubiquitin (see below). In pulse−chase assays, GST-25K$_{153}$ synthesized diubiquitin with a rate constant that was only 3.4-fold less than that obtained for GST-25K$_{200}$ under the same conditions.[4] However, we noticed that significant levels of labeled tri- and tetraubiquitin accumulated in the GST-25K$_{153}$ assays (data not shown). This was unexpected, since with native E2-25K there is no route to chains of $n > 2$ in the pulse−chase assay: the ^{125}I-diubiquitin product can neither be activated (due to the presence of EDTA in the chase) nor serve as an acceptor (*33*). These results suggested that the GST moiety had perturbed the activity or specificity of the 153-residue E2 protein. It will be shown below that the high activity of GST-25K$_{153}$ in the pulse−chase assay indeed reflects a new mechanism for chain synthesis that is strictly dependent upon the presence of the GST moiety.

To eliminate any effects of the GST moiety, we used thrombin to cleave GST-25K$_{153}$ in the GST-E2 linker region. The resulting enzyme, E2-25K$_{153}$, formed a thiol ester with ubiquitin (see below), but had strongly reduced activity in chain synthesis: its activity was 10% of the activity of C170S,F174L-25K (Figure 1C, upright triangles). This result is qualitatively similar to that obtained with E2-25K$_{151}$ (no detectable activity, Figure 3A). Thus, results obtained with both truncated derivatives indicate that the tail is a major determinant of activity in chain synthesis. The lower activity of E2-25K$_{151}$ (relative to E2-25K$_{153}$) may reflect either the low sensitivity of the pulse−chase assay or a slight conformational aberration in E2-25K$_{151}$. The low activities of both truncated enzymes make it unlikely that the presence of 15 extra residues at the N-terminus (Experimental Procedures), *versus* the absence of the tail, is the basis of the abrogated activity of E2-25K$_{153}$. Additional evidence in support of this conclusion was the finding that cleavage of GST-25K$_{200}$ with thrombin generated an enzyme that was fully active despite the presence of these extra N-terminal residues (data not shown).

To test whether the tail of E2-25K could function as an independent element conferring activity toward ubiquitin as target protein, we expressed a chimeric E2 in which residues 154−200 of E2-25K were fused to yeast UBC4 (Figure 2A,B). Given that UBC4 is highly homologous to the core of E2-25K (Figure 2A), the low activities of the two truncated E2-25K derivatives predict that UBC4 should have negligible chain synthesis activity. This was the case: as monitored by continuous assay in a soluble lysate prepared from recombinant *E. coli* cells, UBC4 had no activity in chain synthesis (Figure 3B, lanes 6−9). The chimeric protein was also inactive, under conditions in which 5% of the wild-type activity would have been detected (Figure 3B, lanes 10−13). Similar results were obtained in the pulse−chase assay following partial purification of the respective E2s.[3]

[4] G. Xia and C. Pickart, unpublished experiments.

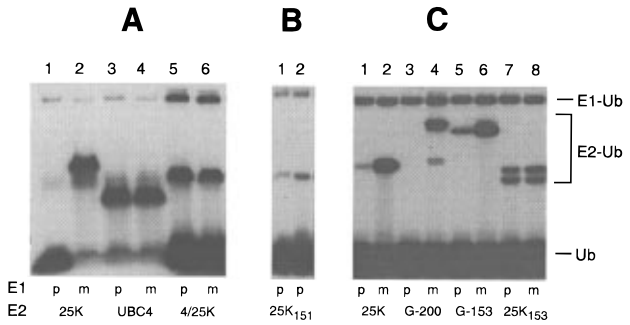

FIGURE 4: E1 specificity of E2-25K and derivatives (autoradiographs). (A) E2-25K, UBC4, and UBC4/25K. Soluble lysates prepared from recombinant *E. coli* cells were assayed for E2−ubiquitin thiol ester formation (Experimental Procedures) with mammalian (m) E1 purified from bovine erythrocytes (lanes 2, 4, 6) or with plant (p) E1 purified from wheat germ (lanes 1, 3, 5). Lanes 1, 2, E2-25K; lanes 3, 4, UBC4; lanes 5, 6, UBC4/25K. In each incubation, the lysate comprised 30−70% of the assay volume; the volume electrophoresed was adjusted to normalize the E2 thiol ester signal. (B) E2-25K$_{151}$. The assay contained ~1 μM purified refolded E2-25K$_{151}$ and 0.1 μM wheat germ E1. Incubation was for 2.5 min (lane 1) or 10 min (lane 2). These assays were carried out at the same time as the assays in Figure 3A. Lanes 6 and 7 of Figure 3A thus provide the mammalian E1 control. (C) Other E2 derivatives. Assays contained ~0.1 μM purified E1 and ~1 μM purified E2: C170S,F174L-25K (lanes 1, 2); GST-25K$_{200}$ (G-200, lanes 3, 4); GST-25K$_{153}$ (G-153, lanes 5, 6); and E2-25K$_{153}$ (lanes 7, 8). Odd-numbered lanes, wheat germ (p) E1; even-numbered lanes, bovine erythrocyte (m) E1. A fraction of the GST-25K$_{200}$ was cleaved at the GST−E2 junction by an *E. coli* protease, giving rise to the lower E2 band in lane 4. The thiol ester of E2-25K$_{153}$ migrates as a doublet, as seen for some other E2s [*e.g.*, (*26*)].

The lack of activity of the chimera was not due to an inhibitor in the lysate, since recombinant E2-25K was active under the same conditions (Figure 3B, lanes 2−5). Nor was lack of activity due to failure to form a ubiquitin thiol ester (see below).

We conclude that the tail of E2-25K is not sufficient to confer polyubiquitin chain synthesis activity on the UBC4 core. Thus, even though the tail is a major determinant of activity, as shown by the inhibitory effect of deleting it (Figures 1C and 3A), core residues that are unique to E2-25K must be present for the tail to exert its stimulatory effect. This conclusion is consistent with the finding that E2-25K$_{153}$ (Figure 1C), but not UBC4 (Figure 3B), had detectable chain synthesis activity. The different properties of the E2-25K and UBC4 cores, both in isolation and in conjunction with the E2-25K tail, must be due to residues that are divergent between them.

The Tail of E2-25K Is a Specificity Element in the Interaction with E1. The above-described experiments implicated the tail as a determinant of activity in chain synthesis. Other experiments showed that the tail contributed to a property of E2-25K that first became apparent when we tried to use E1 purified from wheat germ in our assays. Unexpectedly, E2-25K was virtually inactive in thiol ester formation when assayed with this E1 (Figure 4A,C, lanes 1 *vs* 2). Quantitative experiments with C170S,F174L-25K and GST-25K$_{200}$ showed that the initial rate of thiol ester formation was decreased by more than 100-fold when bovine E1 was replaced by plant E1 (not shown). A 4-fold increase in the concentration of plant E1 did not increase the rate (not shown), indicating that this was a V_{max} effect. The kinetics of E2-25K activation were similar with E1 enzymes purified from human, rabbit, and bovine erythroid cells, and

Table 1: Iodoacetamide Inactivation[a]

enzyme	activity, % of control		
	0.5 mM	2 mM	3 mM
E2-25K	−	51.9 ± 6.0 (3)	37.7 ± 1.5 (3)
C170S,F174L-25K	−	48.1 ± 4.7 (4)	−
C170S,F174L-25K (refolded)	−	46.6 ± 3.5 (4)	−
GST-25K$_{200}$	−	52.4 ± 5.5 (5)	−
E2-25K$_{151}$ (refolded)	<10 (2)	−	−
E2-25K$_{153}$	−	22.9 ± 0.8 (3)	−
UBC4	−	44.4 ± 0.8 (3)	33.6 ± 1.7 (3)
UBC4/25K	−	32.6 ± 3.0 (3)	21.5 ± 1.8 (3)
GST-25K$_{153}$	−	30.5 + 1.6 (3)	−

[a] E2 proteins were incubated with the indicated concentration of iodoacetamide for 10 min at 37 °C. DTT was added to quench the inactivation reaction, followed 10 min later by assay of ubiquitin thiol ester formation. For the control, the enzyme was incubated with prequenched iodoacetamide for 20 min (Experimental Procedures). Purified E2-20K had ≤10% activity following treatment with 1 mM iodoacetamide (not shown). The value for E2-25K$_{151}$ is based on densitometry. Other values are based on band excision and counting. Values shown are mean ± SD of (*n*) determinations.

from rabbit liver, but there was negligible thiol ester formation in assays with yeast E1 (not shown). In contrast, the kinetics of activation of E2-14K, E2-20K, and E2-35K were indistinguishable when these E2s were assayed with plant *versus* mammalian E1 (reactions complete within 2 min, not shown). Therefore, E2-25K discriminates strongly against noncognate (nonmammalian) E1 enzymes in the thiol ester-forming reaction. This behavior is unique to E2-25K among known E2s.

The structure of the E2-25K core is probably similar to the structure of UBC4 (*12*). Since UBC4 did not discriminate against plant E1 in thiol ester formation (Figure 4A, lanes 3 *vs* 4), we tested whether the E1 specificity of E2-25K depended upon the presence of the C-terminal tail. Both E2-25K$_{151}$ and E2-25K$_{153}$ formed thiol esters in the presence of plant E1 (Figure 4B, lanes 1, 2 *vs* Figure 3A, lanes 6 and 7; and Figure 4C, lanes 7 *vs* 8). Loss of selectivity in the case of E2-25K$_{151}$ was not a general consequence of refolding, since E2-25K and C170S,F174L-25K both retained full E1 selectivity after denaturation and refolding (data not shown). These results indicate that the tail of E2-25K is a necessary element in cognate E1 selectivity. However, the tail is not sufficient to confer this property, as shown by the nonselective behavior of the UBC4/25K chimera (Figure 4A, lanes 5 *vs* 6). Thus, core residues that are unique to E2-25K must be present for the tail to confer E1 selectivity. This is similar to the structure−activity correlation seen in polyubiquitin chain synthesis (Figures 1 and 3).

Active-Site Accessibility in Wild-Type and Mutant E2-25K Proteins. To characterize the general active site properties of the truncated and chimeric E2-25K derivatives, we determined the sensitivity of these enzymes to alkylation. The active-site Cys residues of many E2 proteins react rapidly with iodoacetamide (*25*). For example, E2-20K is inactivated with $t_{1/2} \sim 1$ min by 1.5 mM iodoacetamide (*35*). In contrast, incubating E2-25K with 2 mM iodoacetamide for 10 min caused only about 50% inactivation (Table 1). Similar results were obtained with C170S,F174L-25K and GST-25K$_{200}$ (Table 1). In the crystal structure of UBC4, the active-site Cys residue lies in a shallow depression on the surface of the protein (*12*), and appears to be solvent-exposed. Assuming a similar general conformation for the core region of E2-25K, the low reactivity of the E2-25K active-site thiol may reflect an architecture in which the tail of the protein is folded over the active site.

Consistent with this hypothesis, deletion of residues 152−200 engendered increased reactivity of the active-site thiol with iodoacetamide: E2-25K$_{151}$ was completely inactivated during a 10-min incubation with 0.5 mM iodoacetamide (Table 1). Rapid inactivation was not a general consequence of refolding, since refolded C170S,F174L-25K was as resistant to alkylation as its native counterpart (Table 1). E2-25K$_{153}$ was also more sensitive to alkylation than full-length E2-25K, although not as sensitive as E2-25K$_{151}$ (Table 1). As discussed above, the differences between E2-25K$_{151}$ and E2-25K$_{153}$ could reflect a slight conformational aberration in E2-25K$_{151}$. In addition, although the activity of full-length E2-25K was unaffected by the presence of 0.1 M urea (Experimental Procedures), we cannot exclude a specific effect of low concentrations of urea on the folding of E2-25K$_{151}$. (In Table 1, there was ∼40 mM urea in the E2-25K$_{151}$ incubation; in other assays of E2-25K$_{151}$, the urea concentration was ≤20 mM; Experimental Procedures.) However, results obtained with both truncated enzymes are consistent in suggesting that the tail of E2-25K is a positive factor in iodoacetamide resistance.

The behavior of the chimeric enzyme UBC4/25K was intermediate between the behaviors of the full-length and truncated E2-25K enzymes (Table 1). Although this result was initially suggestive of a "partially correct" orientation of the core and tail regions, this interpretation was excluded by the finding that the chimeric E2 was *more* sensitive to alkylation than was UBC4 itself (Table 1). In view of this result, the reactivity of UBC4/25K suggests that the orientation of the core and tail domains of this enzyme differs from the orientation of these elements in E2-25K.

The slow inactivation of UBC4 was unexpected, given the structure of UBC4. Girod and Vierstra (*36*) reported that a plant homolog of UBC4 migrated in gel filtration with an apparent mass of 28−30 kDa, *i.e.*, as a dimer. In addition, Gwozd et al. (*37*) have shown that yeast UBC4 self-associates: one E2 molecule can ubiquitinate another in an intermolecular conjugation reaction, and E2 molecules can be cross-linked to yield dimers and higher oligomers. This self-association was taken to be transient, since yeast UBC4 migrated as an apparent monomer in gel filtration. In our experiments, resistance to alkylation could have arisen from self-association that occluded the active site. Therefore we reexamined the gel filtration properties of yeast UBC4. In our hands, the protein migrated with a molecular mass of 28 kDa (triangles, Figure 5), close to the mass of 32.7 kDa expected for a dimer. E2-25K$_{153}$, whose subunit molecular mass is greater than that of UBC4 (18.7 *vs* 16.4 kDa), migrated on the same column as an apparent monomer with a mass of 17 kDa (open squares, Figure 5). If the folding of E2-25K$_{153}$ indeed resembles that of UBC4 (*12*), then it is unlikely that UBC4 is an asymmetric monomer. These results raise the possibility that yeast UBC4 is dimeric in solution; depending on the orientation of the active sites, dimerization could play a role in resistance to alkylation (see below). We do not know why our gel filtration results differ from those of Gwozd et al. (*37*), or why no dimer was evident in the crystal structure (*12*). Gwozd et al. (*37*) carried out gel filtration in 0.15 M NaCl; Girod and Vierstra (*36*) reported that the plant UBC4 was monomeric at this ionic strength. However, in our hands yeast UBC4 remained dimeric when gel filtration was carried out at 0.2 M NaCl

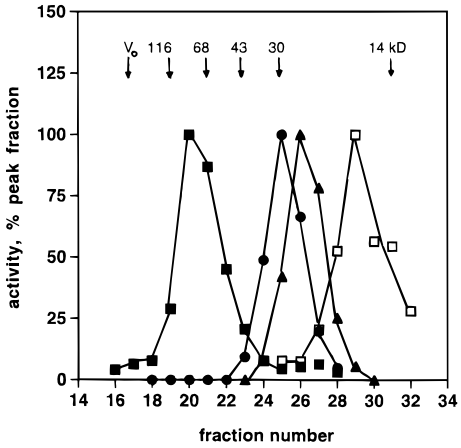

FIGURE 5: Gel filtration of E2 proteins. Purified E2 proteins (GST-25K$_{153}$ and E2-25K$_{153}$) or partially purified E2 proteins (other samples) were chromatographed on a calibrated Sephacryl-200 column (Experimental Procedures). Activity was determined by assay of thiol ester formation with ^{125}I-ubiquitin, and is expressed relative to the activity in the peak fraction of each E2: E2-25K (circles), UBC4 (triangles), GST-25K$_{153}$ (filled squares), and E2-25K$_{153}$ (open squares). Peak fractions of molecular mass standards are indicated (see Experimental Procedures). Data shown here, and discussed in the text, are representative of duplicate runs.

(data not shown). It is possible that two mutations present in the UBC4 used here, S2A and M39V (Experimental procedures), were significant with regard to self-association.

Full-length E2-25K migrated with a molecular mass of 30 kDa, in agreement with previous results [circles, Figure 5 (*7*)]. The UBC4/25K chimera comigrated with E2-25K (data not shown). The simplest interpretation of these data is that both enzymes are asymmetric monomers. If this is true of E2-25K (subunit mass 22.5 kDa), then the tail must make a substantial contribution to asymmetry (*cf.* 17 kDa for E2-25K$_{153}$). However, at present we cannot exclude a model in which E2-25K and UBC4/25K are globular, symmetric dimers.

Dimerization-Dependent Changes in the Mechanism and Specificity of Truncated E2-25K. As mentioned above, E2-25K$_{153}$ had aberrantly high activity in polyubiquitin chain synthesis when assayed as an intact GST fusion protein in the pulse−chase assay. Insight into the molecular basis of this behavior was provided by the results of extended assays of ubiquitin thiol ester formation. Figure 6A (lanes 1−3) shows data from such an experiment carried out at 0.1 μM E1, 5 μM GST-25K$_{153}$, and 10 μM ^{125}I-ubiquitin. The most striking feature of these results is the time-dependent accumulation of E2−ubiquitin adducts of increasing molecular mass. At early times (up to 5 min in this experiment, lane 2), these adducts arise by the accumulation of ubiquitin chains on a thiol group(s) of the fusion protein. This was shown by conversion of the labeled adducts to free di- and triubiquitin following treatment with mercaptoethanol, which cleaves thiol ester bonds (Figure 6A, lanes 5 *vs* 2). At later times, there was also ubiquitin transfer to one or more Lys residues of the fusion protein to generate mercaptoethanol-stable adducts (lanes 5 and 6). Formation of thiol-linked chains was an intrinsic activity of GST-25K$_{153}$, since the rate of appearance of these adducts was directly proportional to the concentration of the fusion protein, and independent of a 5-fold increase in E1 concentration (data not shown). There was no detectable formation of thiol-linked chains in comparable assays of E2-25K$_{153}$, or in assays of the full-length fusion protein GST-25K$_{200}$ (data not shown). Thus,

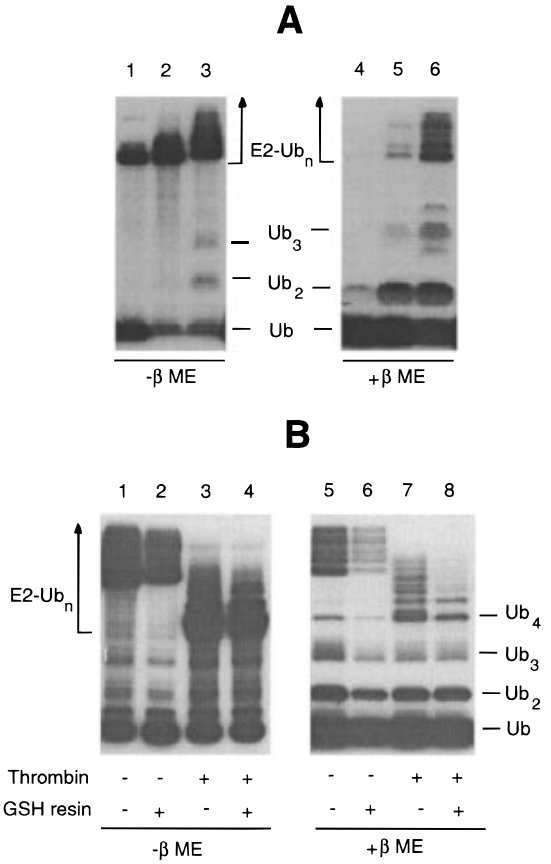

FIGURE 6: New mechanism of chain synthesis by GST-25K$_{153}$ (autoradiographs). (A) Accumulation of fusion protein-linked ubiquitin chains. Incubations (35 μL) contained 10 μM ^{125}I-ubiquitin, 5 μM GST-25K$_{153}$, and 0.1 μM E1: lanes 1, 4, 1 min; lanes 2, 5, 5 min; lanes 3, 6, 30 min. Other conditions were the same as for ubiquitin thiol ester assays (Experimental Procedures). Aliquots of 10 μL were quenched with 10 μL of thiol ester sample buffer; 7 μL of the quenched incubation was electrophoresed (lanes 1−3). A second aliquot of the quenched reaction, 9 μL, was mixed with 9 μL of sample buffer and heated to 100 °C; 14 μL of this mixture was electrophoresed (lanes 4−6). Thus, lanes 1−3 visualize combined thiol- and amide-linked ubiquitin, while lanes 4−6 visualize amide-linked species only. (B) Localization of chains on the E2 moiety of fusion protein. GST-25K$_{153}$ (5 μM) was incubated with 10 μM ^{125}I-ubiquitin and E1 at 37 °C under the conditions in panel A. After 20 min, 10 μL aliquots were distributed to four tubes containing 10 μL of phosphate-buffered saline plus 1 mg/mL ovalbumin, together with buffer alone (lanes 1, 5); 3 μL of GSH−agarose (lanes 2, 6); 0.1 unit of thrombin (lanes 3, 7); or 3 μL of GSH−agarose together with 0.1 unit of thrombin (lanes 4, 8). After incubation for 20 min at room temperature, the tubes were spun in an Eppendorf centrifuge. Aliquots of the supernatants were mixed with thiol ester sample buffer (lanes 1−4) or with normal sample buffer (lanes 5−8; these samples were boiled), followed by electrophoresis and autoradiography.

formation of thiol-linked chains depended *both* on the absence of the E2-25K tail and on the presence of the GST moiety. The thiol-linked chains were assembled through K48, since their formation was abolished by substitution of K48R-ubiquitin (data not shown).

Wild-type E2-25K synthesizes chains by transferring ubiquitin from the active-site Cys to K48 of a free ubiquitin molecule (*19*; see below). This mechanism is unlikely to apply in Figure 6A, where free ubiquitin was $\leq$5 μM. If transfer involved free ubiquitin as an acceptor, there would be <1% conversion to dimer in 5 min (Figure 1A); the conversion seen in Figure 6A was much higher. There are two other possible mechanisms for the synthesis of thiol-linked chains by the GST-25K$_{153}$ fusion protein. One

involves intermolecular transfer of ubiquitin between E2−ubiquitin thiol esters; *i.e.*, K48 of ubiquitin covalently bound to one E2 molecule reacts with G76 of ubiquitin covalently bound to a second E2 molecule. This mechanism would appear to require a high-affinity E2−E2 interaction that juxtaposes appropriate residues of the two E2-bound ubiquitins. A second mechanism involves repeated intramolecular transfer of monoubiquitin from the E2 active site to a Cys (subsequently a Cys-linked ubiquitin) in the GST moiety of the same fusion protein molecule. We show below that the first mechanism predominates.

As required in the first mechanism, GST-25K$_{153}$ is a dimer: all of the fusion protein migrated with a mass of ~80 kDa, nearly twice the subunit mass of 46 kDa (Figure 5, filled squares). Dimerization was driven by self-association of GST, as indicated by the finding that purified recombinant GST (subunit mass, 27 kDa) migrated with a mass of ~50 kDa (data not shown), while E2-25K$_{153}$ migrated as an apparent monomer of ~17 kDa (open squares, Figure 5). Both GST and GST-25K$_{153}$ remained dimeric when gel filtration was carried out in the presence of 0.2 M NaCl.[4] These findings are consistent with the dimeric structure of GST seen in X-ray crystallography (*38*, *39*). As expected, GST-25K$_{200}$ was also a dimer of ~90 kDa.[4]

Dimerization will not necessarily facilitate chain assembly at the active site, as the E2 domains might be oriented in such a way that K48 of one thiol-linked ubiquitin would not react efficiently with G76 of the other thiol-linked ubiquitin. However, two observations suggest that the active sites in the GST-25K$_{153}$ dimer are closely juxtaposed. First, the GST-25K$_{153}$ dimer was modestly protected against iodoacetamide relative to the E2-25K$_{153}$ monomer (Table 1). Second, the dimeric species GST-25K$_{153}$ discriminated strongly against plant E1 in ubiquitin thiol ester formation (≥30-fold rate difference for plant *vs* mammalian E1; Figure 4C, lanes 5 *vs* 6, and data not shown). This discrimination was only about 3-fold less than that shown by full-length E2-25K, and was similarly a V_{max} effect (data not shown). Discrimination by GST-25K$_{153}$ was abolished following thrombin treatment of the fusion protein (lanes 5, 6 *vs* 7, 8), which renders the E2 moiety monomeric (Figure 5). Overall, these results suggest that a close juxtaposition of two E2 active sites, mediated by the dimerization of their respective GST moieties, reduces the accessibility of the active site to alkylating agents and noncognate E1s (see Discussion). An alternative model, in which active-site accessibility is reduced by an intramolecular GST−E2 interaction, can be excluded because the GST moiety is already involved in an intermolecular self-association (*38*). In addition, a GST−active-site interaction would be unlikely to increase activity in polyubiquitin chain synthesis (see below).

These results suggest that the specific structure of GST-25K$_{153}$ could facilitate ubiquitin transfer between E2 active sites. If this is indeed the predominant mechanism, the thiol-linked chains (Figure 6A) should be associated with the E2 moiety of the fusion protein, rather than with the GST moiety. In this case, the thiol-linked chains will not bind to GSH−agarose following cleavage of the GST-25K$_{153}$−ubiquitin thiol ester with thrombin. The first two lanes of each panel in Figure 6B show the positive control for such an experiment. Here the thiol ester adducts were applied to GSH−agarose without prior thrombin treatment; most of the E2-associated ubiquitin (lanes 1 *vs* 2), including about two-thirds of the thiol-linked chains (lanes 5 *vs* 6), bound to the resin. Nonquantitative binding was presumably due to overloading of the resin. Following thrombin cleavage, thiol-linked chains containing up to five ubiquitins were quantitatively recovered in the unbound fraction (Figure 6B, lanes 7 *vs* 8), indicating that these chains were localized on the E2 moiety. The chains must be at the active site, since there is no other Cys residue in truncated E2-25K (Figure 2A). We conclude that thiol-linked chains arise predominantly through ubiquitin transfer between the subunits of a dimeric thiol ester complex. Large conjugated species, which were highly underrepresented in the GSH−agarose flow-through, presumably arise by ubiquitin transfer to Lys residues of GST.

The mechanism whereby chains are assembled at the active site of GST-25K$_{153}$ is of some interest. According to the mechanism proposed here, the product of the first round of reaction would be a GST−E2 dimer in which one E2 domain is free, while the other is linked to diubiquitin. Presumably the unoccupied E2 domain can then be activated by E1; the resulting E2-bound monoubiquitin could then act as the acceptor in the next round of reaction, leading to bound triubiquitin; and so on. The growing chain product would be stoichiometric relative to enzyme until it is released by hydrolysis or reaction with a nucleophile such as DTT.

Wild-type E2-25K strictly utilizes a mechanism in which free (*vs* E2-bound) ubiquitin is the acceptor in chain synthesis. This is shown by two observations: first, Ub$_{74}$ and D77-ubiquitin, which cannot form thiol esters, accept ubiquitin with the same kinetics as does Ub$_{76}$ (*19*; Figure 1); second, we have never observed thiol-linked chains with full-length enzyme, even when it is fused to GST. GST-25K$_{153}$ is more flexible: while it predominantly utilizes the mechanism just discussed, it can also utilize free ubiquitin as an acceptor. This conclusion follows from the activity of GST-25K$_{153}$ in the continuous assay, where the acceptor ubiquitin must come from solution, since the E2-bound ubiquitin lacks K48. The specific activity of GST-25K$_{153}$ in the continuous assay was 10% that of C170S,F174L-25K (Figure 1C, circles *vs*. inverted triangles). The relative specific activity of GST-25K$_{153}$ in the pulse−chase assay was 3.4-fold higher (data not shown), apparently reflecting a strong contribution from the mechanism documented in Figure 6. That this contribution is manifested at the high acceptor concentration in the pulse−chase assay (117 μM) indicates that ubiquitin transfer between the subunits of a dimer can be very efficient.

Although dimerization of truncated E2-25K was necessary to observe inter-E2 ubiquitin transfer (*cf.* no activity with E2-25K$_{153}$), dimerization did not invoke this mechanism generally. As noted above, the dimeric GST-25K$_{200}$ enzyme did not synthesize thiol-linked chains. Nor did UBC4 exhibit this activity, although it may exist as a dimer in which the active sites are near the subunit interface.

DISCUSSION

The Tail of E2-25K Is a Core-Dependent Activity Element: Implications for E2 Structure−Function. The existence of a large family of E2 proteins makes a major contribution to specificity in protein degradation, as shown by the selective functions of individual E2s. This was first evident from assays of *in vitro* ubiquitination (*7*, *26*, *40*), and was subsequently shown convincingly at the level of biological function. To cite just a few examples from *S.*

cerevisiae, RAD6/UBC2 functions in DNA repair and sporulation (*41*, *42*); CDC34/UBC3 and UBC9 in cell cycle regulation (*43*, *44*); and UBC4 and UBC5 in the turnover of short-lived and abnormal proteins (*23*). In some cases, the specific substrates involved in these functions have been identified: *e.g.*, G1 cyclins and cdk inhibitors for CDC34 (*45*−*48*). While it is likely that the selective functions of individual E2 enzymes arise from specificity in their interactions with other proteins, including E3s, these presumptive interacting species remain largely unidentified.

As the relatively high conservation of the ∼150-residue E2 core domain is somewhat confounding to the notion of selective interaction, it is attractive to postulate that the unconserved terminal extensions present in certain E2 proteins act to recruit the core catalytic domain to specific substrates, either directly, through an extension−substrate interaction, or indirectly, by interaction of the extension with *trans*-acting factors, including E3 enzymes (*e.g.*, *10*, *18*). In its simplest form, this model could accommodate functional equivalence for many core domains, especially those which are highly homologous.

Considerable *in vitro* and *in vivo* data support the hypothesis that E2 terminal extensions contribute to specificity. For example, deleting the 23-residue acidic tail of RAD6 selectively abrogates one of the three known functions of this E2 (*49*), and deleting the 125-residue tail of CDC34 eliminates the function of this E2 in cell cycle progression (*50*, *51*). At the biochemical level, the histone-conjugating activities of RAD6 (*52*), CDC34 (*43*), wheat UBC4 (*53*), and human E2-20K/UbcH2 (*54*) depend upon the presence of the tails of these enzymes, which either are globally acidic (RAD6, wheat UBC4, E2-20K) or harbor strongly acidic segments (CDC34). Moreover, transfer of an acidic tail to a different E2 core can produce a chimeric enzyme with histone-conjugating activity (*53*, *54*). The best evidence that a tail can be a transferable specificity element is provided by the finding that a RAD6(core)-CDC34(tail) chimera rescues the cell cycle defect of a *cdc34*Δ yeast strain (*50*, *51*). These biological and biochemical observations provide support for a simple model in which terminal extensions can be the predominant determinants of E2 specificity. However, other data, and the current results, indicate that the structure−function relationship in E2 proteins is more complex.

In structural terms, E2-25K can be viewed as the product of grafting a unique tail onto a generic, UBC4-type core (*20*). Although lack of insight into the cellular function of E2-25K prevents a biological test of this hypothesis, the robust polyubiquitin chain synthesis activity of E2-25K presented an opportunity to test the relevance of the tail for biochemical activity. Since the tail is not highly charged, and is predicted to contain at least two α-helices (*55*; M. Haldeman, unpublished), any role for it in determining activity or substrate specificity is unlikely to derive from simple electrostatic attraction or repulsion. This feature distinguishes the present work from previous *in vitro* studies on the function of E2 tails in substrate selection.

Our results indicate that the tail of E2-25K is a necessary, but not sufficient, element in determining two characteristic properties of E2-25K. The first property is activity in ubiquitin conjugation. Truncated derivatives comprising 151 or 153 residues had polyubiquitin chain synthesis activities that were reduced by an order of magnitude or more. Thus, the tail is necessary for efficient conjugation activity, but it is dispensable for thiol ester formation. However, while UBC4 lacked detectable chain synthesis activity, the 153-residue core of E2-25K had low, but detectable, activity. This latter result provided a preliminary indication that the E2-25K and UBC4 cores were functionally distinct, despite their high homology. The existence of a specific role for the E2-25K core domain in conjugation was confirmed by the finding that the UBC4/25K chimera lacked polyubiquitin chain synthesis activity. The simplest interpretation of these results is that some of the divergent (relative to UBC4) residues in the E2-25K core are highly significant for function in chain synthesis. These residues may function directly in binding or catalysis, or they may stabilize a specific core−tail orientation that is important for function (below).

In the course of the present work, we discovered that E2-25K is virtually inactive in ubiquitin thiol ester formation when assayed with nonmammalian E1 enzymes. This unprecedented selectivity may have its basis in primary sequence divergence between E1 enzymes [human E1 is 60−70% similar to plant and yeast E1s (*56*-*58*)]. Another possibility is that the E1 phosphorylation known to occur in mammalian cells (*59*, *60*) is both unique to higher eukaryotes and significant for the ability of E1 to activate E2-25K. The important point for the present discussion is that, as in polyubiquitin chain synthesis, the tail of E2-25K is a necessary, but not sufficient, element in E1 selectivity. This is shown by the finding that both of the truncated derivatives, and the UBC4/25K chimera, were similarly activated by plant and mammalian E1s.

Our results add to a growing list of findings which indicate that E2 core regions, while highly conserved, have nonetheless evolved to perform specific functions. For example, although the RAD6-CDC34 chimera is functional at the G1-to-S phase transition, a UBC4-CDC34 chimera is not (*50*). Another example is provided by the existence of multiple ∼16 kDa UBC4 homologs in mammals (*61*, *62*). Although the core regions of these enzymes are highly related, the different enzymes apparently carry out selective functions. Thus, a testis-specific UBC4 isoform exhibits unique properties in conjugation, even though it is more than 90% identical to another isoform in the same tissue (*62*). We have identified a point mutation in the E2-25K core region that confers on the full-length protein a biochemical phenotype similar to that shown by the truncated derivatives characterized in the present work.[2] Collectively, these data, and the current results, point to the likelihood that there is a high degree of specificity in the functions of conserved E2 core domains.

Core residues that are required for the proper function of the E2-25K tail in chain synthesis and E1 selectivity are most likely to be poorly conserved between UBC4 and E2-25K. There are many such residues sprinkled throughout the E2-25K core sequence, but there are some regions of concentration, which may be considered in the context of the UBC4 structure (*12*). Residues predicted to comprise the loop connecting helix H1 to β-strand S1 (residues 19−26), the N-terminal end of S1 (27−31), and the N-terminal ends of H3 (126−130) and H4 (138−143) are poorly conserved between E2-25K and UBC4 (Figure 2A). In UBC4, the corresponding residues are distant from the active site, in regions suggested to mediate selective interactions (*12*, *13*). E2-25K and UBC4 are also dissimilar in a small region just N-terminal to the active site (residues 86−90). This region has been suggested to function in interactions common to

all E2s. Interestingly, a mutation in this region of E2-25K dramatically inhibits polyubiquitin chain synthesis.[2] One possibility is that this region interacts with the tail (below).

Molecular Function of the E2-25K Tail. The terminal extensions of E2 proteins apparently contribute to selective function by diverse molecular mechanisms. For example, one function of the acidic tail of yeast RAD6 is to interact with the *UBR1*-encoded E3 enzyme (*63*). The tail of yeast UBC6 targets this E2 to the cytosolic face of the endoplasmic reticulum membrane, where UBC6 may ubiquitinate membrane proteins (*64*). A limited region of the tail of CDC34 mediates self-association, as manifested by cross-linking (*65*); this same region is necessary and sufficient for cell cycle function (*50*, *51*, *65*).

The properties of truncated derivatives of E2-25K indicate that the tail is a critical element in E1 selectivity and catalysis of polyubiquitin chain synthesis; the tail also protects the active site against alkylation. The apparent molecular mass of E2-25K, 30 kDa, is most easily reconciled with an asymmetric monomeric conformation. A simple model to explain our findings invokes a structure in which the tail is folded over the active site in free E2-25K. This would reduce the active-site accessibility to small molecules; E1 selectivity could result from the enhanced ability of a mammalian E1 enzyme to induce a conformational change that displaces the tail and allows access to the active-site Cys residue. In this structural model, there is no immediately evident mechanistic role for the tail in conjugation, since the high K_m for the acceptor ubiquitin, ~600 μM, excludes a function as a simple ubiquitin-binding element. Nonetheless, the high sensitivity of catalytic activity to the environment of K48 in the acceptor ubiquitin, as manifested by discrimination against iodotyrosylubiquitin (*33*), suggests that there is a significant interaction with the acceptor. We tentatively assign this interaction to the core domain, based on the finding that E2-25K$_{153}$ discriminates strongly against iodotyrosylubiquitin.[4] If this assignment is correct, then the catalytic function of the tail may reflect interactions with the E2-bound ubiquitin, rather than with the acceptor ubiquitin. Recent results of Hodgins et al. (*66*) show that an E2 tail can modulate the chain synthesis activity of the core. These authors found that the topology of a polyubiquitin chain assembled on a Lys residue of UBC1 varied, depending on whether the tail was present.

GST-induced dimerization of the 153-residue core domain generates an enzyme with properties apparently resembling those of full-length E2-25K: GST-25K$_{153}$ exhibits pronounced E1 selectivity, enhanced chain synthesis activity, and reduced sensitivity to alkylation. These results immediately suggest an alternative model for the role of the tail; specifically that it mediates dimerization. Results obtained with CDC34 provide precedent for tail-mediated self-association (*65*). In the dimerization model, tail-mediated self-association would serve to strengthen a weak interaction between the E2 core domains. This interaction could block access of alkylating agents and noncognate E1s to the dimeric active sites. The role of the tail in chain synthesis would be indirect; it would serve to place the two thiol-linked ubiquitins near each other.

Despite the suggestive properties of GST-25K$_{153}$, and recognizing that only structure determination can provide an unambiguous resolution, we favor the first of the two models discussed above. First, the apparent molecular mass of E2-25K is ~30 kDa, much less than the 45−50 kDa that is expected for a dimer (Results). In addition, we did not detect chemical cross-linking of E2-25K under conditions in which GST-25K$_{153}$ was almost completely converted to higher-order adducts (data not shown). Second, and more important, although GST-induced dimerization increased chain synthesis activity, the predominant mechanism utilized by the artificial dimer was different from the mechanism utilized by the native enzyme.

It has been reported that the tail of E2-25K exhibits modest similarity to a sequence of ~55 residues, the "UBA domain", that is found in a diverse set of proteins, including several enzymes of the ubiquitin pathway (*55*). Our results indicate that the UBA domain of E2-25K is important for catalysis and specificity, but do not identify any generalized function for this domain. The UBA domain may be a protein interaction element that was separately recruited to diverse proteins, and which evolved, in the case of E2-25K, to perform a specific function in polyubiquitin chain synthesis. Besides this catalytic function, it is possible that the tail of E2-25K also directs a yet-uncharacterized interaction(s) that is (are) significant for biological function. In this regard, the cellular function of E2-25K probably does not involve *unassisted* catalysis of polyubiquitin chain synthesis, because the high K_m for ubiquitin (~600 μM), and low k_{cat} (~0.6 min^{-1}), will render activity negligible at cellular concentrations of ubiquitin ($\leq$20 μM). An interaction with another protein, such as an E3 enzyme, might stimulate E2-25K's activity in chain synthesis, or lead to the ubiquitination of other targets (*15*).[4] The possiblity of other interactions does not diminish the significance of the current results in developing models for E2 structure−function.

Induced Dimerization Can Invoke a Novel Mechanism of Chain Synthesis. The most surprising finding of the present study was that induced dimerization of the E2-25K core dramatically altered the catalytic and specificity characteristics of this domain. At first glance, dimerization appeared to reverse the effects of deleting the tail, although subsequent experimentation showed that this interpretation was oversimplified. Dimerization is driven by the GST−GST interaction (*38*), but there is probably some E2−E2 interaction as well; the acquisition of pronounced E1 selectivity, together with a relatively robust chain synthesis activity, is difficult to ascribe to a random and fluctuating orientation of the E2 domains. Overall, these results clearly indicate that self-association can alter the catalytic properties of E2 proteins, and specifically facilitate the synthesis of polyubiquitin chains by a novel mechanism [see also (*65*)]. Ellison and co-workers have also observed the assembly of polyubiquitin chains on the active-site thiol of a truncated E2, in this case yeast UBC1 (M. Ellison, personal communication). The core of UBC1 is highly related to the core of E2-25K (*20*).

Our results indicate the need for caution in interpreting the activities of GST fusion proteins. In the present case, extensive prior knowledge of the ubiquitin structure−function relationship in the E2-25K reaction led us to question the apparent high activity of GST-25K$_{153}$. All of the special E2-25K-like properties of this derivative ultimately proved to depend on GST-induced dimerization. It is easy to imagine that such induced dimerization, which should hold for all GST fusion proteins, could affect the activity of the fusion partner either positively or negatively. For example, by enhancing the local concentration of the fusion partner, dimerization may contribute to the frequent success of the

"GST capture" technique that is widely used to detect protein−protein interactions.

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