Crystal Structure of the *Saccharomyces cerevisiae* Ubiquitin-conjugating Enzyme Rad6 at 2.6 Å Resolution*

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The *Saccharomyces cerevisiae* ubiquitin-conjugating enzyme (UBC) Rad6 is required for several functions, including the repair of UV damaged DNA, damage-induced mutagenesis, sporulation, and the degradation of cellular proteins that possess destabilizing N-terminal residues. Rad6 mediates its role in N-end rule-dependent protein degradation via interaction with the ubiquitin-protein ligase Ubr1 and in DNA repair via interactions with the DNA binding protein Rad18. We report here the crystal structure of Rad6 refined at 2.6 Å resolution to an R factor of 21.3%. The protein adopts an αβ fold that is very similar to other UBC structures. An apparent difference at the functionally important first helix, however, has prompted a reassessment of previously reported structures. The active site cysteine lies in a cleft formed by a coil region that includes the 310 helix and a loop that is in different conformations for the three molecules in the asymmetric unit. Residues important for Rad6 interaction with Ubr1 and Rad18 are on the opposite side of the structure from the active site, indicating that this part of the UBC surface participates in protein-protein interactions that define Rad6 substrate specificity.

Ubiquitin is a highly conserved 76-amino acid eukaryotic protein that is covalently attached through its C terminus to the ε-amino group of lysine side chains in acceptor proteins (reviewed in Refs. 1–4). The best known role for ubiquitination is to target substrate proteins for degradation by the 26 S protease, an activity that plays a key role in a number of cellular processes including cell cycle progression, signaling pathways, stress responses, removal of damaged or misfolded proteins, and the production of antigenic peptides.

Ubiquitination of substrates is performed by the numerous ubiquitin-conjugating enzymes (UBCs) or E2s. UBCs receive ubiquitin from an E1 ubiquitin-activating enzyme to form a thiol-ester intermediate in which the UBC active site cysteine is linked to the ubiquitin C terminus. The ubiquitin is then transferred from the UBC to form an isopeptide bond through its C terminus to a lysine side chain of the target substrate protein. In some cases this process proceeds directly, whereas in other cases a further level of substrate specificity is provided by a ubiquitin-protein ligase or E3.

The *Saccharomyces cerevisiae* ubiquitin-conjugating enzyme Rad6 (also known as UBC2) is a 172-residue protein that is predominantly localized to the nucleus (5–7). Rad6 has a 149-amino acid core domain common to other E2s and a 23-residue C-terminal “tail” that is comprised almost entirely of acidic residues. A single cysteine, Cys-88, in the core domain serves as the point of attachment of ubiquitin prior to transfer to cellular targets. *rad6* mutants are extremely sensitive to UV light and other DNA damaging agents and exhibit a defect in post-replicative bypass of UV-damaged DNA and in damage-induced mutagenesis. Furthermore, mutations in *Rad6* cause poor growth, a sporulation defect, and an increase in the rate of retrotransposition of yeast Ty elements (6, 8–12). The ubiquitin-conjugating activity is essential for the DNA repair, mutagenesis, and other functions of Rad6, because *rad6* mutants in which the active site cysteine has been replaced with alanine or serine have a *rad6Δ* phenotype (13, 14).

Rad6 substrate specificity is apparently determined, at least in part, through interactions with the ubiquitin-protein ligases or E3 proteins Ubr1 and Rad18. The single strand DNA-binding protein Rad18 appears to mediate the DNA repair functions of Rad6 (15), whereas a separate Rad6 activity, the so called N-end rule degradation pathway, depends upon formation of a specific Rad6 complex with Ubr1 (7, 16, 17). Although Rad6 exists in complex with both Rad18 and Ubr1 in *in vivo* attempts to detect a Rad6-Rad18-Ubr1 ternary complex have failed, indicating that Rad6 associates with these proteins in separate complexes. This is consistent with roles for Rad18 and Ubr1 in defining distinct sets of Rad6 substrates.

To further the understanding of how Rad6 functions in diverse biological processes, we have determined the structure of *S. cerevisiae* Rad6 by x-ray crystallography. The structure closely resembles that of other UBC enzymes, although our analysis reveals a “frame shift” error in a functionally important part of previously reported structures. Residues that are required for binding the Rad6-specific E3 proteins Ubr1 and Rad18 are on the opposite side of the molecule from the active site.

**EXPERIMENTAL PROCEDURES**

Expression and Purification—Rad6 was expressed from plasmid pSCW242 in the *S. cerevisiae* strain CMY135 (11). Cells grown to high density (∆A_{600} > 1.4) in culture medium (6 mg/ml NaOH, 10 mg/ml succinic acid, 3.6 mg/ml ammonium sulfate, 36 mg/ml glucose, 2.9 mg/ml yeast nitrogen base (Difco), 2.6 mg/ml vitamin assay casamino acids (Difco), 7.2 × 10^{-2} mg/ml each of adenine and uracil) were harvested by centrifugation at 11,300 × g and then stored at −80 °C. Frozen cells were thawed and resuspended in lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 10% glycerol, 200 mM KCl, and 10 mM β-mercap-
Structure of Rad6

Rad6 Structure—The crystal structure of Rad6 was determined using a combination of single isomorphous replacement, molecular replacement, and NCS averaging. The molecular replacement solution was used to generate NCS operators and for the calculation of a molecular mask but was not used to estimate phases directly. NCS averaging and other density modifications resulted in a high-quality electron density map devoid of model bias (Fig. 1). A model built into this map has been refined to an $R_{factor}$ of 21.3% ($R_{free}$ of 24.6%) against 2.6 Å data. The refined structure has good geometry (27) (Table II). The N-terminal methionine and C-terminal 18 residues of each molecule are disordered and have been omitted from the model. Note that electrospray mass spectrometry demonstrated that

to ethanol). Purification was by four column chromatography steps with thiomerosal (20–3.0 Å). 

| TABLE I Data statistics | TABLE II Refinement statistics |
|-------------------------|-------------------------------|
| **Quantities in parentheses are statistics for the highest resolution shell, which is 2.64–2.60 Å for native and 3.05–3.0 Å for the derivative.** | **Resolution (Å)** |
| $d_{max}$ (Å) | Native (20–2.6 Å) | 2.6 | 2.0 |
| No. of observed reflections | 76,204 | 58,825 |
| No. of unique reflections | 26,012 | 16,799 |
| Complete (%) | 91.3 (66.9) | 89.3 (55.3) |
| $R_{sym}$ (%) | 7.0 (26.9) | 7.2 (17.9) |
| $R_{merge}$ (%) | 12.9 (3.6) | 14.3 (3.1) |
| No. of heavy atoms | 4 | 22.7 |
| Phasing power (centric)* | 1.18 | 1.30 |
| Phasing power (acentric)* | 0.67 | 0.78 |
| $R_{coll}$ (acentric)* | 0.78 | 0.32/0.73 |
| (FOM) before after DM | 0.30/0.73 |

$^a$ All observed reflections [$(|F_{obs}| > 0)$] were used for refinement.

$^b$ $R_{factor} = 100\Sigma_{|F_{calc}|-|F_{obs}|}/\Sigma_{|F_{calc}|}$.

$^c$ $R_{free} = 100\Sigma_{|F_{calc}|-|F_{obs}|}/\Sigma_{|F_{calc}|}$.

$^d$ $R_{free} = R_{factor}$ for a randomly selected set of reflections (5%) that have not been used in refinement.

$^e$ Geometry was analyzed with PROCHECK (27).

and SCALEPACK (18) (Table I).

Structure Determination and Refinement—Rad6 crystals belong to space group $I4_1$22 with cell parameters of $a = 113.75$ Å, $b = 146.36$ Å, and $c = 199.88$ Å. There are three Rad6 molecules in the asymmetric unit corresponding to 3.9 Å$^2$/dalton (68% solvent). Most crystallographic calculations used programs from the CCP4 suite (19). The mercury atom positions were determined by inspection of difference Patterson and Fourier maps. Initial protein maps using the mercury phases were of poor quality, and although they show protein versus solvent regions, they were otherwise uninterpretable. Molecular replacement using the structure of the Rad6 homolog UBC1 from Arabidopsis thaliana as a search model (20) and the program AMoRe (21) readily located the three molecules in the asymmetric unit. The previously identified mercury positions corresponded to the highest peaks in a difference Fourier calculated with model derived phases. Rigid body refinement of the UBC1 molecular replacement solutions using X-PLOR (22) gave an $R$ factor of 46.1% for 7.0–3.5 Å data. This solution was used to define noncrystallographic symmetry (NCS) operators and to construct a molecular mask (23). The single isomorphous replacement phases were then refined by 3-fold averaging, histogram shifting, and solvent flattening using the program DM (24). The resulting phases, which depended upon the molecular replacement solution only for NCS operators and the molecular mask, gave an electron density map that was readily interpretable (see Fig. 1). Rounds of automated positional and temperature factor refinement using XPLOR were interspersed with model building using the program O (25). 5% of the data were withheld from refinement to optimize weights and to monitor the refinement by cross-validation (26). NCS restraints on 1617 main chain atoms and 1695 side chain atoms (3312 atoms out of a total of 3699 protein atoms) were acquired during automated refinement. As a check of the reliability of the NCS restraints assignment, the coordinates of the structure late in the refinement were subjected to a 3000-degree simulated annealing refinement using torsion angle molecular dynamics and no NCS restraints (22). The resulting structure showed significant deviations between the superimposed monomers only in portions of the molecule where NCS restraints were not applied in the previous refinement cycles, namely residues 81–87 and residues 114–124. The average pairwise root mean square deviations in atomic positions of these superimposed monomers was 0.69 Å using all atoms, and 0.57 Å when residues 81–87 and 114–124 were omitted. Refinement statistics are given in Table II.
the N-terminal methionine and 18 C-terminal residues were retained after purification, and SDS-polyacrylamide gel electrophoresis analysis of washed crystals was consistent with full-length protein. In addition, mobile side chains of four residues have been included with zero occupancy (Lys-14 of molecule A and Lys-131 of all three molecules in the asymmetric unit).

Rad6, which has overall dimensions of approximately \(52 \times 25 \times 37\) Å, is comprised of an antiparallel \(\beta\)-sheet with four strands, four \(\alpha\)-helices, and one short \(3_{10}\) helix (Fig. 2A). The \(\beta\)-sheet is exposed to solvent on one side and is flanked on the other side by \(\alpha\)-helices 1 and 2 and the \(3_{10}\) helix. The active site cysteine is located in a shallow cleft formed by residues 89–95, which includes the \(3_{10}\) helix, which makes a small promontory beneath the cysteine, and residues 115–121, which form a loop above the cysteine. Due to crystal packing interactions, residues 115–121 adopt different conformations in the different molecules of the asymmetric unit. In two of the molecules, the side chain of Asn-117 is hydrogen bonding to the amide nitrogen of Ala-119. In the third molecule this side chain is hydrogen bonded to a symmetry related molecule, resulting in displacement of \(\alpha\)-carbon atoms by up to 3.9 Å for Pro-118. Because these alternate loop conformations affect the exposure of the active site cysteine, it is possible that this mobility is necessary for Rad6 function.

Comparison with Other UBC Structures—The Rad6 structure closely resembles that of other published UBCs. Least squares overlap of Rad6 on the known structure of the Rad6 homolog UBC1 from \(A.\ thaliana\) (20), which was used as the search model in molecular replacement and shares 63% residue identity with Rad6, gave a root mean square deviation of 1.44 Å over 149 C\(_\text{a}\) atoms.

Visual inspection of the superimposed structures reveals that the main differences between the Rad6 and UBC1 \(C\)\(_\text{a}\) positions are near the N termini, with significant divergence before residue 6 of Rad6, and in the loop between \(\alpha\)-helix 2 and \(\alpha\)-helix 3 that comprises the roof of the active site cleft (residues 115–121). There is also an apparent register shift in the first helix of the superimposed structures with respect to the sequence alignment of Fig. 3. Thus Rad6 residues 6–13 appear structurally equivalent to UBC1 residues 7–14. The return to register at Arg-15 of Rad6 occurs because the C-terminal portion of helix 1 (residues 14–16) in the UBC1 model is in a \(3_{10}\) helical conformation. One effect of this N-terminal register shift is to place Leu-9 and Phe-13 of the UBC1 structure at the positions of Arg-8 and Asp-12 of Rad6. Although these two charged Rad6 side chains are buried, their hydrogen bonding potential is saturated. The two residues form a salt bridge with each other, and each has hydrogen bonding interactions with the phenolic hydroxyl of Tyr-63. In addition, the Asp-12 side
chain hydrogen bonds with the backbone nitrogen of Val-102, and Arg-8 hydrogen bonds with the carbonyl oxygens of Tyr-100 and Pro-98 (Fig. 1). Although hydrophobic residues, such as Leu-9 and Phe-13 of UBC1, are commonly found in buried environments, in the context of the UBC fold it appears that this arrangement would leave a number of potential hydrogen bonds unsatisfied. It is noteworthy that Arg-8, Asp-12, and Tyr-63 are invariant residues among Rad6 homologs (Fig. 3).

Similar apparent discrepancies are also present for Rad6 helix 1 and two of the other published UBC structures (28, 29). Since submission of this manuscript, Dr. W. J. Cook (University of Alabama) has used our Rad6 coordinates to guide further refinement of these three previously published structures, which are now found to closely resemble Rad6 with no apparent register shift in helix 1. Arg-8 and Asp-12 are conserved in all published UBC structures, with Asp-12 sometimes substituted by Glu. Recently, crystal structures have been reported for murine and human Ubc9, where Arg-8 and Glu-12 are seen to be buried as they are in the Rad6 structure (30). Thus Arg-8 and Asp/Glu-12 side chains appear to be buried in equivalent environments for all known UBC structures.

The UBC1 structure has been used to guide the design and interpretation of previous mutagenic studies on Rad6. For example, Arg-6, Arg-7, and Arg-8 are surface exposed in the UBC1 model, and their mutation therefore seemed unlikely to have serious consequences for stability of the Rad6 structure. Thus, biochemical and phenotypic effects of mutating these three residues to alanine were assumed to result from the disruption of direct binding interactions to other proteins such as Ubr1 and Rad18 (31). It is now apparent, in light of the Rad6 crystal structure, that the consequences of this triple mutation are likely to be indirect, because the guanidinium groups of Arg-6 and Arg-8 both participate in intramolecular hydrogen bonding, and their mutation is expected to destabilize the protein structure.

Possible E1, Ubr1, and Rad18 Binding Surfaces—There are greater than 60 sequences of either confirmed or putative UBCs available from various organisms and 13 for \textit{S. cerevisiae} alone. The level of conservation between these sequences indicates that they all have a similar fold for the conserved core domain. Because all 13 UBCs of \textit{S. cerevisiae} interact with ubiquitin and with at least one of the two homologous ubiquitin-activating enzymes Uba1 and Uba2, it is expected that this binding surface(s) on the UBC proteins will be formed by conserved residues. On the other hand, because each UBC has a distinct group of substrates that are either recognized
Fig. 4. Molecular surface representation of Rad6. A, the surface has been colored magenta to indicate positions that have 70% or greater sequence identity between the aligned sequences of the 13 UBCs from S. cerevisiae. B, the surface has been colored to indicate N and C-terminal binding determinants for Ubr1 (blue and cyan) and Rad18 (orange and yellow). The three views shown are successive 90° rotations about a vertical axis, with the left image in approximately the same orientation as Fig. 2 and the middle image directly toward Cys-88 (green). Protein sequences were acquired from the Swiss-Prot data base (40). Alignments were performed using MultAlin (41). This figure was made with GRASP (42).

Rad6 effects N-end rule degradation through its interaction with the ubiquitin protein ligase Ubr1 (16, 17). Deletion analysis suggests that this interaction is mediated by residues near the N and C termini of Rad6, which as shown in Figs. 2B and 4B, are on the opposite face of the molecule from the active site Cys-88. Residues 1–9 of the highly conserved N terminus of Rad6 are required for Rad6-Ubr1 interaction in vitro, and cells expressing Rad6Δ1–9 protein are deficient in N-end rule degradation of model substrates (7). The observation that Rad6Δ1–9 forms a thiolester bond with ubiquitin in the presence of E1 and forms a complex with Rad18 in vitro indicates that removal of residues 1–9 does not grossly alter the Rad6 fold (7, 15), although an important caveat for all deletion studies is that propagation of subtle conformational changes may impair activity at some, but not all, distant active sites on a protein structure.

Rad6Δ150–172, a mutant lacking the C-terminal 23 amino acids, also has a reduced efficiency in N-end rule protein degradation and does not form a stable complex with Ubr1 but maintains the ability to form a thiolester bond with ubiquitin in the presence of E1 and to ubiquitinate proteins in vitro (7, 32–34). Because Rad6Δ154–172 shows an essentially wild type phenotype, residues 150–153 also appear to be important for Rad6-Ubr1 interaction (7). All of these residues (Glu-150, Asp-151, Asp-152, and Met-153) are solvent exposed, although none are well conserved among the Rad6 homologs.

It has been shown, both in vivo and in vitro, that Rad6 forms a tight complex with the single strand DNA binding protein Rad18, which likely functions to target Rad6 to sites of DNA damage (35). Rad18 is a member of the Rad6 DNA repair epistasis group (6, 12), and rad18 mutant strains are equally sensitive to UV radiation and are as defective in post-replicative bypass and UV-induced mutagenesis as rad6 mutants, although sporulation and N-end rule proteolysis are not affected (6, 12, 36).

Because Rad6-Rad18 complex formation occurs for the Rad6Δ1–9 or Rad6Δ150–172 mutants but not with the Rad6Δ1–22 or Rad6Δ142–172 mutants, it appears that residues 10–22 and 142–149 are important in this interaction (15). Residues 10–19 are part of α-helix 1, whereas residues 20–22 are in an extended conformation. Exposed or partly exposed residues in this N-terminal Rad18 binding determinant include Met-10, Arg-11, Phe-13, Lys-14, Arg-15, Lys-17, Glu-18, Asp-19, and Pro-22. All of these except Arg-15, Lys-17, Glu-18, and Pro-22 are invariant among Rad6 homologs, with just one Rad6 homolog having a lysine at position 15 instead of arginine. The C-terminal Rad18 binding determinant, residues 142–149, is contained within α-helix 4. Exposed residues here include Lys-142, Glu-143, Glu-146, Lys-147, and Trp-149. Of these, only Glu-146 is invariant among Rad6 homologs, with position 149 always occupied by Trp or Phe. Thus, Glu-146 and Trp/Phe-149, which are adjacent in space on the same exposed face of α-helix 4, may be the critical features of α-helix 4 in the Rad6-Rad18 binding interaction.

Because the Rad6Δ142–172 mutant forms a thiolester bond with ubiquitin in the presence of E1, it is unlikely that failure to bind Rad18 is due to protein misfolding (15). However, the binding study that failed to show complex formation between Rad6Δ1–22 and Rad18 utilized a GST-Rad623–153 fusion protein, and there was no independent check that this construct was correctly folded. Both α-helix 1 and α-helix 4 are on the opposite side of the molecule from the active site cysteine, which would allow binding of Rad18 to Rad6 without hindering access of substrate to the active site of Rad6. A 40-residue domain of Rad18 is sufficient for binding to Rad6 (15). Because the distance between the centers-of-mass of Rad6 residues 10–22 and 142–149 is 28.5 Å and a simple model building exercise suggests that at least 12 residues are required to span these two determinants across the surface of Rad6, the 40-residue Rad18 domain would have to be highly extended to contact both determinants.
In summary, the crystal structure of Rad6 that we have determined at 2.6 Å resolution can serve as a framework for the design and interpretation of mutagenic studies. In particular, clarification of the conformation and solvent accessibility of residues in the N-terminal helix is relevant to deciphering details of Rad6 interactions with Rad18 and Ubr1.

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