Amino-terminal Dimerization, NRDP1-Rhodanese Interaction, and Inhibited Catalytic Domain Conformation of the Ubiquitin-specific Protease 8 (USP8)*

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Ubiquitin-specific protease 8 (USP8) hydrolyzes mono and polyubiquitylated targets such as epidermal growth factor receptors and is involved in clathrin-mediated internalization. In 1182 residues, USP8 contains multiple domains, including coiled-coil, rhodanese, and catalytic domains. We report the first high-resolution crystal structures of these domains and discuss their implications for USP8 function. The amino-terminal domain is a homodimer with a novel fold. It is composed of two five-helix bundles, where the first helices are swapped, and carboxyl-terminal helices are extended in an antiparallel fashion. The structure of the rhodanese domain, determined in complex with the E3 ligase NRDP1, reveals the canonical rhodanese fold but with a distorted primordial active site. The USP8 recognition domain of NRDP1 has a novel protein fold that interacts with a conserved peptide loop of the rhodanese domain. A consensus sequence of this loop is found in other NRDP1 targets, suggesting a common mode of interaction. The structure of the carboxyl-terminal catalytic domain of USP8 exhibits the conserved tripartite architecture but shows unique traits. Notably, the active site, including the ubiquitin binding pocket, is in a closed conformation, incompatible with substrate binding. The presence of a zinc ribbon subdomain near the ubiquitin binding site further suggests a polyubiquitin-specific binding site and a mechanism for substrate induced conformational changes.

The post-translational ubiquitylation system is composed of a cascade of E1, E2, and E3 enzymes that activate and transfer ubiquitin or ubiquitin homologs to target proteins, including those targets that are membrane-anchored (1, 2). Modification by ubiquitin or ubiquitin-like molecules typically affects localization of the protein within the cell. For example, Lys-48-polylubiquitylated targets are recognized by the proteasome, which catalyzes complete target proteolysis (3). By affecting the abundance and/or half-life of signaling molecules, the ubiquitylation system can have dramatic effects on signal transduction pathways and play major roles in cellular biology. Degradation of integral membrane proteins can also be initiated by the ubiquitylation system but subsequent steps differ (4). Ubiquitylated receptors for example are rounded up by multivalent adaptors (e.g. AP2, epsin), which in turn recruit clathrin molecules that induce internalization through membrane budding. Dissociation of clathrin coats releases endosomes, which are either recycled back to the plasma membrane or fused with lysosomes, where membrane proteins undergo proteolytic degradation. Budding, uncoating, trafficking, and fusion events are influenced by the ubiquitylation state of the various intermediates, which is regulated not just by E3 ligases but by deubiquitylases as well.

The human genome contains over 90 deubiquitylases, the specific biological roles of which are mostly unknown. Some deubiquitylases have been implicated in ubiquitin recycling, which typically can occur at the mouth of the proteasome where ubiquitin moieties are salvaged before target proteolysis. Other deubiquitylases are implicated in ubiquitin remodeling, upstream of proteosomal degradation. For example, ubiquitin-specific protease 8 (USP8) is implicated in ubiquitin remodeling and regulation of epidermal growth factor receptors (ErbBs). In yeast, Doa4 is shown to be important for receptor sorting (5–7), and in COS cells, USP8 overexpression delayed, and its underexpression accelerated epidermal growth factor-mediated degradation of ErbBs (8). The exact steps of endocytosis at which USP8 is involved remain unclear; however, RXXK motifs in USP8, which bind specifically and uniquely to the SH3 domains of STAM/HRS/HBP, suggest a function upstream of the endo-

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The atomic coordinates and structure factors (codes 1WHB, 1WFW, 1A9U, 2GWF, 2FZP, and 2GFO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; USP8, ubiquitin-specific protease 8; NRDP1, neuregulin receptor degradation protein-1; DTT, dithiothreitol; r.m.s.d., root mean square deviation; AMC, amionomethyl coumarin.
Structures of USP8 and NRDP1 Domains

cytotic degradation pathway of growth factor receptors (9, 10). Interaction between USP8 and the G protein exchange factor CDC25Mm/RasGrf1, which is implicated in endocytosis, also implies upstream function (11, 12). The E3 ligase, NRDP1 (neu-regulin receptor degradation protein-1), a member of the tripartite or RING, B-box, coiled-coil E3 ligase family, catalyzes ubiquitylation of ErbBs and interacts directly with USP8 (13–15). Although USP8 and NRDP1 appear to be involved in a tug-of-war for receptor ubiquitylation/deubiquitylation, USP8 also enhances NRDP1 stability (15).

To provide insight into the function of USP8, we have determined the high-resolution crystal structures of predicted USP8 domains, the rhodanese and catalytic as well as a novel aminoterminal dimerization domain. The structure of the rhodanese domain was determined in complex with NRDP1, revealing the mode of their interaction.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—The cDNA templates encoding protein products of human USP8 and NRDP1 genes (NM_005154 and NM_005785) were obtained from Origene and Mammalian Gene Collection, respectively, and their fragments encoding residues 1–142, 181–319, and 734–1110 of USP8 and 193–317 of NRDP1 were individually inserted into a modified pET28-LIC expression vector (EMD/Novagen) downstream to the nucleotide sequence encoding His6 tag and thrombin cleavage site. The resulting plasmids were used to transform Escherichia coli BL21 (DE3) gold cells (Stratagene). The transformed cells were grown in TB medium (Sigma) at 37 °C with vigorous air bubbling for 6–7 h until A600 of 5.5–6.0 was reached. The temperature was then decreased to 15 °C, and protein production was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 100 μM. Incubation continued overnight (15–16 h). Selenomethionine derivatives of the recombinant proteins were produced in a similar way using M9 selenomethionine high-yield growth media package (Mediclon). The cells were collected by centrifugation, re-suspended in lysis buffer (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM β-mercaptoethanol, 2 mM imidazole, and 100 μM phenylmethane sulfonyl fluoride) and lysed by passing the cell suspension through a microfluidizer (Microfluidics) at a peak pressure of about 18,000 psi. After centrifugation, the clear supernatant was incubated with TALON metal-affinity resin (BD Biosciences) for 30 min at 4 °C with constant stirring. The affinity resin was sedimented by gentle centrifugation, re-suspended in the lysis buffer containing 10 mM imidazole, transferred into an Econo-Pac column (Bio-Rad) and washed, first, with the same buffer, and then, with the buffer supplemented with 0.05% Tween 20, and finally, with the same buffer without the detergent. His-tagged proteins were eluted with the lysis buffer containing 200 mM imidazole, the tag was cleaved by incubation with bovine thrombin (Sigma) for 2 h at ambient temperature (21–23 °C), and proteins were finally purified by size-exclusion chromatography on a Superdex 200 column at 8 °C using an AKTA purifier (GE Healthcare) and 20 mM Tris-Cl, pH 8, 150 mM NaCl, 2 mM dithiothreitol (DTT) as an elution buffer. Purified proteins were concentrated by centrifugal ultrafiltration using Amicon Ultra-15 concentrators (Millipore). To obtain the USP8-NRDP1 complex, the two proteins were mixed in equimolar ratio (about 0.5 μmol each at a final concentration of ~0.1 mM in the above gel-filtration buffer), incubated for 30 min at ambient temperature and then for 1 h on ice, and chromatographed as above. Aliquots of each sample were analyzed by mass spectrometry using an LC/MSD TOF instrument (Agilent) before and after thrombin cleavage to verify the correct molecular mass of the proteins and the completeness of tag removal; SDS-PAGE was used to evaluate purity of the products. This procedure allowed producing >95% pure recombinant proteins with a yield of 20–30 mg/liter cell culture.

Crystals were grown by the hanging-drop technique, mixing 2 μl of protein solution with an equal volume of reservoir solution. Region 1–142 of USP8 was crystallized using protein concentration of 12 mg/ml and 27% polyethylene glycol 3350, 0.2 M Li2SO4, 0.1 M bis-Tris, pH 5.6, 1 mM DTT as a reservoir solution. NRDP1 was crystallized at a protein concentration of 25 mg/ml using 1.8 M (NH4)2SO4, 0.1 M HEPES, pH 7.0, 0.2 M NaCl, 1 mM DTT as a reservoir solution. In case of USP8-NRDP1 complex, protein concentration was 10 mg/ml, and the reservoir solution contained 14% polyethylene glycol monomethyl ether 5000, 0.1 M bis-Tris, pH 6.5, and 1 mM DTT. Selenomethionine proteins were crystallized under the same conditions as the corresponding native proteins. The catalytic domain of USP8 was crystallized at 10 mg/ml; reservoir solution consisted of 18% polyethylene glycol 3350, 0.1 M bis-Tris, pH 6.3, 0.2 M KSCN, and 1 mM DTT. Crystals were briefly soaked in a reservoir solution supplemented with a mixture of sucrose, glucose, glycerol, and ethylene glycol as the cryoprotectant before being frozen in liquid nitrogen (77 K).

X-ray Data Collection, Phasing, and Refinement—A single-wavelength anomalous diffraction data set at the selenium anomalous peak wavelength was collected at the Advanced Light Source beamline 8.2.1 of the NRDP1-USP8 complex crystals in which the NRDP1-USP8 interaction domain had been labeled with selenomethionine. The data were processed with HKL2000 (16) and then input into the program SOLVE (17) using data from 30 to 2.6 Å. SOLVE located all of the possible 18 selenium sites. Density modification and automatic model building using RESOLVE (18, 19) resulted in good electron density maps and partial models for each of the six subunits and allowed the placement of the NMR model (Protein Data Bank accession code 1WHB) of USP8 rhodanese domain into the density. These models were then refined against a 2.1 Å native data set, also collected at Advanced Light Source beamline 8.2.1, and a rough model of the NRDP1-USP8 interaction domain was built with ARP/wARP (20). This model, containing primarily alanines, was then used as a model to solve the monomeric structure of the USP8 interaction domain of NRDP1 with a 1.9 Å data set collected at the Argonne Photon Source Sector 19BM. ARP/wARP was able to dock the sequence to this model, and refinement of the structure proceeded quickly using Refmac in the CCP4 program suite 5.02 (21) and manual rebuilding and checking with the molecular graphics program O (22).

The refined structure of the USP8 interaction domain of NRDP1 was then placed in the NRDP1-USP8 complex and then further refined against the 2.1 Å native data set using REFMAC. Initially tight noncrystallographic restraints were used in the
refinement, but these were relaxed as the refinement progressed. At the final stage of the refinement for both the monomeric NRDP1 and the NRDP1-USP8 structures, translation/ liberation/screw (TLS) refinement was carried out with parameters generated using the TLSMD web server (23). Both structures have excellent stereochemistry as judged by PROCHECK (24), with no Ramachandran violations.

For USP8 dimerization domain, a single-wavelength anomalous diffraction data set at the selenium anomalous peak wavelength was collected at the Argonne Photon Source beamline 19BM, using crystals containing selenomethionine-labeled protein. The data were processed with HKL2000, and the structure factors were input into the program SOLVE using data from 30 to 2.4 Å. SOLVE located two of the possible four selenium sites (the amino-terminal methionines were disordered and not located in the experiment). Density modification and automatic model building using RESOLVE resulted in good maps and partial models for each of the two molecules in the asymmetric unit. Using the molecular graphics program O, the models were manually adjusted, and after an initial round of refinement using REFMAC, the resulting phases were fed into the program ARP/wARP, which automatically built nearly complete models, including sequence docking and placement of the water molecules. Further checking and refinement of the model was carried out with the aid of REFMAC and O.

For the structure determination of the USP8 catalytic domain, diffraction data from crystals of the space group P2₁ (a = 30.911 Å, b = 53.795 Å, c = 30.640 Å, α = γ = 90°, β = 92.43°) were collected using a Rigaku FR-E generator and a RAXIS-IV++ area detector. The data were integrated and scaled with the program package HKL2000. Statistics of data collection and refinement are provided in Table 1. The molecular replacement program PHASER (25), part of the CCP4 program suite, was used to solve the structure with the coordinates of the Protein Data Bank structure 1Q1C as the search model. Automatic model building with the program ARP/wARP, maximum likelihood refinement using the program REFMAC5, and manual building with the graphics program O led to a model with a working R value of 0.167 and a R-free value of 0.202 for the resolution range from 1.60 to 30.89 Å. All residues of the catalytic domain (90–205) and two amino-terminal residues of the cloning tag were located in the experiment.

Enzymatic Assays—Deubiquitylation assays for determining steady-state kinetics parameters were performed at 28 °C in 50 mM HEPES, pH 7.5, 10 mM DTT, 0.01% Tween 20, 4.8% Me2SO containing 10 nM USP8, and 0.7–12 μM ubiquitin AMC (Boston Biochem). USP8 concentration was determined by its absorbance at 280 nm using a predicted extinction coefficient of 56560 M⁻¹ cm⁻¹. Prior to use in the experiment, USP8 was incubated in the above buffer (without Me₂SO) for at least 10 min to allow for DTT-mediated enzyme activation. Reaction progress was monitored by the increase in fluorescence emission at 450 nm (λₑₓ = 340 nm) using a FluoroDia T70 fluorescence microplate reader (Photon Technology International). Fluorescence measurements were made every 10 s over the time course of the assay, and measurements made during the first 2 min of the reaction were used to calculate initial velocities. Fluorescence intensity values were converted to moles of AMC using an equation generated by fitting the fluorescence intensity measurements of a range of AMC concentrations prepared in 50 mM HEPES, pH 7.5, 0.01% Tween 20. Initial reaction velocities in quadruplicate were used as input for fitting the Michaelis-Menton equation using the SigmaPlot v9.01 enzyme kinetics module (Systat Software Inc.). kcat was calculated using the equation kcat = Vmax/[E₀], where [E₀] is the total enzyme concentration. Deubiquitylation assays used to establish substrate preference of USP8 were performed at 28 °C in 50 mM HEPES, pH 7.5, 10 mM DTT containing 50 nM USP8 and 0.5 μM of either ubiquitin-AMC, SUMO-AMC, NEDD8-AMC (Boston Biochem), or RLGG-AMC (BACHEM) (26), and fluorescence measurements were made as described above.

RESULTS AND DISCUSSION

Amino-terminal USP8 Domain—The amino terminus of USP8 contains a 30-residue predicted coiled-coil segment that could mediate oligomerization of the protein (27) (supplemental Fig. 1a). We purified a larger polypeptide that contained this region of USP8 (residues 1–142) and determined the high-resolution crystal structure of a novel domain (Table 1). Consistent with its behavior during size-exclusion chromatography (data not shown), the structure of the amino-terminal fragment, which contained two molecules in the asymmetric unit, revealed an extended, antiparallel homodimer (Fig. 1). Dimerization is mediated by a swapping mechanism; the first structural element of the complex, lined with six basic residues (three from each molecule, Lys-42, Arg-58, and Lys-65) that hints at the potential for ligand binding, possibly by a negatively charged, symmetric molecule. The carboxyl-terminal helices protrude in an antiparallel fashion so that the carboxyl-terminal residues (Lys-138) are separated by about 112 Å. This architecture appears to be unique allowing dimerization concomitant with optimization of intermolecular distances and suggests that catalytic domains are prevented from coming too close to each other.

Our data show, for the first time, that USP8 contains a novel dimerization domain located at the amino terminus. Although we could not check within the framework of this study whether full-length USP8 is also a dimer (because of insufficient expression in bacterial cells), the amino-terminal domain likely contributes to full-length protein multimerization. Putative USP8 dimerization suggests that its substrates may also be dimeric or multivalent with regularly spaced ubiquityl units. Furthermore, dimerization of USP8 may be consistent with observations that the ubiquitylation machinery only recognizes dimeric receptors (28). Oligomerization is also characteristic of other deubiquitylases: USP7 (29), DUB1 (29), UCHL1 (30), and UCHL5.3. A related deubiquitylase, AMSH/STAMBP, which has been implicated in endosome biology (31) by virtue of its RXXK

3 G. V. Avvakumov, J. R. Walker, S. Xue, P. J. Finerty, Jr., F. Mackenzie, E. M. Newman, and S. Dhe-Paganon, unpublished observation.
motifs, contains an amino-terminal region that, despite weak sequence similarity, is reminiscent of the amino-terminal dimerization region of USP8. An intriguing possibility is heterodimerization among deubiquitylases.

**Rhodanese and NRDP1 Domains**—A 40-residue linker separates the amino-terminal dimerization domain from the rhodanese domain (supplemental Fig. 1a), which is analyzed in conjunction with NRDP1. It has been shown that the rhodanese domain of USP8 interacts with the carboxyl-terminal region of NRDP1 molecule that includes a predicted coiled-coil region (15). However, our data show that the coiled region is not required for the interaction with USP8. When a protein spanning residues 193–317 of NRDP1 was incubated with the USP8 rhodanese domain (residues 181–319) prior to size-exclusion chromatography, a single peak was observed with an elution volume corresponding to ∼30-kDa globular protein; mass spectrometry analysis confirmed that this gel-filtration peak contained both proteins (supplemental Fig. 2). A strong interaction between the two proteins is suggested by the observation that no significant dissociation was confirmed that this gel-filtration peak contained both proteins (supplemental Fig. 2). A strong interaction between the two proteins is suggested by the observation that no significant dissociation

![FIGURE 1. Structure of USP8 dimerization domain.](image)

TABLE 1

| Data collection, phasing, and refinement statistics | USP8 dimerization domain (residues 1–142) | USP8 rhodanese domain (residues 181–319)/NRDP1 USP8-interaction domain (residues 193–317) | NRDP1 USP8-interaction domain (residues 193–317) | USP8 catalytic domain (residues 734–1110) |
|---------------------------------------------------|------------------------------------------|---------------------------------------------------------------|-------------------------------------------------|----------------------------------------|
| PDB code*                                         | 2A9U                                    | 2GWf                                                          | 2FZP                                           | 2GFO                                   |
| Space group                                       | P2,2,2,1                                 | P2,2,2,1                                                      | P2,2,2,1                                       | P6                                      |
| Unit cell                                         | 34.68/64.81/151.89                      | beta = 91.2                                                   | APS 19BM                                        | APS 19BM                               |
| Wavelength                                        | 0.97883 (peak)                          | 0.97957 (peak)                                               | 1.00768 (peak)                                 | 0.97896 (peak)                         |
| Resolution                                        | 2.1                                     | 2.6                                                           | 1.9                                             | 2                                      |
| Unique reflections                                | 19987                                   | 29587                                                        | 12100                                          | 32281                                  |
| Data redundancy                                   | 6.5                                     | 3.7                                                           | 4.3                                             | 5.4                                    |
| Completenessa                                      | 90.5 (80.2)                             | 93.9 (99.4)                                                  | 99 (99.6)                                      | 99.4 (99.9)                            |
| l/σl                                            | 16.6 (3.2)                              | 12.4 (2.4)                                                   | 9.3 (1.8)                                      | 20.03 (1.4)                           |
| Rfree/a                                          | 0.09 (0.44)                             | 0.12 (0.46)                                                  | 0.14 (0.56)                                    | 0.08 (0.72)                            |
| Refinement                                        | Resolution: 33.8-2.1                    | 30.0-2.3                                                     | 27.3-1.9                                       | 18.2-2.0                               |
| Reflections used                                  | 17952                                   | 39806                                                        | 10951                                          | 31452                                  |
| All atoms (solvent)                               | 2274 (88)                               | 6388 (218)                                                   | 1146 (99)                                      | 2938 (191)                            |
| Rwork/Rfree                                      | 20.8/25.9                               | 20.6/23.7                                                    | 16.9/23.2                                      | 16.8/21.0                             |
| r.m.s.d. bond length                             | 0.016                                   | 0.008                                                        | 0.017                                          | 0.015                                  |
| r.m.s.d. bond angle                              | 1.3                                     | 1.1                                                          | 1.5                                            | 1.498                                  |
| Figure of merit                                   | 0.8                                     | 0.83                                                         | 0.86                                           | 0.87                                   |
| Average B-factors                                 | 31.1                                    | 41.4                                                         | 25.1                                           | 48.1                                   |
| Ramachandran plot                                  | Most favoured                           | 94.7                                                         | 89.3                                           | 89.6                                   |
|                                    |                                           | Additionally allowed | 5.3                             | 10.7                                     | 10.4                                     | 7.8                                     |
|                                    |                                           | Generously allowed | 0                             | 0                                         | 0                                         | 0                                         |
|                                    |                                           | Disallowed         | 0                             | 0                                         | 0                                         | 0                                         |

*Highest resolution shell is shown in parentheses.

b $R_{	ext{free}} = 100 \times \sqrt{(I - \langle I \rangle)} / \langle I \rangle$, where $I$ is the observed intensity and $\langle I \rangle$ is the average intensity from multiple observations of symmetry-related reflections.

c $R_{\text{free}}$ value was calculated with 5% of the data.
Noncatalytic rhodanese domains are also common, found as substrate-targeting domains of dual-specificity phosphatases and chaperone-like domains of stress-response foldases. The substrate-targeting domain of dual-specificity phosphatases is distantly related to the USP8 rhodanese domain, having an r.m.s.d. = 5.1 Å when comparing Cα atoms. The mechanism by which noncatalytic rhodanese domains form protein-protein interaction is unknown. Although the surface characteristic of the primordial active site of the USP8 rhodanese domain is consistent with the possibility of a protein-protein interaction site, no interaction in this area is seen with the NRDP1 domain in the asymmetric unit.

**Rhodanese-NRDP1 Complex**—In this space group, there are multiple contacts between molecules in the unit cell. Homomeric interactions likely represent crystal contacts because both the rhodanese domain and NRDP1 carboxyl-terminal domain individually migrated as monomeric proteins during gel filtration (supplemental Fig. 2). USP8 and NRDP1 interact with each other through a number of surfaces, but one interface is dominant and unlikely to be simple crystal contacts. For this interface, the association between the USP8 rhodanese domain and the carboxyl-terminal NRDP1 domain is best characterized as a domain-domain interaction. A peptide loop of USP8 connecting helices α3 and α4 (residues 237–242) is nestled in a pocket of NRDP1 that is roughly centered on residue Ile-218 (Fig. 3a). The USP8 binding site of NRDP1 covers about 2100 Å² and includes residues Ile-218, Asp-222, Glu-252, Leu-262, Arg-265, and Arg-269. Compared with the remaining surface of NRDP1, the USP8 interaction surface is conserved among known orthologous sequences (Fig. 2c and supplemental Fig. 1c). Three prominent ionic bonds are formed between the two molecules: H238USP8–D222NRDP1, D241USP8–R265NRDP1, and D242USP8–R269NRDP1 (Fig. 3). Helix α5, which is long and unusually twisted, forms a significant portion of the binding site, providing both interacting arginines (Arg-265 and Arg-269). Additionally, hydrogen bonds occur between the USP8 rhodanese peptide and NRDP1: Ala-237 backbone carbonyl with the Thr-220 hydroxyl; Asn-241 backbone amide nitrogen with the Met-217 backbone carbonyl; Asp-245 carboxyl with the Gln-266 side chain amide; Lys-244 amine with the Thr-220 hydroxyl; Lys-244 amine with the Ser-219 hydroxyl (USP8 and NRDP1 residues, respectively); and a network of hydrogen bonds that include two adjacent water molecules sandwiched between Leu-239 and Pro-240 of USP8 and the NRDP pocket. Proline-240 is in the trans-configuration and lays flat against NRDP1 residues Ile-218 and Ala-311. This region of USP8 does not bind in a completely extended configuration; the backbone of the second aspartic acid residue (Asp-242) is turned up and away from the rest of the peptide, forming the beginning of helix α4. Although rhodanese helix α2 comes close to the binding pocket, it provides only weak interactions with NRDP1. The rhodanese domain may provide a scaffold to help keep the overall conformation of the USP8 rhodanese domain hidden from the crystal contacts. Unlike the USP8 rhodanese domain in the asymmetric unit.

**Structures of USP8 and NRDP1 Domains**

![Diagram of the USP8-NRDP1 complex.](image)

**Figure 2. Structure of the USP8-NRDP1 complex.** a, ribbon and surface depictions of the complex were generated by PyMOL. USP8 rhodanese domain is yellow, and NRDP1 is green. Red and blue surfaces represent simple display of acidic and basic residues, respectively. α-Helices and β-strands are labeled. b, electrostatic surface representation (−10 kT/e, red to +10 kT/e, blue) of NRDP1 was generated by the APBS software package and displayed in PyMOL. Active site residues of NRDP1 are labeled green, c, conserved residues of NRDP1 are in green and form a prominent patch coincident with the rhodanese binding pocket. Nonconserved residues are in red. Species included in this analysis are man, mouse, rat, frog, and fly. The USP8 rhodanese segment is shown in yellow with side chains that interact with the NRDP domain.

Lys-278/Glu-292. Although NRDP1 is devoid of a single, deep active site, there are a number of clefts and cavities that could mediate protein-protein interactions, including patches centered on Arg-265, Met-308, and Leu-306.

The structure of the USP8 rhodanese domain (residues 185–310) is composed of an open, twisted five-stranded β-sheet surrounded by α helices, characteristic of a classic Rossmann fold (Fig. 2). Alignment with the NMR structure of the USP8 rhodanese domain (Protein Data Bank accession code 1WHB) shows an r.m.s.d. of 1.24 Å for 130 Cα atoms. The rhodanese fold is found in all. Catalytic versions of the rhodanese-fold include phosphatases and sulfurtransferases, and non-catalytic versions are implicated in protein-protein interactions. The catalytic forms have a well defined and conserved active site (centered on the N terminus of helix α2) and contain a reactive cysteine located at the N terminus of strand β3. Unlike the phosphatase active site, the corresponding site of the USP8 rhodanese domain is collapsed and does not contain a cysteine residue. Moreover, a helical segment that normally forms a wall of the phosphatase active site is missing in USP8. Noncatalytic rhodanese domains are also common, found as substrate-targeting domains...
Residues of the USP8 binding site in USP8-bound and unbound NRDP1 structures are also superimposable (data not shown). Significant difference was only seen in helix \( /H_{9251}/H_9251\), which is more ordered in the apo structure; the first four residues of the NRDP1 construct and nine vector-derived residues are observed only in the apo structure. Likewise, no major conformational changes are noted between bound and unbound forms of the rhodanese domain (Protein Data Bank accession code 1WHB).

The rhodanese domain may also contribute to multimerization of USP8 through an indirect mechanism. Because NRDP1 also contains a region predicted to be coiled coil (residues 135–178), it likely exists as a dimer. Recruitment of dimeric NRDP1 by the rhodanese domain could also contribute to USP8 multimerization. Unfortunately, because all our attempts to produce polypeptides containing the coiled-coil region of NRDP1 failed, we could not assess the latter hypothesis.

**USP8 Catalytic Domain**—At the carboxyl terminus of the molecule, a 376-residue fragment containing the catalytic domain of USP8 was crystallized (Fig. 4). Crystals diffract to 2.0 Å resolution with an average nonsolvent B-factor of 38.2 Å² (Table 1), producing a model with no Ramachandran violations that includes all residues of the predicted catalytic domain plus 12-residues amino-terminal to the catalytic domain. A loop region within the domain (residues 889–897) is disordered. The overall fold of USP8 is similar to that of USP7 (r.m.s.d. 3.5 Å over 244 C\(/H_{9251}/H_{9251}\) positions) and USP14 (r.m.s.d. 3.7 Å over 177 C\(/H_{9251}/H_{9251}\) positions), having a papain-like architecture plus an extended, fingers-like domain that forms the ubiquitin binding pocket (Fig. 4). Despite low sequence similarity (19 and 15% compared with USP7 and USP14, respectively), the structure of USP8 supports the notion that this domain architecture is conserved among members of the USP family. Nevertheless, a number of significant differences exist, including an additional 30-residue hinge-like, helical region that buttresses the papain-like core and the fingers region and the presence of an occupied zinc ribbon at the tip of the fingers.

The active site of USP8 is composed of a cleft between helix \( /H_9251\) and a wall formed by a \( \beta\)-sheet (\( \beta\)-11, \( \beta\)-14 – \( \beta\)-19). The amino portion of helix \( /H_9251\) contains the catalytic cysteine (Cys-786), and the \( \beta\)-sheet contains part of the catalytic triad (His-1067 and Asp-1084). The catalytic triad appears to be in a catalytically competent configuration, where H1067-ND1 is 3.4 Å away from C786-SG; H1067-ND2 is 2.5 Å away from D1084-OD1 (Fig. 5). Asn-781 is poised to stabilize the oxyanion intermediate; a water or chloride, which would mimic the oxyanion intermediate, is stabilized by Asn-781 as well as Gln-866 (Fig. 5).
The peptide binding site appears to be blocked. Two loops, referred to as blocking loops 1 and 2 (BL1, 1014–1023; BL2, 1060–1067), which have been shown to recognize the P positions of the substrate and form a lid over the active sites of USP7 and USP14, are positioned in a closed conformation in the USP8 structure (Fig. 5). Notably, two residues apparently draw BL2 toward the catalytic cysteine, and a water molecule sits in the active site, hydrogen bonding with various residues (Fig. 5). Sitting on top of the catalytic cysteine, Gln-866 forms a network of hydrogen bonds including the backbone carbonyl of Gly-1066 in BL2, and the aliphatic side chain of the BL2 Leu-1063 sits in a hydrophobic pocket generated by Phe-1014 and the backbone atoms of Gln-867 and Asp-868. These interactions must be broken, and the loops must adopt more open conformations to allow substrate binding.

The structure of USP8 also reveals an additional feature that is inconsistent with substrate binding; the fingers appear to be tightened inward such that there is insufficient room for a ubiquitin molecule (Fig. 6). The fingers of USP8 comprise three prominent antiparallel β-strands (β4, β5, and β10), capped by a zinc-binding module, where the zinc is tetrahedrally coordinated by four cysteines (Cys-936–X–Cys-939...Cys-985–X–Cys-988) in a two β-hairpin subdomain, consistent with a zinc ribbon fold (32). As shown previously, the finger domain of USP7 and USP14 holds the core domain of ubiquitin, forming specific interactions along most of the inner surface of the fingers. USP7 and USP14 have fingers in the same relative orientation, and no significant structural change occurs upon ubiquitin binding (33, 34). In contrast, when the thumb and palm sections of USP8 and USP7 (or USP8 and USP14) are aligned, the tips of the USP8 fingers are nearer to the active site by about 9 Å (Fig. 6). The dramatic shift of the fingers can be better explained by a hinge as opposed to a curling mechanism, the finger domain moving as a rigid body. The hinge appears to occur at the base of the fingers, along the long axis of strand β6. This could be an additional layer of regulation of USP8 activity. A number of features could mediate the stabilization of the closed finger conformation observed in USP8. A unique 25 Å helix α9 (902–918) juxtaposes the fingers and may act as a brace to hold the fingers in the closed configuration. This helix extends along the first finger (β5) such that its amino-terminal end interacts through an extensive interface with the back of the fingers. Notably, a salt bridge, two hydrogen bonds, and a large aromatic patch form the bulk of interactions. The carboxyl end of helix α9 is anchored to helix α10, a small conserved helix at the base of the fingers, which is tightly packed against the papain-like core. Residues 888–898, which run antiparallel to helix α9, are disordered. Alternatively, the short helix α12 and its associated loops could account for finger closure, as it makes contact with the bracing helix α9 and has extensive connections with the back of the fingers (Fig. 6). Finally, electrostatic surface analysis of USP8 reveals significant positive charge at the inner surface of the fingertips and significant negative charge at the opposite side of the ubiquitin pocket (Fig. 6). Although separated by about 18 Å, this strong dipole could promote attraction of the fingertips toward the papain-like core.
Despite having an apparently inactive conformation in our crystals, the isolated catalytic domain of USP8 is active in solution (Fig. 7). Analysis of the Michaelis-Menten plot of ubiquitin-AMC gives a $k_{cat}$ of $2.4 \text{ s}^{-1}$ and $K_m$ of $10.2 \pm 1.4 \text{ M}$. A number of putative mechanisms may explain this apparent contradiction. One possibility relates to substrate-induced conformational changes. Because the substrate is large and forms many contacts with USP8, the binding of a portion of the substrate could cause conformational changes in USP8 that would open up the substrate-recognition site and allow the remaining parts of the substrate to bind. Although not well defined, this mechanism was invoked to explain the auto-inhibited structures of otherwise active deubiquitylase, including USP7 (33), UCHL1 (35), and UCHL3 (36). The fact that USP8 cannot hydrolyze the bond between AMC and the short peptide corresponding to the ubiquitin tail (Fig. 7, inset) further suggests that substrate-induced activation is mediated by the binding of the ubiquitin core to the fingers domain. Another possibility is that in solution, BL1 and BL2 are flexible, and crystallization of USP8 traps a conformation that is most compatible with crystal packing. Indeed, the fingers subdomain of a symmetry-related molecule is juxtaposed against BL2 and may contribute to the observed closed conformation. However, relatively low B-factors for BL2 (an average value of 35.84 Å$^2$) are only slightly higher than the overall average. On the other hand, BL1, the average B-factors (47.2 Å$^2$) of which are significantly higher than the rest of the molecule, adopts a conformation incompatible with substrate binding but is not restricted by crystal contacts. The entire BL1 loop is solvent-exposed having no significant interactions aside from a loose salt bond between its Lys-1021 and Asp-1064 of the BL2. Although crystal contacts may play a role in the observed conformation of the USP8 fingers, this flexibility is likely to be relevant to the biological function of this enzyme. Whether this flexibility is a part of the auto-inhibitory repertoire of USP8 is yet to be elucidated.

The function of the USP8 zinc ribbon is not clear. An orthologous USP, papain-like protease from the severe acute respiratory syndrome coronavirus, also contains a zinc ribbon that is necessary for its catalytic activity and contributes to efficient protein folding when expressed in bacteria (37, 38). Based on their sequence, about half of human USP is predicted to contain the zinc ribbon at the fingertips. Because USP8 efficiently catalyzes hydrolysis of polyubiquitin chains, whether Lys-48- or Lys-63-linked (8), as well as the AMC derivative of ubiquitin, it is possible that the zinc ribbon mediates binding of a second ubiquityl moiety of a polyubiquitin substrate. In this case, binding to ubiquitin could mimic that of the zinc finger of Npl4, the complex structure of which reveals interaction between the solvent-exposed cysteines of the zinc finger and Ile-44 of ubiquitin, the latter residue being a common site for protein-ubiquitin interaction. A similar interaction between ubiquitin and USP8 would suggest preference for binding to polyubiquitin chains and may provide
Data are the means ± S.D. for four determinations. Inset, time course of hydrolysis by USP8 (50 nM) using the ubiquitin homologs, SUMO-AMC, and Nedd8-AMC as well as a peptide corresponding to the carboxyterminal five residues of ubiquitin (RLRGG) coupled to AMC, all at 0.5 μM.

**FIGURE 7.** Michaelis-Menten plot of the activity of USP8 catalytic domain using Ubiquitin-AMC as substrate. Ubiquitin-AMC was incubated with 10 nM USP8 at 28 °C, and product (AMC) was measured using a FluorDia T70. Initial rates (v) and \( k_{\text{cat}} \) and \( K_m \) values were calculated using the SigmaPlot enzyme kinetics module. Data are the means ± S.D. for four determinations. Inset, time course of hydrolysis by USP8 (50 nM) using the ubiquitin homologs, SUMO-AMC, and Nedd8-AMC as well as a peptide corresponding to the carboxy-terminal five residues of ubiquitin (RLRGG) coupled to AMC, all at 0.5 μM.

an additional basis for substrate-induced conformational rearrangements needed for catalysis.

Substrate specificity is an important parameter of catalytic efficiency, and its understanding can significantly aid drug discovery and development. We have also tested the ability of USP8 to catalyze hydrolysis of fluorescent ubiquitin-like analogs, SUMO and Nedd8, as well as the carboxy-terminal pentapeptide of ubiquitin. The inset of Fig. 7 shows that only ubiquitin-AMC was hydrolyzed by USP8. The high-resolution structure can provide an understanding for the basis of this specificity. Almost all polar backbone atoms of the ubiquitin tail interact with the amino acid residues in the active site of USP7 and USP14, and the crystal structure of USP8 shows that, except for a few residues in BL1 and BL2, most active site residues, namely Gln-867, Asp-868, Glu-871, His-1059, and Tyr-1068, are conserved and not distorted significantly (Fig. 5). Clamping down by BL2 would appear to be the basis of the strict requirement for glycines at the P1 and P2 positions of ubiquitin, as their Ca atoms are within van der Waals distances of the backbones of BL2 and the loop immediately amino-terminal to the catalytic cysteine. A notable divergence between USP8 and USP7/14 is the peptide binding face of BL1, which in USP8 appears to lack residues capable of hydrogen bonding with the carbonyl group of Arg-72 of ubiquitin (Fig. 5). Nevertheless, a hydrophobic pocket in BL1 could provide space for ubiquitin Leu-73. This divergence could be explained by differences observed for kinetic parameters of USP8. Although the catalytic efficiency of USP8 (0.239 M\(^{-1}\) s\(^{-1}\)) is similar to that of USP2 (0.252 M\(^{-1}\) s\(^{-1}\)) and USP7 (0.250 M\(^{-1}\) s\(^{-1}\)), USP8 has ∼20-fold higher \( K_m \) and \( k_{\text{cat}} \) values compared with USP2 (33, 39). Apparently, specificity is not expressed in the P' side of the substrate. Except for a shallow acidic patch, the absence of any significant S' pocket in USP8 suggests a lack of target specificity (Fig. 6). Whether the marked specificity of the catalytic domain of USP8 toward ubiquitin substrates can be extended to the full-length protein in vitro or in vivo will require further studies.

Based on experiments using COS7 cells, the USP8 catalytic domain, independent of the rhodanese domain, is proposed to also interact with NRDP1 (15). To test this, we conducted size-exclusion chromatography of the catalytic domain with or without the carboxy-terminal domain of NRDP1 and found no evidence of interaction (data not shown). A possible interpretation of these data is that the catalytic domain may interact only with ubiquitylated forms of NRDP1 in an enzyme-substrate fashion.

**Conclusion**—This is the first report of a possible USP8 dimerization mediated by the amino-terminal region. Our results also reveal the mechanism by which USP8 interacts with the E3 ligase NRDP1. A docking interaction occurs between domains from each protein. The 120-residue NRDP1 domain has a novel, globular fold with a conserved active site that recognizes the rhodanese domain of USP8. Our data show the detailed ionic and hydrogen bonds that occur between the active site of NRDP1 and the 5-residue loop of the rhodanese domain. This interaction appears to be dominated by salt bridges. An alignment of orthologous USP8 sequences (residues 238–242 in human sequence; supplemental Fig. 1a) reveals potential consensus sequences, which are also found in BRUCE (BIR repeat containing ubiquitin-conjugating enzyme) and ErbB4, both of which have been shown to interact with the carboxy-terminal portion of NRDP1 (13, 14, 40). Although neither BRUCE nor ErbB4 has a rhodanese domain, NRDP1 may interact with these targets via its carboxy-terminal domain in a manner similar to the interaction with USP8. Finally, the structure of the USP8 catalytic domain described here shows an auto-inhibited state, which is likely reversed by substrate binding.

The x-ray structures of the three domains of USP8 provide an insight into the function of each domain and a better understanding of the architectural features of this multi-domain protein. There is no a priori reason to believe that the conformation of individual USP8 domains determined here will differ significantly in the full-length protein. However, interdomain USP8 regions will definitely contribute to the formation of the overall protein structure and its function. Additionally, some structural changes within the domains may occur as part of
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regulatory mechanisms induced by USP8 interactions with other proteins (e.g. STAM/HRS/HBP). The structures reported here can provide the basis for further study of such effects.

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