Structural and Functional Analysis of the Human Mitotic-specific Ubiquitin-conjugating Enzyme, UbcH10*

Received for publication, September 28, 2001, and in revised form, March 22, 2002
Published, JBC Papers in Press, April 1, 2002, DOI 10.1074/jbc.M109388200

Yaqiong Lin, William C. Hwang, and Ravi Basavappa‡
From the Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14642

Cell cycle progression is controlled at several different junctures by the targeted destruction of cell cycle regulatory proteins. These carefully orchestrated events include the destruction of the securin protein to permit entry into anaphase, and the destruction of cyclin B to permit exit from mitosis. These destruction events are mediated by the ubiquitin/proteasome system. The human ubiquitin-conjugating enzyme, UbcH10, is an essential mediator of the mitotic destruction events. We report here the 1.95-Å crystal structure of a mutant UbcH10, in which the active site cysteine has been replaced with a serine. Functional analysis indicates that the mutant is active in accepting ubiquitin, although not as efficiently as wild-type. Examination of the crystal structure reveals that the NH₂-terminal extension in UbcH10 is disordered and that a conserved 3₁₀-helix places a lysine residue near the active site. Analysis of relevant mutants demonstrates that for ubiquitin-adduct formation the presence or absence of the NH₂-terminal extension has little effect, whereas the lysine residue near the active site has significant effect. The structure provides additional insight into UbcH10 function including possible sites of interaction with the anaphase promoting complex/cyclosome and the disposition of a putative destruction box motif in the structure.

Ubiquitin-mediated proteolysis regulates cell cycle progression at several key control points. At least two such control points occur in mitosis. One is at the transition from metaphase to anaphase and the other is at the exit from mitosis (for reviews, see Refs. 1–5). At the transition from metaphase to anaphase, the securin protein in the securin-separase protein complex is destroyed to release separase. The freed separase cleaves the protein complexes binding the sister chromatids together. Cleavage of these protein complexes is thought to facilitate sister chromatid segregation, and hence entry into anaphase. To exit from mitosis, cyclin B in the cyclin B-cdc2 complex must be destroyed. Destruction of cyclin B results in the inactivation of the cdc2 kinase. The inactivation of cdc2 is an essential event for resetting the cell cycle machinery (6).

To accomplish the ubiquitination of securin and cyclin B (and other proteins targeted for ubiquitin-mediated destruction), three enzyme activities, designated E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme, Ubc), and E3 (ubiquitin ligase), must work in concert (for review, see Ref. 7). The E1 protein activates ubiquitin and then transfers it to the E2 protein. The ubiquitin forms an adduct to the E2 protein via a thiol ester linkage between the active site cysteine of E2 and the carboxyl terminus of ubiquitin. The E2 then donates the ubiquitin to the target protein, either directly or in conjunction with the E3 activity. In some instances, the same protein possesses both the E2 and E3 activity. Ultimately, a polyubiquitin-target protein conjugate is formed that is recognized by the proteasome. The proteasome hydrolyzes the target protein and releases free ubiquitin. Whereas ubiquitin and E1 are highly conserved proteins, each eukaryotic organism contains different E2 and E3 activities. The various E2 and E3 proteins function in cognate pairs and provide specificity in target protein ubiquitination.

In the case of mitotic destruction of securin and cyclin B, the same E2 and E3 activities are thought to be responsible for the destruction of both proteins. The E3 activity is contained in a large multisubunit complex, termed the anaphase promoting complex or cyclosome (APCC). The target protein specificity for ubiquitination seems to be conferred by different particular subunit compositions of the APCC. The mitotic E2 proteins have been identified in several organisms, including human (UbcH10) (8), clam (E2-C) (9), mouse (mE2-C) (10), Xenopus (UbcX) (11), Schizosaccharomyces pombe (Ube4) (12), and goldfish (E2-C) (13). These mitotic proteins are essential for cell cycle progression since mutation of the active site cysteine confers a dominant negative phenotype (8, 14).

The E2 protein is remarkable in that, despite its relatively small size (typically ~20 kDa), it must interact with 3 or 4 different proteins, namely ubiquitin, E1, E3, and perhaps the target protein. Therefore, the E2 protein must maintain structural features that allow interactions with the common elements of the system, ubiquitin and E1, and yet specify interactions with its cognate E3 and target protein. Although the crystal structures of several Ubc proteins have been determined and examination of their structures has given much insight into the function of this family of proteins (15–23), much still remains to be understood about the stereochomical basis of E2 function. Here we report the 1.95-Å crystal structure determination of the mitotic specific E2 protein from humans, UbcH10, an essential protein for cell cycle progression. We also report complementary functional analysis of select mutants. These studies provide new insight into E2 function.

*This work was supported in part by National Institutes of Health Grant GM 57536 and a Scholar Award from the Leukemia and Lymphoma Society of America (to R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1I7K) of the mUbcH10 crystal structure have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).‡ To whom correspondence should be addressed. Tel.: 585-273-4799; Fax: 585-275-6007; E-mail: Ravi_Basavappa@urmc.rochester.edu.

1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Ubc, ubiquitin-conjugating enzyme; APC/C, anaphase promoting complex/cyclosome; MES, 4-morpholineethanesulfonic acid.
**Human Mitotic Ubiquitin-conjugating Enzyme, UbcH10**

**EXPERIMENTAL PROCEDURES**

DNA Constructs—BL21(DE3)pLysS Escherichia coli strains containing pT7-7 plasmid, encoding either wild-type or mutant (C114S) UbcH10 genes, were provided by Dr. J. V. Ruderman (Harvard Medical School, Boston, MA). The sequence encoding the wild-type UbcH10 protein was subcloned into a pET28 vector (Novagen) at the NdeI and HindIII sites. The UbcH10 sequence contains an internal NdeI cleavage site, therefore, limited restriction digestion with NdeI enzyme was used and the fragment of the desired length was isolated by agarose gel electrophoresis. The pET28-UbcH10 construct has an NH$_2$-terminal His$_6$ tag and a thrombin cleavage site two residues upstream of the insertion site. Nt-UbcH10, the NH$_2$-terminal deletion mutant of UbcH10 (comprising residues 28–179), was subcloned by standard techniques into the pET28 vector at the NheI and SstI sites. The K19A mutant of UbcH10 was prepared using the QuikChange kit (Strategene) with the pET28-UbcH10 as the starting template.

Protein Expression and Purification—The purification protocol for wild-type and mutant UbcH10 used for crystallization trials were the same. Overnight cultures were grown in LB containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) with continuous shaking at 37 °C. Fresh media (2 liters) containing antibiotics was inoculated with overnight culture (20 ml). The cells were grown until the A$_600$ reached ~0.6, at which point protein expression was induced by adding isopropyl-1-thio-β-D-galactoside to a final concentration of 1 mm. Cells were harvested 3 h later by centrifugation (~4000 × g, 15 min).

Cells were lysed by tip sonication. Resulting cell lysates were centrifuged at 27,000 × g for 20 min and the supernatant was collected. The protein was purified by a three-step procedure. First, polyethyleneimine (PEI) was added to the final concentration of 0.5% (v/v), and the sample was incubated on ice for 10 min. This polyethyleneimine precipitation step was repeated once. Second, anion exchange resin DEAE-52 (Whatman) was added to the supernatant (1 g of DEAE-52 per 10 ml of supernatant) and incubated on ice for 30 min. The column was washed with buffer (200 ml) and the sample was then applied to the column (200 ml). The pH of the supernatant was adjusted to 7.0. Third, supernatant was applied to a POROS HS20 cation exchange column (1.7 ml) buffered with 33.3 mM MES, 33.3 mM HEPES, 33.3 mM Na acetate, pH 7.0, on a BioCad Sprint Chromatography System (PerSeptive Biosystems). UbcH10 was eluted by ~250 mM NaCl in a NaCl gradient which was developed from 0 to 1.5 in a 40-column volume at a flow rate of 6 ml/min. For crystallization, purified mutant UbcH10 was concentrated using Centriprep YM-10 and Centricon YM-10 (Amicon) to a 6 ml of reaction mixture containing 40 mM Tris-HCl, pH 7.6, 5 mM MgCl$_2$, 2 mM ATP, 1 mM phosphocreatine, 2 unit/ml phosphocreatine kinase, 0.2 mM biotinylated-ubiquitin (prepared as above), 1 mg/ml bovine serum albumin, 5 mg/ml Ubch10, and wheat E1 ~0.2 μg/ml purified by an ubiquitin affinity column was incubated at 20 °C for various times. The reaction was stopped by adding SDS loading buffer either with or without 5% β-mercaptoethanol and boiled for 2 min at 100 °C. Samples were electrophoresed on 20% polyacrylamide gels (Phast system, Amersham Biosciences) followed by blotting with streptavidin-horseradish peroxidase (Pierce) for detecting biotinylated ubiquitin. In the activity assays of Nt-UbcH10 and K119-UbcH10, the activity reaction mixtures were prepared using 0.2 mM H$_2$O$_2$, pH 7.5. Samples then were electrophoresed on 20% polyacrylamide gels.

Cristalization—Despite extensive efforts, well diffracting crystals of wild-type UbcH10 were not obtained. However, well diffracting crystals of mUbcH10 were grown. Using the vapor diffusion method, first small crystals were grown by mixing 6 μl of protein (10 mg/ml) and an equal volume of precipitant (30% polyethylene glycol 1500) and equilibrating against precipitant for 5 days at 20 °C. Then, to improve crystal size, a droplet containing equal volumes (6 μl of protein (10 mg/ml) and a solution containing 24% polyethylene glycol 1500, 50 mM Tris, pH 7.0) was equilibrated against a reservoir containing the same solution without the protein for 2 days. Small crystals were then seeded into the pre-equilibrated droplet. Crystals grew to an average size of 0.3 mm × 0.2 mm × 0.3 mm by 1 week after seeding.

Data Collection—A nearly complete set of data was obtained by combining diffraction data collected from 2 capillary-mounted crystals at room temperature using an R-AXIS II area detector (Molecular Structure Corp., MSC) with a rotating copper anode on a Rigaku RU200 generator operating at 50 kV and 100 mA and an Osram mirror system. The oscillation method was used with an exposure time of 5 min and sweeps of 2 degrees. Data were processed and reduced by the HKL package (26). The crystals showed diffraction beyond a d-spacing of 1.90 Å, but only data to 1.95 Å were used based on data quality. Data collection and reduction statistics are summarized in Table I.

Structure Determination and Refinement—The problem was solved using the molecular replacement method. The search model was based on the E2-C (PDB ID 2E2C) structure, which shares 61% sequence identity with UbcH10. To prepare the search model, non-homologous residues were changed to alanine and the side chains of homologous residues were truncated to contain the common atoms. Identical residues were unchanged. Considerations based on the Matthews coefficient (4.45 Å$^3$/dalanin for 1 molecule in the asymmetric unit) indicated the presence of 2 molecules in the asymmetric unit. The rotation solutions for both molecules were obtained using the CNS program suite (27). These solutions were confirmed using the AMoRe program (28) in the X-PLOR program suite (29). The space group is P1, then the translation function solution was not necessary for the first monomer. The location of the second monomer in the unit cell was found by fixing the first monomer and performing the translation search as implemented in CNS.

The models placed by the molecular replacement procedure had an R-factor of about 41% using data to 2.3 Å. During the refinement, 10% of the data were reserved for R$_{free}$ cross-validation, whereas the remainder was used in refinement. Rigid body refinement of the CNS reduced the R$_{working}$ to 35.32%, and R$_{free}$ to 35.14. A σa-weighted 2F$_{o}$ − F$_{c}$ electron density map (30) was calculated using phases derived from the model. Model rebuilding was performed using O (31). At this stage, residues in the model were changed to the mUbcH10(C114S) sequence (residues 22–177). Further model refinements were performed by repeating simulated annealing following by visual inspection of the agreement between model and electron density map. The use of NCS restraints resulted in increased R$_{free}$ and so, NCS was not maintained in the refinement. Because of the unstructured nature of the NH-terminal (13–29) and COOH-terminal (176–179) region, they were invisible during refinement. A total of 196 water molecules were added to the model. Four residues had alternate side chain conformations, Glu$^{55}$, Ile$^{66}$, and Ser$^{124}$ in molecule A, and Lys$^{167}$ in molecule B. The refined structure was validated by composite omit maps (27), PROCHECK (32), and WHAT_CHECK (33). Statistics of the refined structure are listed in Table I. Secondary structural elements were determined using the program PROMOTIF (34).
RESULTS AND DISCUSSION

We have determined the crystal structure of a mutant UbcH10, in which the active site cysteine has been changed to a serine. Extensive efforts to obtain well diffracting crystals of wild-type UbcH10 were not successful. However, the C114S mutant (designated mUbcH10) should be nearly isosteric with the wild-type, and therefore can provide insight into the stereochemical basis of Ubc function in general and UbcH10 function in particular.

Activity of Mutant UbcH10—We tested the ability of mutant UbcH10 to accept ubiquitin as an adduct. The linkage between mUbcH10 and ubiquitin should be an ester bond rather than the thiol ester bond that occurs with wild type. In our assay, as shown in Fig. 1A, mUbcH10 indeed can form an adduct with ubiquitin, although the formation of adduct is much slower with mUbcH10 than with wild-type UbcH10. As is expected of ubiquitin, although the formation of adduct is much slower with mUbcH10 than with wild-type UbcH10. As is expected of

ubiquitin, although the formation of adduct is much slower with mUbcH10 than with wild-type UbcH10. As is expected of with mUbcH10 than with wild-type UbcH10. As is expected of an ester bond, the ubiquitin linkage to mUbcH10 is resistant to β-mercaptoethanol reduction but not to alkaline hydrolysis (Fig. 1, B and D). The control reaction with wild-type UbcH10 indicates that such treatment with β-mercaptoethanol is sufficient to cleave a thiol ester bond (Fig. 1B). Our observation of the ability of the cysteine to serine Ubc mutant being able to accept ubiquitin is consistent with most previous reports of similarly mutated Ubes being able to accept ubiquitin or ubiquitin-like proteins (35–39). However, one published report indicates that similarly mutated C114S in the mouse homolog of UbcH10 is not able to accept ubiquitin (14). This discrepancy could be due to differences in the sensitivities of ubiquitin detection methods used (streptavidin-horseradish peroxidase/ECL detection of biotinylated ubiquitin used here versus 125I-labeled ubiquitin detected by autoradiography used in the previous report). Whereas the mUbcH10 is active in accepting ubiquitin as demonstrated here, it is a dominant-negative mutant (8). The dominant-negative nature of the mutant can be explained in part by the low free energy of hydrolysis of the ester bond compared with that of the thiol ester bond, thereby making transfer of the ubiquitin from the mUbcH10 to the target protein relatively less favored. In such a case, the mUbcH10-ubiquitin adduct may remain bound unproductively to the APC/C. In addition, the slow rate of mUbcH10-ubiquitin adduct formation suggests that perhaps the mUbcH10, even without the linked ubiquitin, is able to sequester components of the ubiquitination machinery.

An intriguing observation we note is that after longer incubation times, we detect what apparently are polyubiquitin chains being formed on the UbcH10 (Fig. 1A). The linkage to UbcH10 is resistant to reduction by β-mercaptoethanol treatment (Fig. 1B). This suggests that the polyubiquitin chain is linked to UbcH10 by an isopeptide bond and not with a thiol ester linkage. Autoubiquitination of other Ubes has been reported before (40, 41). Although this autoubiquitination may represent nonspecific transfer of ubiquitin to a nearby primary amine (42), it may be relevant to the recently reported findings that cellular levels of UbcH10 are cell cycle dependent (10, 14), and that UbcH10 apparently is destroyed in an APC-facilitated manner (14).

Overall Fold of UbcH10—We have solved and refined the crystal structure of the active site C114S mutant of the mitotic-specific ubiquitin-conjugating enzyme from human using data to 1.95-Å resolution. The data quality and model refinement statistics are presented in Table I. The UbcH10 protein is an α+β protein with one 4-stranded antiparallel β sheet and 4 α-helices (Fig. 2). The topology of the β sheet falls into group B (up-and-down meander motif) as defined by Zhang and Kim (43). The residues forming the β sheet are located in the primary sequence between the residues forming the first and second helices. The NH2-terminal helix lays diagonally across one broad face of the sheet, whereas the other three helices flank two opposite edges of the sheet. Almost two turns of a 310-helix (residues 115–119) are located between the fourth strand of the sheet and the second α-helix. The active site residue (114) is situated in a set of β turns and adjacent to the NH2 terminus of the 310-helix. The overall shape of the UbcH10 protein is roughly that of an elongated triangular prism. The side chain of the active site residue is situated on one long edge of the prism.

Comparison with Other Ubc Structures—The secondary structural elements are highly conserved in all known Ubc structures (Fig. 3A and B). In particular, β strands 3 and 4 are strictly conserved in length, whereas deviations in the lengths of β strands 1 and 2 lead to a local breakdown of structural equivalence (Fig. 3A). All four α-helices display small variations in terms of length, whereas the 310 helix is extremely well conserved. When the various Ubc structures are superimposed, it is seen quite distinctly that the continuous polypeptide segment consisting of β strands 2, 3, 4, the 310 helix, helix 2, and the intervening turns are relatively highly conserved in backbone position. The remaining regions display more variability in disposition. As can be seen from Fig. 3B, these more variable regions flank the more highly conserved region. Moreover, the active site residue, although situated in the highly conserved segment, is near a set of turns connecting helices 2 and 3 that are relatively poorly conserved. The variable regions on the face opposite to that containing the active site could be involved in interactions with E3 activities that function by providing a scaffolding for interaction of the Ubc-ubiquitin adduct. The variable regions near the active site could represent sites of interaction with E3 activities that involve ubiquitin transfer to the E3 prior to the target protein or could be important for target protein recognition. The rather striking structural conservation of β strands 2–4 and helix 2 is not reflected in the sequence (Fig. 3C). Overall, this region is not better conserved in the sequence than the other parts of the protein. The strong evolutionary pressure to maintain structure and the relatively low pressure to maintain sequence suggest that this region is more important for intrinsic structural reasons than for specific protein-protein interactions. This comparison makes clear that the Ubc structure is exquisitely structurally tuned to perform its function.

NH2-terminal Extension—The full-length UbcH10 contains 179 residues. This Ubc belongs to the class III Ubc proteins, characterized by an NH2-terminal extension followed by the “core” Ubc fold. However, electron density is absent for the residues in the NH2-terminal extension (1–29) and residues 176–179. To determine whether the absence of density is due to disorder or proteolytic degradation, mass spectrometric analysis was performed on a sample prepared from a dissolved crystal. The mass spectrometric analysis yielded results that are consistent with cleavage at residue Arg12 (data not shown). Therefore, the first 12 residues have been removed by proteolytic cleavage, whereas residues 13–29 and 176–179 are present but disordered.

To determine whether or not the N-terminal extension is necessary for UbcH10 to accept ubiquitin, we prepared a mutant lacking most of the NH2-terminal extension (up to residue 27). The activity assay of this deletion mutant reveals that these residues are not important for ubiquitin-adduct formation (Fig. 4). Moreover, substitution of the NH2-terminal sequence with that derived from the pet28 cloning construct also does not impede ubiquitin-adduct formation (Fig. 4). The sequence of the NH2-terminal extension is fairly well conserved among mitotic E2, indicating some function, for example, in-
interaction with the APC or other components of the network regulating mitotic destruction. Experiments to pursue these ideas are underway.

**Oligomeric State of UbcH10**—An intriguing question still not answered satisfactorily concerns the oligomeric state of E2 proteins. This is an important question since the oligomeric state can have significant implications for the function of E2s in ubiquitination processes.

**FIG. 1.** A, activity assay of mUbcH10 and characterization of mUbcH10-ubiquitin linkage. A comparison of wild-type and mUbcH10 activity. The mUbcH10 protein was assayed for its ability to accept ubiquitin from the E1 protein, as described in the text using biotinylated ubiquitin. Reactions were incubated with samples taken at the times as indicated. Samples were electrophoresed in 20% acrylamide gels under nonreducing conditions. The presence of ubiquitin was detected by streptavidin-horseradish peroxidase coupled with enhanced chemiluminescence (ECL, Amersham Biosciences). Migration distances of ubiquitin (Ub), UbcH10-ubiquitin (E2-Ub), and E1-ubiquitin (E1-Ub) were corroborated by running samples on gels in parallel but visualizing with silver stain (not shown). The immunoblots indicate that mUbcH10 is able to accept ubiquitin as adduct, although less efficiently than wild-type UbcH10. B, stability of wild-type and mutant UbcH10 to reduction. Reactions performed as above were electrophoresed under reducing or non-reducing conditions. The wild-type UbcH10-ubiquitin adduct is very labile to β-mercaptoethanol (βME) treatment, although at later times some stable complex was formed and is likely to be ubiquitinated UbcH10 (isopeptide linkage). The mUbcH10-Ub adduct is relatively much more stable to βME treatment, as is expected for an ester bond. C, control reactions for the assay. Control reactions, incubated for 1 h, were performed in the absence of E1, ATP, or UbcH10 to verify that the emergence of the ubiquitin-containing band depended on the presence of all three of these components. As seen, no band corresponding to E2-Ub forms when any one of these components is missing. D, alkaline hydrolysis of the mUbcH10-Ub adduct. The stability of the mUbcH10-Ub adduct to alkaline treatment was determined by incubating the adduct for 10 min in various concentrations of NaOH as indicated and then neutralizing the reaction. The mUbcH10-Ub bond can be broken upon treatment with NaOH (100 mM), which is consistent with the linkage being an ester bond. An isopeptide linkage would remain stable even under these conditions. BSA, bovine serum albumin.
The active site residue (114) is depicted in ball-and-stick rendering. This and all other structure figures were prepared using the Swiss-PDB Viewer (53) and in certain cases post-processed with POV-Ray stick rendering. The active site residue (114) is shown in green.

Human Mitotic Ubiquitin-conjugating Enzyme, UbcH10

Table I

| Summary of crystallographic and model data |
|--------------------------------------------|
| **Unit cell (space group P1)**              |
| Edges a, b, c (Å)                           |
| Angles α, β, γ (deg)                        |
| Resolution (Å)                             |
| Data quality                               |
| No. of unique reflections                  |
| Completeness (%)                           |
| Average I/σ(I)                             |
| Rsym (%)                                   |
| No. of residues in model monomer           |
| No. of protein atoms in the model monomer  |
| No. of solvent atoms                       |
| R-factor                                   |
| Stereochemistry                            |
| Root mean square deviation from ideal values |
| Bond lengths (Å)                           |
| Bond angles (deg)                          |
| Dihedrals (deg)                            |
| Ramachandran plot                          |
| Residues in most favored regions           |
| Residues in additional allowed regions     |
| Residues in generously allowed regions     |
| Residues in disallowed regions             |

\[ R_{\text{sym}} = \frac{\sum_{h}(I_{h}) - \langle I(h) \rangle}{\sum_{h}I_{h}}, \]
\[ R_{\text{work}} = \frac{\sum_{h}(I_{h}) - \langle I(h) \rangle}{\sum_{h}I_{h}}, \]
\[ R_{\text{free}} = \frac{\sum_{h}(I_{h}) - \langle I(h) \rangle}{\sum_{h}I_{h}}, \]

where \( I_{h} \) is the observed intensity of reflection with index \( h \) and \( \langle I(h) \rangle \) is the average value of all observations of \( I(h) \) including its Friedel mates.

\[ R_{\text{free}} = \frac{\sum_{h}(I_{h}) - \langle I(h) \rangle}{\sum_{h}I_{h}}, \]

The numbers in parentheses are for the highest resolution shell (1.94–2.01 Å).

The overall fold of mUbch10 (divergent stereoview). The α-helices, 3_{10}-helix, and β-strands are shown in red, green, and blue, respectively. The active site residue (114) is depicted in ball-and-stick rendering. This and all other structure figures were prepared using the Swiss-PDB Viewer (53) and in certain cases post-processed with POV-Ray (www.povray.org) or MegaPOV (nathan.kopp.org/patched.html).

The α-helices, 3_{10}-helix, and β-strands are shown in red, green, and blue, respectively. The active site residue (114) is depicted in ball-and-stick rendering. This and all other structure figures were prepared using the Swiss-PDB Viewer (53) and in certain cases post-processed with POV-Ray (www.povray.org) or MegaPOV (nathan.kopp.org/patched.html).

FIG. 2. The overall fold of mUbch10 (divergent stereoview). The α-helices, 3_{10}-helix, and β-strands are shown in red, green, and blue, respectively. The active site residue (114) is depicted in ball-and-stick rendering. This and all other structure figures were prepared using the Swiss-PDB Viewer (53) and in certain cases post-processed with POV-Ray (www.povray.org) or MegaPOV (nathan.kopp.org/patched.html).

State can have fundamental implications in the stereochemistry of the ubiquitination reaction. For example, the artificially induced dimerization of a E2–25K mutant produced by expressing as a glutathione S-transferase fusion dramatically alters its activity (44). Sometimes the same E2 protein, such as yeast Ubc4, has been reported to be a monomer as assessed by gel filtration chromatography (45) and crystal packing analysis (16) or as a dimer also by gel filtration chromatography (44) and chemical cross-linking (45). In the case of UbcH10, analysis of the crystal packing reveals a rather large interaction surface that results in a total surface area of 1270 Å² becoming solvent excluded. The interaction surface is formed by residues 36, 39, 40, 43, 44, 51, 53–57, 63, 65, 78, and 80. The presence of this large contact surface might suggest that the UbcH10 acts as a dimer. Moreover, a very similar interaction surface is seen in the crystal packing of the clam mitotic E2 protein, E2-C (21). To determine the quaternary structure of UbcH10 in solution, analytical ultracentrifugation studies were performed at protein concentrations of 0.94, 0.5, and 0.11 mg/ml. The results indicate a molecular weight of 19,260 (data not shown), which corresponds very well to the theoretical molecular weight of 19,652. Since the intracellular concentration of UbcH10 certainly is less than 1 mg/ml, it is likely that UbcH10 functions as a monomer and that the significant interactions observed in the crystal lattices are not functionally important.

Active Site Environment—The active site residue (114) is next to the NH₃ terminus of a 3_{10} helix. Unlike most 3_{10} helices, which are situated at the terminus of an α-helix (46), this 3_{10} helix is entirely separate from any α-helix. This 3_{10} helix is formed by residues 115–119 and has the sequence LDILK. The 3_{10} helix and the identity of residues forming this helix are highly homologous among E2 proteins (21). Why 3_{10} helix formation in proteins is favored in certain instances instead of α-helices is not well understood. In isolation, the α-helix is less strained energetically than the 3_{10} helix due to more favorable main chain hydrogen bonding geometry in the α-helix as well as slight steric hindrance of the side chains in the 3_{10} helix. The presence of 3_{10} helices in proteins might be explained in part by interactions of the side chains in the helix with the rest of the protein that would disfavor the α-helix and favor the 3_{10} helix. Such steric constraints are indicated in the case of the 3_{10} helix in the E2 proteins. The 3 residue per turn geometry of the 3_{10} helix places the charged residues Asp^{116} and Lys^{119} in-phase with each other, whereas the hydrophobic residues are oriented in different directions. In the context of the neighboring parts of the E2 structure, this allows the charged residues to be relatively solvent exposed and allows the hydrophobic residues to pack primarily against hydrophobic atoms (Fig. 5). If these residues instead were in an α-helical conformation, then these side chains would be in a much less favorable environment. The formation of the 3_{10} helix may have functional significance since it places the last residue of the helix (Lys^{119}) in proximity to the active site residue (Fig. 5). The positive charge of the ε-amino group of this residue could be important for reducing the pKₐ of the active site cysteine to make it more reactive. To test whether this lysine residue indeed is important for ubiquitin-adduct formation, a mutant (K119A-UbcH10) was prepared in which this residue has been changed to an alanine. Ubiquitin-adduct formation assay with this mutant indicates greatly diminished activity when compared with wild-type. Therefore, lysine 119 seems to play an important role in the mechanism of ubiquitin-adduct formation in UbcH10. It is probable that this role is more electrostatic (as proposed above) than purely structural in nature since the lysine is highly solvent exposed and therefore mutation to an alanine would be unlikely to cause significant structural perturbation of the active site region.

In addition to the 3_{10} helix, the active site is situated in the neighborhood of four β turns (Fig. 6). These turns are important since they (together with the 3_{10} helix) provide almost all the contacts with the active site residue. The only other residues contacting the active site residue are Leu^{138} and Ile^{113} (which is just NH₃-terminal to the active site residue). Moreover, the residues in these turns give rise to much of the surface features surrounding the active site. In UbcH10, the turns are
formed by residues 104–107 (turn A), 108–111 (turn B), 143–146 (turn C), and 147–150 (turn D). These turns are present in other known Ubc structures (Fig. 6). In all cases, turn A is nearly a canonical type I turn, turns with \((\phi, \psi)\) of residue \(i + 1 = -(-60, -30)\) and \((\phi, \psi)\) of residue \(i + 2 = -(90, 0)\). The turn B conformation is also quite well conserved among Ubc proteins and falls usually in the type I category. However, turns C and D vary considerably in conformation.

Turns A and B are adjacent, as are turns C and D. Because of the \(\phi, \psi\) angles of residues 107 and 108, the consecutive A and B turns form an S-shaped configuration that has features reminiscent of a two-stranded \(\beta\)-sheet. However, the \(\phi, \psi\) angles at the junction of turns C and D are such that the overall direction of the main chain is roughly maintained. Indeed, an additional turn containing residues from both turns C and D is nested within this adjacent pair of turns. The consecutive nature of these two pairs of turns suggests that they serve more than merely to connect secondary structural elements. The turns

![Fig. 3. Comparison of Ubc structures. A, structure-based sequence alignment. The structures of the Ubc proteins were superimposed and structurally equivalent residues were identified using the program STAMP (54, 55). The bar graphs above the sequences indicate the root mean square deviation (rmsd) of the structurally equivalent x-carbon atoms from their average position. Helical regions and \(\beta\) strand regions are indicated in gray and black backgrounds, respectively, and are labeled. B, divergent stereoview of superimposed Ubc structures. Only the x-carbon atoms are shown. The loop extending from a single structure near the 3_{10} helix is in the Ubc7 structure. The secondary structural elements are labeled. The most highly structurally conserved region corresponds to strands S2, S3, S4, and helix 2. C, divergent stereoview of the UbcH10 structure depicted as a ribbon. The ribbon is color coded according to sequence conservation with blue being the most conserved and red the least conserved. The most highly conserved region in the structure (panel B) is not uniformly the most highly conserved region in sequence. The sequence alignment was performed using the ConSurf server (Ref. 56, bioinfo.tau.ac.il/ConSurf).](http://www.jbc.org/21918)
almost certainly are important for providing both the requisite local molecular topography and the necessary surface distribution of functional groups. If structural diversity reflects functional diversity, then turns A and B would contribute to a function common to all types of Ubc proteins, whereas turns C and D would contribute to function specific to the particular species of Ubc.

Possible Sites of Interaction with APC/C—Crystal structures of UbcH7 with E6-AP (a HECT-domain containing E3) and UbcH7 with c-Cbl (a RING-domain containing E3) have been solved recently (47, 48). Surprisingly, in both cases the major sites of interaction of the functionally distinct E3 proteins with UbcH7 involve residues Phe63, Pro97, and Ala98, implicating these residues as general sites for E3 interaction, with Phe63 being especially critical. The corresponding residues in UbcH10 are Tyr91, Ala124, and Leu125. Residues Tyr91 and Ala124 were proposed previously to be sites of interaction with APC/C based on sequence comparison (21) and indeed form a contiguous patch on the surface. Recent studies have shown that the APC11 subunit of the APC/C is a RING domain protein, that in...
the absence of any other subunit of the APC/C is able to support ubiquitination of cyclin B and securin with E1 and mitotic E2 (49, 50). However, specificity for target protein ubiquitination is diminished compared with when the entire APC/C participates in the reaction. Based on the two crystal structures of E2-E3 complexes, it is likely that the APC11 contacts UbcH10 at residues including Tyr<sup>27</sup> and Ala<sup>124</sup> (Fig. 7). The most striking observation resulting from a sequence comparison of mitotic E2 proteins is a long ridge of residues on the face opposite to the active site (Fig. 7). Given that the APC11 is a small protein (−10 kDa), it is unlikely that the APC11 can also contact these residues. Thus, it seems that APC11 interacts with UbcH10 on one side, whereas other APC/C subunits make more substantial contacts on the opposite side. This conjecture is consistent with the prevailing notion that APC/C provides the scaffolding to recruit and optimally orient the mitotic E2-ubiquitin adduct and the target protein for specific but efficient ubiquitin transfer.

**Structure of Putative UbcH10 Destruction Box**—A recent study by Yamanaka and colleagues (14) indicates that UbcH10 abundance varies in-phase with the cell cycle, being expressed in G<sub>2</sub>/M phase and destroyed in late M phase. The targeting for destruction apparently proceeds by autoubiquitination by UbcH10 that is augmented by the APC/C. The autoubiquitination seems to be dependent on a destruction box-like motif in the UbcH10 sequence. The destruction box is the motif that is recognized by the mitotic-specific ubiquitination machinery. The consensus sequence for the destruction box often is given as Arg-X-X-Leu-X-(Leu/Ile)-X-Asp with the requirement for Asp in the final position relaxed for some proteins such as cyclin A and some securins (51, 52). Yamanaka et al. (14) suggest that a destruction box exists in the UbcH10 that includes residues 129–132 with the sequence Arg-Thr-Ile-Leu. Mutation of residues in the putative destruction box stabilizes UbcH10 against targeted destruction. Examination of the UbcH10 crystal structure shows that this sequence is contained within a helix that packs on the surface and is oriented such that the consensus residue Arg<sup>129</sup> is exposed, whereas the hydrophobic consensus residues Leu<sup>132</sup> and Ile<sup>135</sup> are buried within the protein (Fig. 8). Of the 9 amino acid residues 129–137 that comprise the putative destruction box, all but the hydrophobic consensus residues and Ile<sup>131</sup> are solvent exposed. Therefore, these residues, in principle, can readily interact with other proteins. The disposition of these residues as seen in the crystal structure lends credence to the provocative hypothesis that the UbcH10 is both an essential component of the mitotic-specific destruction machinery as well as a target.

**Acknowledgments**—We thank Dr. Joan Ruderman for the generous gifts of expression plasmids encoding UbcH10 and mUbcH10 and Dr. Richard Vierstra for expression plasmids encoding wheat ubiquitin-activating enzyme. We are grateful to Dr. Leslie Poole for performing the analytical ultracentrifugation analysis.

**REFERENCES**

1. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996) Science 274, 1652–1659
2. Townsley, F. M., and Ruderman, J. V. (1998) Trends Cell Biol. 8, 238–244
3. Hershko, A. (1999) Philos. Trans. R. Soc. Lond. B Biol. Sci. 354, 1571–1575
4. Nasmyth, K., Peters, J. M., and Ulmann, F. (2000) Science 288, 1379–1385
5. Amon, A. (2001) Nat. Cell Biol. 3, E12–E14
6. Noton, E., and Difley, J. F. (2000) Mol. Cell 5, 85–95
7. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
8. Townsley, F. M., Aristarkhov, A., Beck, S., Hershko, A., and Ruderman, J. V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2362–2367
9. Aristarkhov, A., Eytan, E., Moghe, A., Admon, A., Hershko, A., and Ruderman, J. V. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4294–4299
10. Arvand, A., Bastians, H., Welford, S. M., Thompson, A. D., Ruderman, J. V.,
