Core Domain Mutation (S86Y) Selectively Inactivates Polyubiquitin Chain Synthesis Catalyzed by E2-25K†

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ABSTRACT: The mammalian ubiquitin conjugating enzyme known as E2-25K catalyzes the synthesis of polyubiquitin chains linked exclusively through K48−G76 isopeptide bonds. The properties of truncated and chimeric forms of E2-25K suggest that the polyubiquitin chain synthesis activity of this E2 depends on specific interactions between its conserved 150-residue core domain and its unique 50-residue tail domain [Haldeman, M. T., Xia, G., Kasperek, E. M., and Pickart, C. M. (1997) *Biochemistry 36*, 10526−10537]. In the present study, we provide strong support for this model by showing that a point mutation in the core domain (S86Y) mimics the effect of deleting the entire tail domain: the ability to form an E2∼ubiquitin thiol ester is intact, while conjugation activity is severely inhibited (≥100-fold reduction in k_{cat}/K_m). The properties of E2-25K enzymes carrying the S86Y mutation indicate that this mutation strengthens the interaction between the core and tail domains: both free and ubiquitin-bound forms of S86Y-25K are completely resistant to tryptic cleavage at K164 in the tail domain, whereas wild-type enzyme is rapidly cleaved at this site. Other properties of S86Y-26K suggest that the active site of this mutant enzyme is more occluded than the active site of the wild-type enzyme. (1) Free S86Y-25K is alkylated by iodoacetamide 2-fold more slowly than the wild-type enzyme. (2) In assays of E2∼ubiquitin thiol ester formation, S86Y-25K shows a 4-fold reduced affinity for E1. (3) The ubiquitin thiol ester adduct of S86Y-25K undergoes (uncatalyzed) reaction with dithiothreitol 3-fold more slowly than the wild-type thiol ester adduct. One model to accommodate these findings postulates that an enhanced interaction between the core and tail domains, induced by the S86Y mutation, causes a steric blockade at the active site which prevents access of the incoming ubiquitin acceptor to the thiol ester bond. Consistent with this model, the S86Y mutation inhibits ubiquitin transfer to macromolecular acceptors (ubiquitin and polylysine) more strongly than transfer to small-molecule acceptors (free lysine and short peptides). These results suggest that unique residues proximal to E2 active sites may influence specific function by mediating intramolecular interactions.

The Ub[1]-proteasome pathway is the principal mechanism for turnover of short-lived proteins in eukaryotic cells. In this pathway, the covalent attachment of Ub to the substrate confers recognition by the 26S proteasome (*1*). Ubiquitination is initiated by the formation of an isopeptide bond between the ϵ-amino group of a substrate Lys residue and the C-terminal carboxylate of Ub (G76). Substrates are most efficiently recognized when additional Ubs are conjugated to this first Ub, to form a polyUb chain linked by K48−G76 isopeptide bonds (*2*−*4*).

Synthesis of the Ub−substrate bond is usually accomplished in three steps (*1*, *5*). Ub activating enzyme or E1 catalyzes the ATP-dependent formation of an E1∼Ub thiol ester. Ub is next transferred to a Cys residue of a Ub conjugating enzyme or E2. Finally, Ub is transferred from the E2 to the substrate Lys residue by a Ub-protein ligase or E3. For E3s which are characterized by the presence of a catalytic region known as the "hect" domain, the final ligation step occurs through the formation of an E3∼Ub thiol ester intermediate (*6*).

Substrate specificity in the Ub-proteasome pathway arises primarily at the level of ubiquitination. Specificity is thought to depend mainly upon the properties of the E3, particularly its ability to recognize a primary sequence element (ubiquitination signal) in the substrate. Consistent with this model, the known E3s show a high degree of substrate selectivity, and the ability of certain substrates to be ubiquitinated depends on the presence of a ubiquitination signal, such as the "destruction box" of the mitotic cyclins (*7*). Only a few E3 enzymes have been identified so far (*1*), but in view of the diversity of substrates of the pathway, it is likely that the number of E3s is large.

Multiple E2 enzymes also exist (*1*, *5*, *8*). In the yeast *Saccharomyces cerevisiae*, the ablation of individual E2 genes elicits specific phenotypes, as a result of the impaired

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[1] Abbreviations: DTT, dithiothreitol; E1, Ub activating enzyme; E2, Ub conjugating enzyme; E3, Ub-protein ligase; MEL, murine erythroleukemia; SUMO-1, Ub-like protein also known as GMP-1 and sentrin (see text); Ub, ubiquitin; Ub_n, K48-linked polyUb chain containing *n* Ubs.

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turnover of specific substrates (*1*). The existence of substrate selectivity at the level of the E2 enzyme can be accommodated by a model in which the E3 enzyme is the primary specificity factor (above) if there is a high degree of specificity in the E2−E3 interaction, as has been observed in several cases (*9*−*14*). But since multiple E2s would be unnecessary if the E3 is the *sole* specificity factor, it is likely that the E2 plays some role in substrate selection. The molecular basis of this role is poorly understood. In some cases, it may be based in a direct E2−substrate association, as suggested by the isolation of E2 genes in interaction screens with pathway substrates [e.g., (*15*−*17*)]. The specificity of a given E3 may also be modulated by the formation of distinct complexes with different E2s [e.g., (*9*, *18*, *19*)]. These models are not mutually exclusive.

E2 proteins are characterized by the presence of a conserved core domain of ∼150 amino acids which harbors the active site Cys residue; certain E2s have unique N- or C-terminal extensions which may be as much as ∼125 residues in length [reviewed in (*20*)]. Substantial evidence indicates that these extensions are specificity elements which function in mechanistically diverse ways [see (*21*)]. However, regions of modest divergence within the core domain can also be highly significant for function. For example, two Ubc4 isozymes expressed in mouse testis, whose sequences diverge at only a few positions, differ strongly in their conjugation properties (*19*). Since the core domains of the six structurally characterized E2s are virtually superimposable [(*22*−*26*); R. Basavappa, personal communcation], unique residues within E2 core domains are presumed to affect function by mediating interactions, rather than by altering folding. Consistent with this idea, nonconservative substitutions in the core domains are largely restricted to one face of the molecule, opposite the active site (*22*−*26*).

The mammalian enzyme E2-25K has a 50-residue tail which is dissimilar to the tails of other known E2s (*27*). Uniquely among mammalian E2s, E2-25K synthesizes long K48-linked polyUb chains in an E3-independent manner (*28*). This robust and well-characterized in vitro activity makes E2-25K well-suited for structure−function analysis. We showed previously that the tail of E2-25K is necessary, but not sufficient, for activity in polyUb chain synthesis (*21*). Here we show that the effect of mutating the core residue S86 (to Tyr) is similar to the effect of deleting the tail: conjugation, but not thiol ester formation, is severely inhibited. The S86Y mutation is shown to strengthen the interaction between the core and tail regions of E2-25K; such an enhanced interaction could provide an explanation for the deleterious effect of the mutation. These findings have implications for the structure−function relationship in E2-25K and other E2 proteins.

EXPERIMENTAL PROCEDURES

Materials, Enzyme Preparations, and General Methods. Reagents and proteins were from Sigma unless stated otherwise. The peptide Ac-AGKQL-NH_2 was obtained from Quality Controlled Biochemicals, Inc. (Hopkinton, MA). K48R-Ub was expressed and purified as described (*4*). Bovine Ub was radioiodinated to ∼8000 cpm/pmol with chloramine-T (*5*). E1 was purified to electrophoretic homogeneity from bovine erythrocytes or rabbit reticulocytes (*29*). SDS−PAGE was carried out by the Laemmli method (*30*).

Plasmids. As described under Results, an I76T,S86Y-25K DNA fragment was obtained by PCR amplification of a MEL cell λgt11 library (Clontech) using E2-25K flanking primers C1 and C2 which harbor sites for *Nco*I and *Bam*HI, respectively (*27*). The fragment was first cloned into pGEM3Zf(−) and sequenced (fmol kit, Promega). The *Nco*I/*Bam*HI fragment was then subcloned into pET3d. To separate the mutations, pET3d-25K (*21*) and pET3d-I76T,-S86Y-25K were digested with *Ssp*I, which cuts once in the region separating the codons for residues 76 and 86, and once in the vector. Large (3.9 kb) and small (1.1 kb) fragments from each digestion were purified. To generate pET3d-I76T-25K, the large fragment from the mutant plasmid (carrying the I76T mutation) was ligated to the small fragment from the wild-type plasmid. Results of digestion with *Nco*I and *Bam*HI were used to identify clones in which the small fragment had ligated in the correct orientation. Separately, the small fragment from the mutant plasmid (carrying the S86Y mutation) was ligated to the large fragment from the wild-type plasmid to give pET3d-S86Y-25K.

In the course of this work (see below), we resequenced the plasmid pET3d−C170S,F174L-25K (*21*). Unexpectedly, the plasmid was found to specify Phe rather than Leu at residue 174. Since the F174L mutation was specified by a PCR primer, and was present when the plasmid was first sequenced (*21*), we assume that the mutation reverted over several years of plasmid propagation in *E. coli*. We showed as part of the present work that C170S-25K is indistinguishable from wild-type E2-25K with regard to activity in polyUb chain synthesis (Results).

The I76T and S86Y mutations were combined with the C170S mutation as follows. Empty pET3d vector was digested consecutively with *Nco*I and *Bam*HI. The plasmid pGEM3Zf(−)I76T,S86Y-25K (above) was digested with *Nco*I and *Pst*I to generate a 360 bp fragment carrying the T76 and Y86 codons. The plasmid pGEM3Zf(−)C170S-25K was digested with *Pst*I and *Bam*HI to generate a 440 bp fragment carrying the S170 codon. The two fragments were combined with the vector in a three-way ligation to generate pET3d-I76T,S86Y,C170S-25K. This plasmid was sequenced (Hopkins Core facility) to verify the presence of the mutations.

Expression and Purification of Recombinant Proteins. The recombinant proteins encoded by the above-described plasmids, as well as wild-type E2-25K, were expressed in *E. coli* strain BL21(DE3)pLysS. Induction, cell lysis, soluble extract preparation, and E2-25K purification were carried out as described (*21*). The electrophoretic purity of each recombinant protein was ≥90%. The presence of the S86Y mutation altered the behavior of E2-25K during gradient elution from an FPLC MonoQ column (Pharmacia-LKB Biotech); forms of the enzyme with Tyr at residue 86 eluted at 0.1 M NaCl, while forms with Ser at residue 86 eluted at 0.13 M NaCl. The divergent behavior reflected a change in charge due to altered folding, because all forms of E2-25K used here comigrated during two-dimensional IEF/SDS−PAGE (not shown).

Pulse−Chase Assays. The pulse (10−30 μL) contained E2 (1−5 μM) and ^{125}I-Ub (4−5 μM) under conditions as

described previously (*28*). After removing an aliquot to monitor thiol ester formation, the chase was initiated. For assay of Ub_2 synthesis, the chase contained unlabeled wild-type Ub at 1 mg/mL. For assay of Ub transfer to small molecules (see Table 3, Results), the chase incubation contained 1 mg/mL K48R-Ub (to dilute ^{125}I-Ub without permitting Ub_2 synthesis), and one of the following: (1) 20 mM EDTA (to detect hydrolysis); (2) 20 mM EDTA and 1 mM DTT; (3) 30 mM lysine; or (4) the pentapeptide Ac-AGKQL-NH_2 at 6.7 mM. In all cases, aliquots were quenched at increasing times with sample buffer lacking β-mercaptoethanol. Disappearance of the E2∼Ub adduct was monitored by band excision and counting (following electrophoresis and autoradiography).

Iodoacetamide Inactivation. E2 (∼1 μM) was reacted for 10 min with 3 mM iodoacetamide (37 °C), followed by quenching with DTT and assay by thiol ester formation (*21*). For the control, iodoacetamide and DTT were preincubated for 10 min prior to E2 addition.

[E1] Dependence of the Rate of E2∼Ub Thiol Ester Formation. Incubations of 10 μL (37 °C) contained 25 mM Tris-HCl (24% base), 2.5 mM $MgCl_2$, 0.15 unit/mL pyrophosphatase, 0.15 unit/mL creatine phosphokinase, 5 mM phosphocreatine, 5 μM E2, 8 μM ^{125}I-Ub, 1 mM ATP, and E1 at concentrations ranging from 10 nM to 1 μM. Reactions were initiated by addition of labeled Ub. After 2 min, a 3 μL aliquot was quenched by addition to 7 μL of thiol ester sample buffer. In several incubations, a second aliquot was quenched at 5 or 10 min, providing a value for the end point, which was independent of [E1] and of the presence of any mutation(s) in E2-25K. Aliquots of the quenched incubations were electrophoresed; the E2∼Ub bands were excised from the dried gel and counted. Preliminary studies confirmed that the appearance of the E2∼Ub adduct was first-order at several concentrations of E1.

Assay of Ub Conjugation to Polylysine. Polylysine with an average molecular mass of 7.5 kDa was purchased from Sigma (P-6516). Assays were carried out in a volume of 12−20 μL, at pH ∼8.0 and 37 °C, under the following conditions: 50 mM Tris-HCl (50% base), 5 mM $MgCl_2$, 1 mM ATP, an ATP-regenerating system, 5 μM ^{125}I-K48RUb, 2 mg/mL ovalbumin, 0.2 mM DTT, 0.1 μM E1, ∼2 μM purified E2, and polylysine at 7.6 mg/mL (∼1 mM polylysine). After 60 min of incubation, replicate aliquots of 5 μL were quenched by dilution into 100 μL of a 10% (v/v) slurry of S-Sepharose (Pharmacia−LKB Biotech) in wash buffer containing 50 mM Tris-HCl (50% base), 50 mM NaCl, 0.1 mM EDTA, 0.2 mg/mL ovalbumin, and 8 mM DTT. The total radioactivity in each tube was determined in a γ counter. The resin was pelleted, and the supernatant was aspirated. The resin pellet was washed twice with 100 μL portions of wash buffer, and the final pellet was counted in a γ counter. Determinations were done in duplicate or triplicate, and were corrected by subtracting blanks obtained by omitting the E2 from the incubation. Control experiments showed that bound radioactivity increased in direct proportion to time and [E2] (data not shown). In a typical experiment, 2.2 ± 0.1% of the radioactivity was bound in the blank, whereas 15−30% (±0.5−1%) was bound in the reaction containing C170S-25K (Mt-D).

Native Trypsin Digestion. Free wild-type and mutant E2-25K proteins (1 mg/mL) were digested with 14% (w/w) trypsin in buffer containing 250 mM Tris (24% base), 0.5 mM EDTA, and 250 mM NaCl (37 °C). Aliquots were removed at increasing time points and quenched with sample buffer containing β-mercaptoethanol. Digested samples were electrophoresed on 13.5% gels and visualized by Coomassie staining. In some cases, fragments bound to Immobilon were visualized by Ponceau staining, excised, and sequenced by automated Edman degradation at the Johns Hopkins core facility. In digests to be analyzed by mass spectrometry, [trypsin] was reduced to 8% w/w, and [NaCl] was reduced from 250 mM to 20 mM. Lowering the salt concentration increased the rate of digestion without altering the mobility of the stable fragment produced from each protein. Soybean trypsin inhibitor (equimolar with trypsin) was added after 60 min. Each digestion mixture was then analyzed by mass spectrometry. For digestion of E2∼Ub thiol esters (and in some digests of free E2s), the preincubation was similar to the pulse in pulse−chase assays (above), except [E2] was 10 μM (0.25 mg/mL), [E1] (if added) was 0.15 μM, and (unlabeled) [K48R-Ub] was 20 μM (0.2 mg/mL). After 4 min (37 °C), NaCl was added to a final concentration of 250 mM, followed by trypsin to 14% w/w (calculated with respect to the sum of the Ub and E2 concentrations).

Mass Spectrometry. Matrix-assisted laser desorption time-of-flight mass spectrometry was carried out as described previously (*4*), using α-cyano-4-hydroxycinnamic acid as the matrix compound.

RESULTS AND DISCUSSION

I76T,S86Y-25K Is Inactive in PolyUb Chain Synthesis. In an attempt to isolate a murine E2-25K cDNA, we carried out PCR screening of a λgt11 library from MEL cells, using flanking primers complementary to the bovine E2-25K cDNA sequence (*27*). By this method, we isolated an 800 bp DNA fragment with >99% sequence identity to the original published bovine cDNA sequence (Figure 1). The following nucleotide changes were found: (1) CG to GC at nucleotides 68−69; (2) T to C at nucleotide 230; (3) C to A at nucleotide 258; and (4) T to C in the 3′ untranslated region (at nucleotide 700 in the numbering of Figure 1).

To confirm these changes, the bovine cDNA was resequenced from the first plasmid generated in our laboratory, pUC19-25K (*27*). The results showed that the previously reported sequence at positions 68 and 69 was incorrect, and that the correct sequence is, in fact, GC as in the clone described above. Therefore residue 23 is Ser rather than Thr (*15*, *21*). However, the other nucleotide changes were verified. They result in two amino acid changes relative to the bovine E2-25K sequence: I76T and S86Y.

E2-25K is very highly conserved in mammals; for example, there is 95% nucleotide and 100% amino acid identity between the bovine and human coding sequences (*15*). To address whether the unique bases in the above-described DNA sequence represented evolutionary divergence between the cow and the mouse, we performed ribonuclease protection assays on RNA prepared from MEL cells, using a probe encompassing the first 400 bp of the potential murine sequence (Figure 1). The results indicated that the divergence between the (authentic) murine and bovine nucleotide sequences was greater than the divergence between the newly cloned sequence and the bovine sequence

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-3   GACATGGCCAACATCGCGGTGCAGCGAATCAAGCGGGAGTTCAAGGAGGTGCTG
        M  A  N  I  A  V  Q  R  I  K  R  E  F  K  E  V  L    17
                              H1
51   AAGAGCGAGGAGACGAGCAAAAATCAAATTAAAGTAGATCTTGTAGATGAGAAT
     K  S  E  E  T  S  K  N  Q  I  K  V  D  L  V  D  E  N    35
                                +     S1
105  TTTACAGAATTAAGAGGAGAAATAGCAGGACCTCCAGACACACCATATGAAGGA
     F  T  E  L  R  G  E  I  A  G  P  P  D  T  P  Y  E  G    53
                   S2
159  GGAAGATATCAACTAGAGATTAAAATACCAGAAACATATCCATTTAATCCCCCT
     G  R  Y  Q  L  E  I  K  I  P  E  T  Y  P  F  N  P  P    71
                   S3
213  AAGGTCCGGTTTACCACTAAAATATGGCATCCTAATATTAGTTACGTCACAGGG
     K  V  R  F  T  T  K  I  W  H  P  N  I  S  Y  V  T  G    89
        S4    (I)                          (S)
267  GCTATTTGTTTGGATATCCTGAAAGATCAATGGGCAGCAGCAATGACTCTGCGC
     A  I  C  L  D  I  L  K  D  Q  W  A  A  A  M  T  L  R   107
              ◊                                   H2
321  ACGGTATTGTTGTCATTACAAGCTCTGCTGGCAGCTGCAGAACCGGATGATCCA
     T  V  L  L  S  L  Q  A  L  L  A  A  A  E  P  D  D  P   125
                   H2
375  CAAGATGCAGTAGTAGCAAATCAGTACAAACAAAATCCTGAAATGTTCAAACAG
     Q  D  A  V  V  A  N  Q  Y  K  Q  N  P  E  M  F  K  Q   143
                   H3                      H4
429  ACAGCTCGACTTTGGGCACATGTGTATGCTGGAGCACCAGTTTCTAGTCCAGAG
     T  A  R  L  W  A  H  V  Y  A  G  A  P  V  S  S  P  E   161
                H4
483  TACACCAAAAAAATAGAAAACCTATGTGCTATGGGCTTTGATAGGAATGCGGTA
     Y  T  K  K  I  E  N  L  C  A  M  G  F  D  R  N  A  V   179
           +     Hi                      Hii
537  ATAGTGGCCTTGTCTTCAAAATCATGGGATGTAGAGACTGCAACAGAATTGCTT
     I  V  A  L  S  S  K  S  W  D  V  E  T  A  T  E  L  L   197
        Hii                         Hiii
591  CTGAGTAACTGAAACATAAAGAGAGATGCTGATATAGTCAAGCTTGCCCCTTCT
     L  S  N  *                                             200
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FIGURE 1: Nucleotide and deduced amino acid sequences of I76T,-S86Y-25K. Nucleotide changes as compared to the bovine E2-25K sequence (*27*) are underlined. The corresponding amino acids of wild-type bovine/human E2-25K are shown in parentheses. The active-site Cys is indicated by a diamond. The stop codon is represented by an asterisk. The crosses designate the tryptic cleavage sites discussed in the text. The secondary structural elements seen in the published E2 crystal structures (*22*−*26*) are indicated (S, β-strand; H, α-helix). The proposed α-helices in the tail domain (*39*) are also shown.

Table 1: E2-25K Mutants

designation	mutations
Mt-A	I76T,S86Y
Mt-B	I76T
Mt-C	S86Y
Mt-D	C170S
Mt-E	I76T,S86Y,C170S

(data not shown). Thus, it is likely that the above-described cDNA (above) arose from unfaithful amplification of a trace of bovine cDNA.

Nonetheless, it was of interest to characterize this mutant protein. To simplify the discussion below, the mutant forms of E2-25K analyzed in this study are designated as shown in Table 1; I76T,S86Y-25K is Mt-A. The purified, bacterially expressed Mt-A protein (Experimental Procedures) was assayed for activity in polyUb chain synthesis. As shown in Figure 2A (lanes 4−6 versus 1−3), the Mt-A enzyme was devoid of activity in a steady-state assay. Thus, changing two amino acids in the core region completely abolished catalytic acitivity in chain synthesis. This result was surprising, because both of the mutations present in the Mt-A protein occur naturally in the core domains of other E2s. For example, Thr is present in yeast Ubc5p at the position

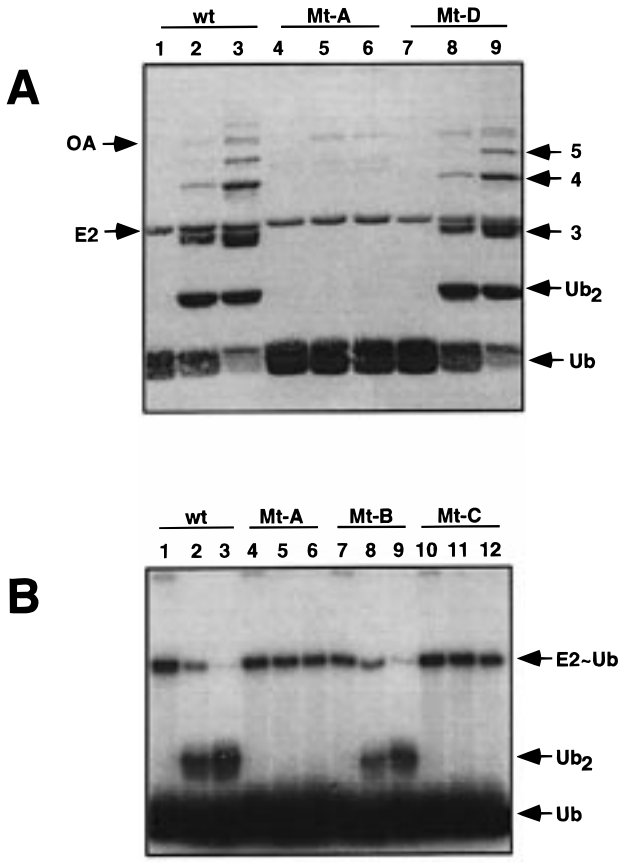

FIGURE 2: S86Y mutation abolishes activity in polyUb chain synthesis. (A) Steady-state chain synthesis (Coomassie-stained gel): wild-type, Mt-A (I76T,S86Y), and Mt-D (C170S). Assays were carried out at pH 8.0 and 37 °C as previously described (*4*), except that the concentrations of E2 and E1 were 10 μM and 0.15 μM, respectively, and [Ub] was 3 mg/mL. An aliquot containing 6 μg of Ub was run in each lane of a 13.5% gel. Lanes 1−3, wild-type E2-25K; lanes 4−6, Mt-A; lanes 7−9, Mt-D. Lanes 1, 4, and 7, zero time (before E1 addition); lanes 2, 5, and 8, 15 min; lanes 3, 6, and 9, 30 min. OA, ovalbumin (carrier in E1). The migration of E2, Ub, and Ub chains composed of 2−5 Ub units is shown. (B) Pulse−chase assays (autoradiograph): wild-type, Mt-A (I76T,-S86Y), Mt-B (I76T), and Mt-C (S86Y). Assays of the indicated forms of E2-25K were sampled at the end of the pulse (lanes 1, 4, 7, 10), or at 10 min (lanes 2, 5, 8, 11) or 30 min (lanes 3, 6, 9, 12) after initiating the chase with unlabeled Ub (Experimental Procedures). The migration of the E2∼Ub adduct, Ub_2, and Ub is indicated.

corresponding to residue 76 in E2-25K (*31*), while Tyr is present at residue 86 (E2-25K numbering) in yeast Cdc34p (*32*), *Arabidopsis* Ubc1 (*22*), and yeast and human Ubc9p (*25*, *33*). [Although Ubc9 is in fact a conjugating enzyme for the Ub-like protein known as SUMO-1 (*20*, *34*−*37*), it is structurally conserved relative to Ub conjugating enzymes (*25*).]

S86Y Mutation Is Responsible for the Mutant Phenotype. To determine whether the loss of chain synthesis activity could be ascribed to a single mutation, we generated plasmids encoding proteins which carried one or the other mutation (Experimental Procedures). As shown in Figure 2B, the purified single mutant proteins Mt-B (I76T) and Mt-C (S86Y), as well as the original double mutant and wild-type E2-25K, formed thiol ester adducts with ^{125}I-Ub (lanes 1, 4, 7, and 10). However, only the wild-type and Mt-B enzymes transferred the labeled Ub to unlabeled Ub during the chase in this pulse−chase assay (Figure 2B, lanes 2−3 and lanes

Table 2: Iodoacetamide Sensitivity[a]

enzyme	mutations	activity remaining, % control
wild-type	−	32.4 ± 0.6 (n = 3)
Mt-A	I76T,S86Y	47.6 ± 4.0 (n = 3)

[a] The indicated form of E2-25K was treated with 3 mM iodoacetamide for 10 min prior to quenching iodoacetamide with DTT. The concentration of active enzyme remaining was then determined by E2∼Ub formation (Experimental Procedures). The concentration of active enzyme is expressed relative to a control in which iodoacetamide was prequenched with DTT.

8−9). In quantitative pulse−chase experiments, the rate of Ub_2 synthesis observed with Mt-B was identical to the rate observed with wild-type enzyme (data not shown). The biochemical defect of the Mt-A protein (Figure 2A) is thus due solely to the S86Y mutation. Moreover, this defect is confined to the second step in the catalytic sequence: transfer of Ub from the E2 thiol to the acceptor Ub. Based on our failure to detect chain synthesis in any of the several assays (*21*) available to us, we conclude that the S86Y mutation reduces k_{cat}/K_m in this second step by >100-fold.

Previous studies showed that the core and tail regions of E2-25K are both important for activity in chain synthesis. Specific residues within the core domain are necessary for the tail to execute its function in chain synthesis: a chimeric E2, in which the E2-25K core domain was replaced by the highly homologous yeast Ubc4 core domain, showed neither chain synthesis activity nor several other properties characteristic of E2-25K (*21*). Thus, at least some of the properties of E2-25K apparently reflect core−tail interactions that are mediated by unique residues in the core domain. The defect caused by the S86Y mutation is similar to the defect caused by deleting the tail of E2-25K (*21*): k_{cat}/K_m in conjugation is strongly reduced (∼10-fold reduction due to truncation, versus ≥100-fold reduction observed here), but thiol ester formation is little affected (below). Thus, we considered whether the S86Y mutation affected core−tail interactions in a way that was detrimental to function.

S86Y Mutation Protects the Active Site against Alkylation. We have shown that the active site of wild-type E2-25K is relatively occluded, as manifested in a reduced rate of alkylation of the active-site thiol (in comparison to other E2s). This property depends on the presence of the tail, and on its proper interaction with the core (*21*). As an initial test of whether the S86Y mutation affected the interaction between the core and tail domains, we determined the sensitivity of the Mt-A enzyme to alkylation. As shown in Table 2, the Mt-A enzyme was significantly less susceptible to alkylation. If inactivation occurred with first-order kinetics, as expected (*38*), $t_{1/2}$ would be ∼10 min for the mutant enzyme and ∼6 min for the wild-type enzyme; i.e., the mutation decreased the alkylation rate constant by about 2-fold. This effect can be explained if, in the mutant enzyme, the tail is more closely associated with the core. It is most likely that this effect is due to the S86Y (versus I76T) mutation, because the I76T mutation was catalytically benign (above), and had no effect on other structural and catalytic properties that were examined (below).

S86Y Mutation Reduces the Efficiency of Ub Transfer from E1 to E2. Intact E2-25K can accept Ub only from mammalian E1 enzymes, and this specificity depends on the presence of appropriate core−tail interactions (*21*). As expected based on the presence of normal or strengthened core−tail interactions (above and below), forms of E2-25K carrying either the I76T or the S86Y mutation (or both) could not accept Ub from wheat germ E1 (not shown). However, forms of E2-25K carrying the S86Y mutation showed a reduction of ∼4-fold in apparent affinity for mammalian (bovine) E1, relative to forms of E2-25K in which the core domain either was unmutated or carried the I76T mutation ($K_{0.5}$ ∼50 nM versus ∼210 nM; filled versus open symbols, Figure 3). These results are consistent with a model in which the S86Y mutation inappropriately enhances the core−tail interaction, leading to the shielding of one or more determinants of E1 binding.

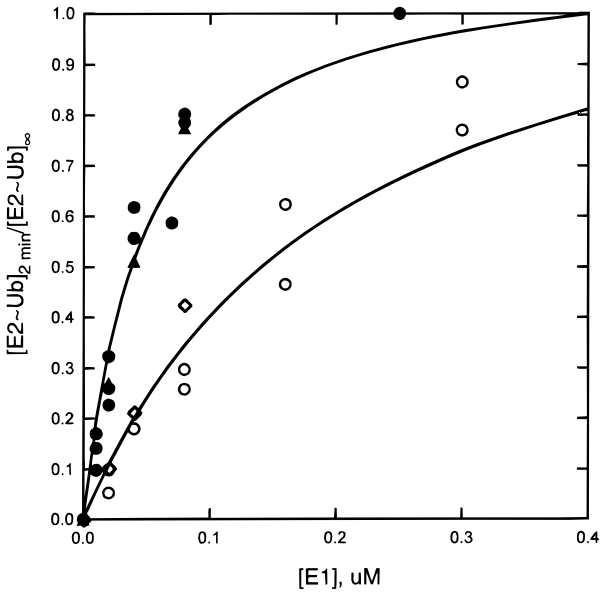

FIGURE 3: S86Y mutation reduces affinity for E1. Assays of E2∼Ub formation were carried out as described under Experimental Procedures. The concentration of E2∼Ub present after 2 min of incubation with the indicated concentration of E1 is expressed relative to the final concentration of E2∼Ub formed at infinite time (5−10 min; this concentration was identical for all of the E2s). Filled circles, Mt-D (C170S); open circles, Mt-E (I76T,S86Y,-C170S); filled triangles, Mt-B (I76T); open diamonds, Mt-C (S86Y). The lines were derived using the program SigmaPlot, assuming $K_{0.5}$ = 47 ± 6 nM (filled symbols) or $K_{0.5}$ = 206 ± 31 nM (open symbols).

S86Y Mutation Prevents Tryptic Cleavage in the Tail Domain. A preliminary indication that the S86Y mutation affected the folding of E2-25K was provided by the observation that forms of the enzyme carrying this mutation (Mt-A, Mt-C, and Mt-E) eluted earlier during anion exchange fractionation (Experimental Procedures). To address more directly whether the S86Y mutation modulated the folding of E2-25K, we carried out tryptic digestion under nondenaturing conditions. Initial studies revealed that wild-type E2-25K gave complex digestion results, apparently due to incomplete cleavage at a C-terminal site(s) (data not shown). We think it likely that the irreproducible digestion pattern was due to oxidation (or other modification) of C170, because the C170 side chain was found to be resistant to reaction with iodoacetamide even under denaturing conditions, as expected if this site was already blocked.[2] In view of these complexities, we carried out the experiments below

[2] C. Pickart, unpublished experiments.

exclusively with forms of E2-25K in which C170 was mutated to Ser. The catalytic properties of C170S-25K (Mt-D) are indistinguishable from those of wild-type E2-25K (Figure 2A, lanes 7−9 versus 1−3). We combined the C170S mutation with the I76T and S86Y mutations to generate the Mt-E protein (Experimental Procedures; Mt-D and Mt-E were also used in Figure 3, circles). The catalytic properties of the Mt-E protein were indistinguishable from those of the Mt-A protein (data not shown).

The Mt-D and Mt-E proteins (0.25 mg/mL = 10 μM) were digested with trypsin (Experimental Procedures). Ub (0.2 mg/mL ~ 20 μM) was included in these incubations to allow direct comparison with the results of other experiments, below. The active Mt-D protein was rapidly digested, through a series of transient intermediates, to a stable fragment with a molecular mass of ~17 kDa (Figure 4A, lanes 1−6). The inactive Mt-E protein also gave rise to a stable fragment, but of a larger mass, ~20 kDa (Figure 4A, lanes 7−12). The size of each stable fragment and the digestion kinetics were unaffected if Ub was omitted from the digest (data not shown). To define the N-termini of the fragments, they were resolved by SDS−PAGE, blotted, and subjected to 4 cycles of N-terminal sequencing (Experimental Procedures). The sequence determined for each fragment was identical: VDLV, corresponding to residues 29 through 32 of E2-25K (Figure 1). As expected, the preceding residue (K28) defines a tryptic cleavage site, which is predicted to occur at the start of the first β-strand in the core domain (*22*−*26*).

Because the stable fragments produced from the two proteins had identical N-termini, the smaller fragment produced from the active Mt-D protein must have undergone (an) additional cleavage(s) at the C-terminus. To define the respective carboxyl termini, the Mt-D and Mt-E proteins were digested by a slightly modified procedure, and the digests were analyzed by mass spectrometry (Experimental Procedures). In the case of Mt-D, the results revealed the presence of a polypeptide with a mass of 15 187 ± 12 Da, which coincides with that expected if the stable fragment encompasses residues V29 through K164 (15 184 Da). The corresponding polypeptide in the Mt-E digest had a mass of 19 157 ± 16 Da, which coincides with the mass expected if the stable fragment encompasses residues K29 through the C-terminus, N200 (19 154 Da). Thus, the tail domain is completely refractory to cleavage in the Mt-E protein. We assigned this property to the presence of the S86Y mutation, versus the I76T mutation, based on the results of qualitative studies with Mt-B (I76T) and Mt-C (S86Y), in which only the Mt-B protein produced the smaller stable fragment (data not shown).

The tail of E2-25K is homolgous to a functionally uncharacterized region, the "UBA domain," which is present in a number of proteins (*39*). In so far as the entire tail domain is resistant to tryptic digestion when the S86Y mutation is present in the core domain, the digestion data support the suggestion that the UBA domain has a defined globular structure, possibly consisting of several α-helices [Figure 1 (*39*)]. An extended helical conformation in the tail domain could allow for a close approach between some portion of this tail domain and the active site. The tryptic resistance of S86Y-25K can be explained if the presence of Tyr at position 86 alters the core−tail interaction such that the tail associates more closely with the core (above), thereby rendering K164 inaccessible to trypsin. The proposed

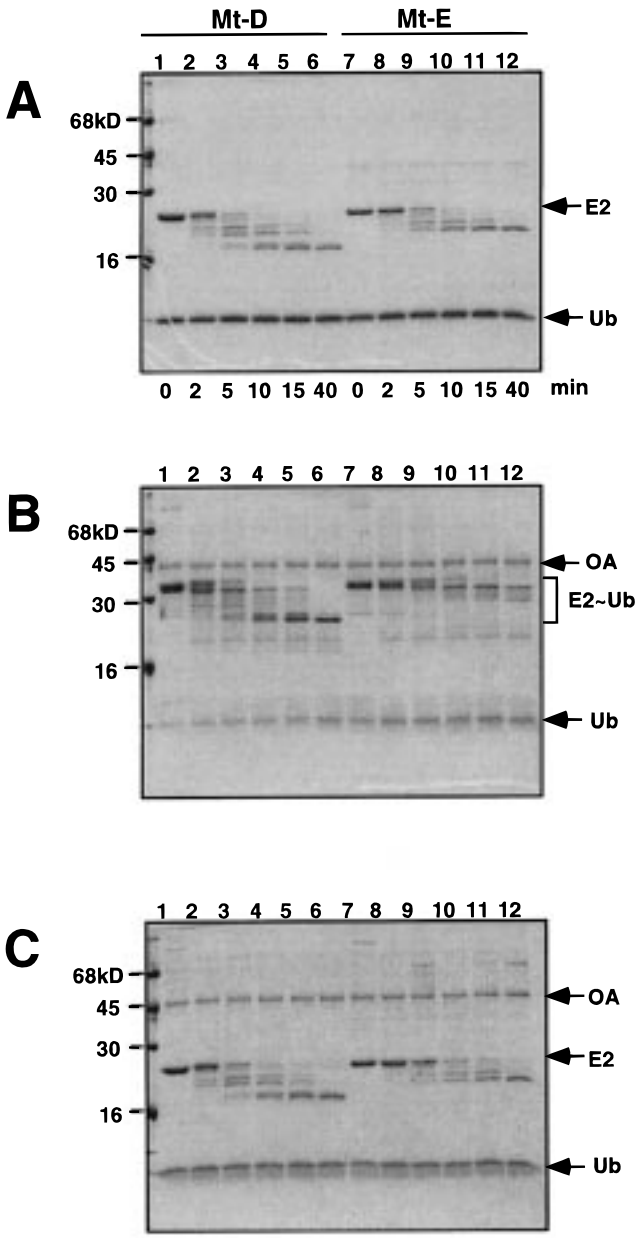

FIGURE 4: S86Y mutation blocks tryptic cleavage within the tail domain (Coomassie-stained gels). (A) Free enzymes: Mt-D (C710S) versus Mt-E (I76T,S86Y,C170S). The indicated form of E2-25K (0.25 mg/mL) was preincubated with 1 mM ATP, 2.5 mM $MgCl_2$, and 0.2 mg/mL K48R-Ub. After 4 min, NaCl was added to 250 mM, followed by trypsin (14% w/w with respect to the combined concentrations of E2 and Ub; Experimental Procedures). Digests were sampled before trypsin addition (lanes 1 and 7), or at 2 min (lanes 2 and 8), 5 min (lanes 3 and 9), 10 min (lanes 4 and 10), 15 min (lanes 5 and 11), or 40 min (lanes 6 and 12); aliquots corresponding to 1 μg of E2-25K were resolved on a 13.5% gel. The positions of the (undigested) E2 and Ub are indicated. (B) E2~Ub adducts: quenching without mercaptoethanol. The preincubation contained E1 (0.15 μM); the experiment was otherwise identical to that shown in panel A. Aliquots were quenched in sample buffer without mercaptoethanol. The region containing the E2~Ub adduct is bracketed; OA designates ovalbumin (carrier in E1). (C) E2~Ub adducts: quenching with mercaptoethanol. Part of each quenched sample from panel B was mixed with an equal volume of sample buffer containing mercaptoethanol and boiled prior to electrophoresis. In each panel, part of a lane containing molecular weight standards is shown at the extreme left.

enhanced association may reflect a new interaction involving solely Y86, or Y86-induced changes in folding that allow for several new interactions. Our results also show that the entire core domain except for the N-terminal helix is refractory to digestion in both forms of E2-25K. This property is likely to be characteristic of E2 core domains, given the structural conservation of this region (*22*−*26*).

To test whether an altered conformation persisted in S86Y-25K when Ub was covalently bound at the active site, we subjected the thiol ester forms of the Mt-D and Mt-E enzymes to tryptic digestion. For this purpose, the E2 protein was preincubated with E1, MgATP, and K48R-Ub to allow formation of the E2∼Ub adduct. Trypsin was then added, and aliquots were quenched at increasing times in sample buffer containing β-mercaptoethanol (which cleaves the thiol ester bond), or in sample buffer lacking mercaptoethanol (which permits detection of the thiol ester adduct). The samples were analyzed by SDS−PAGE and Coomassie staining.

Inspection of Figure 4B (no mercaptoethanol) shows that in each case the E2∼Ub adduct persisted throughout the 40-min incubation. Inspection of Figure 4C (plus mercaptoethanol) shows that the Mt-D and Mt-E proteins each gave rise to a stable fragment of the same molecular weight as that which appeared during the digestion of the free E2 (compare to Figure 4A). Thus, the tail of the Mt-E protein was protected against tryptic cleavage even when Ub was covalently bound at the active site. These results indicate that altered interactions between the core and tail domains, due to the S86Y mutation, persist in the E2∼Ub thiol ester. The persistence of an electrophoretically detectable thiol ester adduct throughout the E2∼Ub digestions (Figure 4B) indicates that the R74−G75 bond in Ub was only minimally cleaved, even though this site is extremely trypsin-susceptible in free Ub (*40*). The inference that the R74−G75 bond of Ub is protected in the E2∼Ub adduct remains to be confirmed by determining the rate of cleavage of this bond (in free Ub) under the conditions of Figure 4B. Comparison of Figure 4A and Figure 4B indicates that there is little, if any, protection of the E2 by covalently bound Ub.

Effect of S86Y Mutation on Ub Transfer from the E2∼Ub Adduct. We considered whether the tighter core−tail interaction that is induced by the S86Y mutation could abrogate conjugation by reducing the accessibility of the thiol ester bond to the incoming Ub acceptor. As a first test of this hypothesis, we carried out pulse−chase experiments to determine the rate of Ub transfer to several small-molecule acceptors. Ub transfer to one of the simplest such acceptors, namely, free lysine, is catalyzed, because the reaction is abolished following denaturation of the E2∼Ub adduct with urea [see (*8*); we have confirmed this result with the purified Mt-D protein; data not shown]. The S86Y mutation reduced k_{cat}/K_m for transfer to lysine by 2.8-fold (experiment 1, Table 3). A similar factor of 3.5-fold was observed with a peptide, Ac-AGKQL-NH_2, in which the Lys residue is in the same sequence context as K48 of Ub (experiment 2, Table 3). The peptide is a marginally better substrate than free lysine for wild-type E2-25K (∼2-fold larger k_{cat}/K_m).[3] These effects, while significant, are much less dramatic than the ≥100-fold reduction in k_{cat}/K_m for transfer to Ub (above). Thus, rather than providing an explanation for the very severe inhibition of polyUb chain synthesis, these results suggest that the structure of the active site is largely intact in forms of E2-25K carrying the S86Y mutation. Correspondingly, it is unlikely that the side chain of S86 plays an important catalytic role which cannot be fulfilled by a Tyr side chain.

Table 3: Reactivity of E2∼Ub Thiol Esters[a]

expt	acceptor	E2-25K	residue 86	k_{obsd} (min^{-1})[b]	ratio[b]
1	lysine (30 mM)	Mt-D	Ser	0.089	2.8
		Mt-E	Tyr	0.032	
2	Ac-AGKQL-NH_2 (6.7 mM)	Mt-D	Ser	0.035	3.5
		Mt-E	Tyr	0.010	
3	DTT (1 mM)	wild-type	Ser	0.131	2.8
		Mt-A	Tyr	0.047	
4	H_2O[d]	wild-type	Ser	0.039	1.8
		Mt-A	Tyr	0.022	
5	polylysine (∼1 mM)	Mt-D	Ser	0.01	7.0
		Mt-E	Tyr	0.0014	(6.5 + 1.5)[e]

[a] In experiments 1−4, transfer of ^{125}I-Ub to the indicated acceptor was monitored by pulse−chase (Experimental Procedures). Values of k_{obsd} were obtained from semilog plots of remaining thiol ester radioactivity versus time. Values shown are for single experiments comparing active and inactive (S86Y) enzymes with a given nucleophile. All results were confirmed in at least one other independent experiment. The conditions of experiments 1 and 2 correspond to k_{cat}/K_m. In experiment 5, steady-state conjugation was monitored as described under Experimental Procedures; k_{obsd} was calculated by dividing the initial rate by the enzyme concentration. The concentration dependence of the polylysine reaction is uncharacterized. [b] Rate constant ratio for S86 enzyme/Y86 enzyme. [c] In experiments 1−3, the value of k_{obsd} has been corrected by subtracting the value of k_{hyd} measured in the same experiment (k_{hyd} applies to condition of no added nucleophile). [d] No nucleophile added (traces of DTT originating in the E1 and E2 preparations may have contributed to the observed reaction). [e] Mean ± SD of 3 independent determinations.

We also characterized Ub transfer to a thiol acceptor, DTT. This reaction is uncatalyzed, as indicated by the finding that the DTT reaction was accelerated by 10−20-fold when the wild-type E2∼Ub adduct was denatured with urea (data not shown). It should be noted that this observation supports a model in which the active site is occluded even in the E2∼Ub adduct formed from wild-type E2-25K. Under native conditions, the presence of the S86Y mutation decreased the rate constant for Ub transfer to DTT by a factor of 2.8-fold (Table 3, experiment 3). The E2∼Ub adducts of the wild-type and Mt-A enzymes reacted with DTT at an identical rate following denaturation with urea (data not shown). Because denatured E2 oxy esters react slowly with thiols [in comparison to thiol esters (*41*, *42*)], this result excludes a model in which the lower reactivity of the mutant E2∼Ub adduct was due to migration of the Ub from the thiol group of C92 to the hydroxyl group of Y86. Hydrolysis of the thiol ester, as inferred from the rate of decay in the absence of added acceptor, was ∼2-fold slower with the mutant enzyme (Table 3, experiment 4). The quantitative reductions in the rates of reaction with DTT and water (and lysine, above) were similar to the factor of ∼2-fold seen in alkylation of the free enzyme (above). These data, and the tryptic digestion results (Figure 4), support a model in which the presence of the S86Y mutation similarly reduces active site accessibility in the free enzyme and the E2∼Ub adduct. However, as noted above, these results do not provide an

[3] J. Piotrowski and C. Pickart, unpublished experiments.

explanation for the very severe inhibition of transfer to the normal acceptor, Ub.

A potential explanation for the differential effect on Ub transfer to small molecules, versus Ub, can be developed based on acceptor size. Specifically, if the side chain of Y86 creates a steric block in the active site, either directly or by promoting an inappropriate interaction with the tail domain (see below), then reactions involving macromolecular acceptors (e.g., Ub) might be more strongly inhibited than reactions involving small-molecule acceptors (e.g., lysine). E2-25K has no known protein substrates besides Ub (*27*, *28*). However, a preliminary test of this hypothesis was made possible by the finding that E2-25K conjugates Ub to lysine side chains in amino acid polymers. These conjugation reactions could not be reliably detected by electrophoretic methods due to precipitation of the polymers in SDS (data not shown), but they could be monitored by absorbing the conjugate products onto cation-exchange resin (Experimental Procedures). As shown in experiment 5 of Table 3, the presence of the S86Y mutation reduced the rate of the conjugation reaction observed with polylysine (∼7.5 kDa, ∼1 mM) by a factor of 6.5-fold. The stronger impairment of transfer to the polymer, versus free lysine, is most simply attributed to the larger size of the polymer, given that the same (lysine−Ub) bond is formed with both substrates.

Implications for E2 Structure−Function. We have shown that the mutation of a residue within the core domain of E2-25K mimics the effect of deleting the tail domain: activity in Ub thiol ester formation is substantially intact, but transfer of the thiol-linked Ub to a second molecule of Ub is severely inhibited. The S86Y mutation is conservative in the sense that the mutant residue retains the hydroxyl functional group, and Tyr occurs at the corresponding position in several other E2s. The latter enzymes include two E2s whose crystal structures have been determined. The structures show that the side chain of residue 86 is within 6 Å of the active-site thiol group, but the side chain of the Y86-homologous Tyr does not engage in specific interactions with other residues (*22*, *25*).

Two general explanations may be considered for the deleterious effect of the S86Y mutation. The first is that the side chain of S86 plays a direct and important role in catalysis which cannot be fulfilled by the side chain of Y86. We consider this explanation to be unlikely because of the substantial catalytic competence of S86Y-25K in reactions involving small-molecule acceptors (above). The second explanation is that the presence of Tyr at residue 86 creates a steric blockade in the active site. We favor this explanation because it is consistent with known features of the active sites of the mutant and wild-type enzymes, and because it can accommodate the differential impairment of reactions involving macromolecular versus small-molecule acceptors.

Several observations suggest that the active site of wild-type E2-25K is rather occluded: the free enzyme is resistant to alkylation [(*21*) and this work]; the E2∼Ub adduct is protected from nucleophilic attack by DTT (this work); and the E2∼Ub adduct is unable to transfer Ub to iodotyrosyl-Ub, apparently due to a negative steric effect from the bulky iodine moieties [this negative effect is specific for the presence of iodotyrosine in the acceptor Ub (*43*)]. Thus, we propose that replacing the small hydroxymethyl side chain of Ser with the bulky phenolic side chain of Tyr impairs activity by decreasing the accessibility of the thiol ester bond to the K48 side chain of the acceptor Ub molecule.

We favor a model in which the postulated steric effect is indirect, because we have shown that the S86Y mutation enhances the physical interaction between the core and tail domains (Figure 4). However, we cannot exclude a direct steric effect. We showed previously that active site occlusion, as manifested in alkylation resistance, depends on the specific orientation of the core and tail domains of wild-type E2-25K (*21*). In the present study, we have shown by several criteria that the S86Y mutation increases this occlusion. We therefore propose that the steric hindrance caused by the presence of Y86 is due to the induction of an inappropriately tight interaction between the core and tail domains. We hypothesize that this hindrance is weakly manifested with small-molecule acceptors such as lysine and short peptides, but strongly manifested with macromolecular acceptors such as polylysine and Ub.

In principle, additional evidence that the effect of the S86Y mutation is mediated through the tail could be obtained by showing that this mutation is benign when the tail is absent (*21*). In practice, the probable insight to be gained from such an experiment is low, due to the very weak conjugation activity of the wild-type core domain (*21*). A function for S86 in stabilizing the core−tail interaction in the wild-type enzyme is consistent with the finding that the core domain of yeast Ubc4p, which lacks a hydroxyl-containing side chain immediately N-terminal to the active-site Cys residue (*33*), cannot interact with the tail domain of E2-25K in a functionally productive manner (*21*). However, it is perhaps more likely that S86 plays a different role in the wild-type enzyme, and placing a Tyr at this position establishes a deleterious new contact through the aromatic ring.

The tail domains of E2 proteins have been suggested to influence function by mediating *inter*molecular interactions, for example, with E3 enzymes or substrates [see (*21*) and references cited therein]. The results of the present study suggest that tail domains may also mediate *intra*molecular interactions with their cognate core domains. The current findings reinforce our suggestion that core−tail interactions make an important contribution to the activity of wild-type E2-25K in polyUb chain synthesis (*21*). This conclusion is also consistent with previous results obtained with other E2 proteins. For example, certain core domain mutations in Rad6/Ubc2p inhibit histone ubiquitination and sporulation without affecting other activities of this E2 (*44*); the two affected activities are dependent on the tail of Rad6p (*45*, *46*). Similarly, certain point mutations near the active site of yeast Cdc34/Ubc3p (*47*, *48*) strongly modulate an autoubiquitination reaction in which Ub moieties are transferred from the active-site Cys to a specific Lys residue in the tail domain (*49*). Core−tail interactions also contribute to the essential cell cycle function of Cdc34p (*50*, *51*).

It is likely that that E2 core domains perform specific functions by participating in both intermolecular and intramolecular interactions [see (*21*) and references cited therein]. The majority of the nonconservative substitutions in known E2s are clustered on the face of the core domain that is opposite the active site (*22*−*25*). It has been suggested that the active-site face is involved in interactions that are common to all E2s, e.g. interactions with the thiol-bound Ub or with E1, while the opposite face is involved in

interactions that are specific to a given E2, e.g., interactions with substrates or E3s (*22*−*25*). The present results indicate that a residue proximal to the active site has potential to interact with a C-terminal extension, and thereby influence specific E2 function.

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