BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Mechanism of Recruitment and Activation of the Endosome-Associated Deubiquitinase AMSH

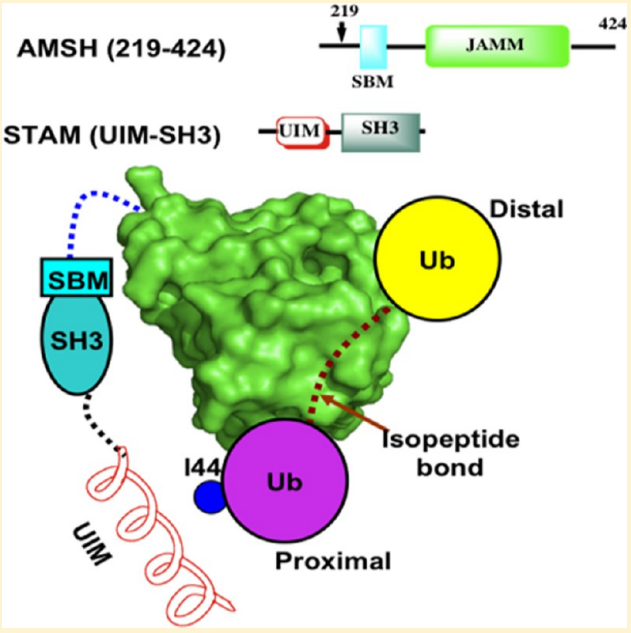
Christopher W. Davies,[†] Lake N. Paul,[‡] and Chittaranjan Das*[,†]

[†]Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, United States

[‡]Bindley Biosciences Center, Discovery Park, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: AMSH, a deubiquitinating enzyme (DUB) with exquisite specificity for Lys63-linked polyubiquitin chains, is an endosome-associated DUB that regulates sorting of activated cell-surface signaling receptors to the lysosome, a process mediated by the members of the endosomal sorting complexes required for transport (ESCRT) machinery. Whole-exome sequencing of DNA samples from children with microcephaly capillary malformation (MIC-CAP) syndrome identified recessive mutations encoded in the AMSH gene causatively linked to the disease. Herein, we report a number of important observations that significantly advance our understanding of AMSH within the context of the ESCRT machinery. First, we performed mutational and kinetic analysis of the putative residues involved in diubiquitin recognition and catalysis with a view of better understanding the catalytic mechanism of AMSH. Our mutational and kinetic analysis reveals that recognition of the proximal ubiquitin is imperative for the linkage specificity and catalytic efficiency of the enzyme. The MIC-CAP disease mutation, Thr313Ile, yields a substantial loss of catalytic activity without any significant change in the thermodynamic stability of the protein, indicating that its perturbed catalytic activity is the basis of the disease. The catalytic activity of AMSH is stimulated upon binding to the ESCRT-0 member STAM; however, the precise mechanism and its significance are not known. On the basis of a number of biochemical and biophysical analyses, we are able to propose a model for activation according to which activation of AMSH is allowed by facile, simultaneous binding to two ubiquitin groups in a polyubiquitin substrate, one by the catalytic domain of the DUB (binding to the distal ubiquitin) and the other (the proximal ubiquitin) by the ubiquitin interacting motif (UIM) from STAM. Such a mode of binding would stabilize the ubiquitin chain in a productive orientation, resulting in an enhancement of the activity of the enzyme. These data together provide a mechanism for understanding the recruitment and activation of AMSH at ESCRT-0, providing biochemical and biophysical evidence that supports a role for AMSH when it is recruited to the initial ESCRT complex: it functions to facilitate the transfer of ubiquitinated receptors (cargo) from one ESCRT member to the next by disassembling the polyubiquitin chain while leaving some ubiquitin groups still attached to the cargo.



AMSH [associated molecule with a Src homology 3 domain of signal transducing adaptor molecule (STAM)] is a member of the JAMM (JAB1/MPN/MOV34) family of deubiquitinating enzymes (DUBs),[1] which regulates ubiquitin signaling by catalyzing the hydrolysis of isopeptide (or peptide) bonds between ubiquitin and target proteins or within polymeric chains of ubiquitin. The JAMM family, being one of the five families of mammalian DUBs, consists of metalloproteases, whereas the others (UCHs, USPs, OTUs, and MJDs) consist of cysteine proteases.[2−4] Members of the JAMM family show substantial variation in their overall amino acid sequence but share, as the name suggests, a conserved JAMM motif as the catalytic domain. Mechanistically, they share distinct similarities to the extensively studied metalloprotease, thermolysin. Like thermolysin, these enzymes have a Zn^{2+} in their active site, which is involved in their mechanism of catalysis. The Zn^{2+} ion is coordinated within the active site usually by two histidines, an acidic residue (aspartic acid or glutamic acid), and a water molecule that eventually is used as the nucleophile for attacking the scissile peptide bond. This catalytic water is held in place by

Received: August 14, 2013
Revised: September 26, 2013
Published: October 2, 2013

dx.doi.org/10.1021/bi401106b | *Biochemistry* 2013, 52, 7818−7829

Zn^{2+} and another acidic residue (glutamic acid in AMSH), which provides a hydrogen bond stabilizing the water. Sequence analysis of the members of the JAMM family reveals that only 7 of 14 proteins have the conserved zinc binding capabilities,[5] while only 6 of those 7 exhibit isopeptidase activity toward ubiquitin and ubiquitin-like proteins, AMSH, AMSH-LP (AMSH like protein), BRCC36, RPN11 (POH1), MYSM1, and CSN5.[5−7]

AMSH is one of the two DUBs, the other being UBPY [also known as ubiquitin specific protease 8 (USP8)],[8] known to be important regulators of the ESCRTs (endosomal sorting complexes required for transport).[9] The ESCRT machinery consists of four protein−protein complexes (ESCRT-0, -I, -II, and -III) and the AAA ATPase Vps4[10,11] that serve several important functions within the cell: endosomal sorting, trafficking, viral budding, cytokinesis, transcriptional regulation, and autophagy.[12] The ESCRTs were initially discovered in yeast in the context of their role in endosomal sorting and trafficking of cell-surface receptors to the lysosome for degradation, as a means of downregulating their signals.[11] These receptors are first ubiquitinated and then shuttled through the ESCRT machinery, until their internalization within endosomes, which then can fuse with the lysosome delivering the receptors for proteolysis.[11]

Of the four complexes, only ESCRT-0 and ESCRT-III can specifically recognize DUBs.[9] ESCRT-0 recognition is conducted through the binding of the SH3 binding motif (SBM) of DUBs to the SH3 domain of the ESCRT-0 member STAM,[13] while ESCRT-III recognition is conducted via the MIT (microtubule-interacting and transport) domain of DUBs binding to the C-terminal MIT-interacting motifs (MIMs) of charged multivesicular body proteins (CHMPs).[9] Previous studies have shown that once AMSH binds to STAM, its activity is enhanced;[13,14] however, the precise mechanism is not known.[13] The X-ray crystal structure of the MIT domain of AMSH bound to the C-terminal MIM fragment of the ESCRT-III member CHMP3 has been determined.[15] The structure reveals that the AMSH−CHMP3 complex is stabilized mainly by polar interactions, manifesting into tight binding between the two proteins, with a dissociation constant (K_D) of 60 nM, the highest reported K_D for an ESCRT-III MIM−MIT interaction.[15]

A clear role for AMSH has not been identified yet; however, whole-exome sequencing analysis has shown that recessive mutations in AMSH lead to microcephaly capillary malformation (MIC-CAP) syndrome.[16] MIC-CAP is discovered at or shortly after birth in which children diagnosed with the disease have severe microcephaly with progressive cortical atrophy, intractable epilepsy, profound developmental delay, and multiple small capillary malformations on the skin.[16−19] The microcephaly phenotype is attributed to the accumulation of ubiquitinated proteins, suggesting a lost of enzymatic function as was seen in knockout mice studies.[20] Of the 10 patients that were screened, six had missense mutations, two had nonsense mutations, two had translational frameshift mutations, and three had intronic mutations.[16] Interestingly, five of the six missense mutations were found within the MIT domain of AMSH, and the sixth, Thr313Ile, was found within the JAMM domain.[16]

Although the exact *in vivo* function of AMSH is not well understood, its exquisite specificity for Lys63-linked polyubiquitin chains, the same type of chain used for endosomal−lysosomal targeting, is well-characterized.[21] The structural basis for this recognition was elucidated with the homologous protein, AMSH-LP, bound to the Lys63-linked dimer of ubiquitin.[22] This structure reveals that the specificity arises from a tripeptide sequence within the proximal ubiquitin (Gln62, Lys63, and Glu64) interacting directly with a threonine, two phenylalanines, and a serine residue[22] on the proximal binding site of the enzyme (in a diubiquitin motif, the proximal ubiquitin is defined as the one that contributes a Lys residue to be linked to Gly76 of the other ubiquitin, defined as the distal ubiquitin). The sequences of AMSH and AMSH-LP are 54% identical and 73% similar; however, AMSH-LP does not have a functional SBM[9,23] or MIT domain[24] and, therefore, does not have the ability to bind to the members of the ESCRT machinery. We recently determined the X-ray crystal structure of the catalytic domain of AMSH and found that the catalytic domains of AMSH and AMSH-LP are structurally very similar; however, much to our surprise, AMSH is thermodynamically less stable than AMSH-LP, which was attributed to the structural plasticity of the former.[25] This idea of structural plasticity is supported by the second X-ray crystal structure of the catalytic domain of AMSH bearing the active-site glutamate to alanine mutation, which was expected to cause the release of the active-site Zn^{2+}. The structure, however, shows that the tetrahedral coordination around the active-site Zn^{2+} in this mutant is still maintained by a nearby aspartate residue moving in to provide the fourth ligand for the metal in place of the lost water.[25] Though AMSH and AMSH-LP are able to localize to the endosomes in a similar manner through their ability to bind to clathrin,[13,26] the differences between them arising from the inability of AMSH-LP to bind to any of the ESCRT complexes suggest that AMSH and AMSH-LP are not functionally redundant. Moreover, the active-site cleft of AMSH features three substitutions relative to AMSH-LP, substitutions of residues that are predicted to be used for ubiquitin binding. One of them, Thr313, which in AMSH-LP corresponds to Met325, is also the site of a MIC-CAP mutation.

In this study, we aim to further establish a role for AMSH as an important regulator of the ESCRT machinery. We have conducted extensive mutational and kinetic analyses of residues within the catalytic domain of AMSH to understand their role in ubiquitin binding to better understand the catalytic mechanism of the enzyme. Also, we have studied the effect of the Thr313Ile mutation found in children with the MIC-CAP syndrome on the activity and thermodynamic stability of AMSH with the hope of understanding the molecular basis of the disease. Our results confirmed that residues within the proximal ubiquitin recognition site of AMSH are the basis of specificity for the Lys63-linked polyubiquitin chain. Furthermore, we found that of the three residue differences between AMSH and AMSH-LP in the distal ubiquitin-binding site, Thr313 and Glu316 had the most significant effects on AMSH activity. Finally, using the minimal domains required for AMSH activation, we have shed light on the mechanism of AMSH recruitment and activation at ESCRT-0; we found that AMSH is activated because of an intact SBM−SH3 interaction and an intact UIM from STAM. On the basis of our results, we have proposed a mechanism and role for AMSH in the context of ESCRT-0.

MATERIALS AND METHODS

Cloning, Expression, and Purification. The DNA encoding the catalytic domain of AMSH was amplified by polymerase chain reaction (PCR) using a plasmid that

contained full-length DNA as the template (pGEX-6p1-AMSH, a kind gift from S. Urbé, University of Liverpool, Liverpool, U.K.) and was subcloned into pGEX-6p1 (GE Biosciences) by using standard cloning protocols. The resulting N-terminally fused glutathione *S*-transferase (GST)-tagged protein was expressed in *Escherichia coli* Rosetta cells (Novagen) and purified with a glutathione-Sepharose column (GE Biosciences) following the manufacturer's instructions. After removal of the tag by PreScission protease (GE Biosciences), the protein was further purified by size-exclusion chromatography (SEC) using a Superdex S75 column (GE Biosciences).

The series of individual point mutations were introduced into the AMSH catalytic domain gene by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. DNA sequencing confirmed the presence of the mutations. The resulting proteins were purified using standard GST affinity chromatography followed by SEC (Superdex S75 column).

The DNA encoding the SH3 and UIM-SH3 domains was amplified by PCR using a plasmid that contained the full-length DNA as the template [pGW1Myc2c-STAM2, a kind gift from C. Blackstone, National Institutes of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), Bethesda, MD] and was subcloned into pGEX-6p1 (GE Biosciences) using standard cloning protocols. The resulting N-terminally fused GST-tagged protein was expressed in *E. coli* Rosetta cells (Novagen) and purified with a glutathione-Sepharose column (GE Biosciences) following the manufacturer's instructions. After removal of the tag by PreScission protease (GE Biosciences), the protein was further purified by size-exclusion chromatography (SEC) using a Superdex S75 column (GE Biosciences).

The A176G mutation was introduced into the UIM-SH3 gene by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. DNA sequencing confirmed the presence of the mutations. The resulting proteins were purified using standard GST affinity chromatography followed by SEC (Superdex S75 column).

Human ubiquitin was subcloned into pGEX-6p1 and purified using GST affinity chromatography, and the GST tag was removed by PreScission Protease. The protein was further purified using SEC (Superdex S75 column). Lys63-diubiquitin was enzymatically synthesized from ubiquitin using ATP, human E1, and the E2 complex (Ubc13 and Uev1a) following previously reported procedures.[22] The reaction mixture was incubated at 37 °C for 2 h and then the reaction quenched by dilution with buffer A [50 mM sodium acetate (pH 4.5)]. The quenched reaction mixture was subjected to ion-exchange chromatography on a Mono-S column (GE Biosciences), yielding Lys63-diubiquitin.

Determination of Kinetic Parameters and the DUB Assay. The kinetic parameters were determined by incubating the enzymes (25 nM T313A; 100 nM C282A, N312A, E317A, and F320A; 2 μM E316A and F395A; 3 μM T341A and S346A; and 10 μM F343A and S345A) with four concentrations of diubiquitin, ranging from 20 to 177 μM, in reaction buffer [50 mM TRIS-HCl (pH 7.0), 20 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT]. The reaction was conducted at 20 °C for 10−75 min depending on the activity for initial velocity measurements. Reactions were quenched by the addition of 5× sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer followed by boiling. The reaction mixtures were visualized by SDS-PAGE gels and scanned. Bands corresponding to monoubiquitin were integrated using ImageJ (http://rsb.info.nih.gov/ij/). Ubiquitin standards in amounts ranging from 4 to 12.3 μg were used to draw calibration plots, which were used to quantify the amount of ubiquitin produced. Kinetic parameters were calculated by fitting the data in SigmaPlot (Systat Software, San Jose, CA).

The *in vitro* DUB assay was conducted by incubating AMSH (residues 219−424) at a final enzyme concentration of 100 nM with 1 μM SH3 domain of STAM2 or UIM-SH3 gene of STAM2 and 20 μM Lys63-diubiquitin in a total reaction volume of 20 μL. All reactions were conducted in reaction buffer [50 mM TRIS-HCl (pH 7.0), 25 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT] for 5 h at 20 °C. The reaction was quenched by the addition of 5× SDS−PAGE sample buffer followed by boiling and then analyzed by SDS−PAGE.

Analytical Ultracentrifugation. Sedimentation velocity experiments were conducted at 50000 rpm using the Beckman Coulter XLA and XLI instruments (Beckman Coulter, Fullerton, CA). The samples were monitored by both interference and absorbance optics at 254 and 280 nm. The proteins were dialyzed in 50 mM TRIS-HCl (pH 7.6) and 50 mM NaCl. Three concentration series for AMSH (residues 219−424) were conducted to evaluate the formation of higher-order species at 24, 48, and 96 μM. The AMSH−SH3 complex was characterized using a constant AMSH concentration of 23.5 μM and three concentrations of SH3 (24, 47, and 70 μM). The AMSH−UIM-SH3 complex was characterized using a constant AMSH concentration of 23.5 μM and three SH3 concentrations (48, 144, and 288 μM). The AMSH−ubiquitin complex was characterized using a constant AMSH concentration of 23.5 μM and two concentrations of ubiquitin (23 and 92 μM). The AMSH−diubiquitin complex was characterized using a constant AMSH concentration of 23.5 μM and three diubiquitin concentrations (24, 48, and 96 μM). The solvent density (1.00170 g/mL), viscosity (0.01022 P), and partial specific volumes that were used for the analyses, 0.73387 mL/g (AMSH219), 0.71870 mL/g (AMSH−SH3), 0.71701 mL/g (AMSH−UIM-SH3), 0.72934 mL/g (AMSH−diubiquitin), and 0.72479 mL/g (AMSH−ubiquitin), were calculated by SEDNTERP version 20120828 beta (http://bitcwiki.sr.unh.edu/index.php/Main_Page).[27] The sedimentation coefficients and apparent molecular weights were calculated from size distribution analyses [$c(s)$] using SEDFIT version 14.3e.[28,29] The figures were prepared using GUSSI version 1.0.7b beta with the sedimentation coefficients standardized to $s_{20,w}$, and the data were normalized to the peak area of the complexes.

Sedimentation equilibrium experiments were conducted at 20 °C using a six-channel centerpiece in an AN-60 Ti rotor spun at speeds of 13200, 29900, and 42000 rpm for the AMSH−ubiquitin complex and 11600, 21000, and 36000 rpm for the AMSH−diubiquitin complex. The molar ratios of the AMSH−ubiquitin complex were 1:2, 1:4, and 1:8, and the molar ratios of the AMSH−diubiquitin complex were 1:2, 1:4, and 1:5, which were used to determine the molecular weights of the complexes. Absorbance scans at 280 nm were taken every 2 h for 60 h. The samples were tested for equilibrium using Sedfit.[28,29] Calculations of the molecular weights were conducted with SEDPHAT version 10.58d[30−35] using the Species Analysis and Species Analysis with Mass Conservation Constraints. Errors were calculated using one-dimensional error surface projections. Final figures were generated in GUSSI.

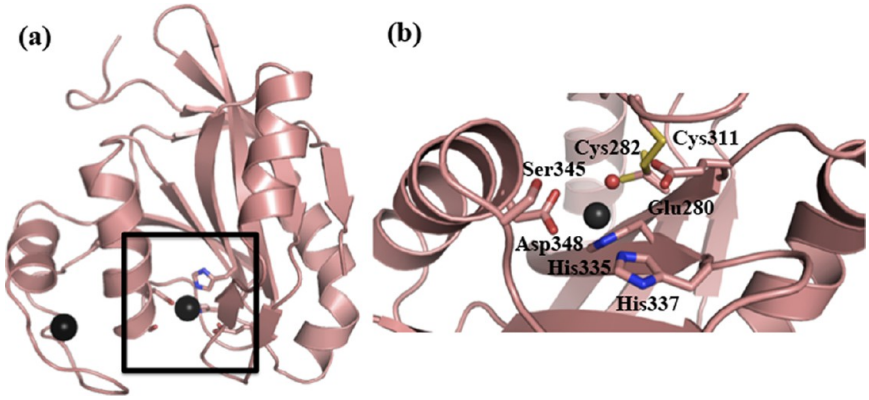


Figure 1. DUB domain of AMSH. (a) Ribbon diagram of the crystal structure of the catalytic domain of AMSH (PDB entry 3RZU). The active site is highlighted by the black square. (b) Expanded view of the active-site residues of AMSH. The black spheres represent Zn^{2+}, and the red sphere represents the active-site water molecule.

Isothermal Titration Calorimetry. To determine the K_D values of binding of AMSH to ubiquitin, ITC experiments were conducted using the MicroCal ITC200 instrument (GE Healthcare Life Sciences). The proteins were dialyzed in the same buffer that was used for AUC. For the AMSH−diubiquitin experiment, 10 μM AMSH was in the cell and 500 μM diubiquitin was in the syringe. The AMSH−ubiquitin titration had 100 μM AMSH in the cell and 1 mM ubiquitin in the syringe. The F320A mutant of AMSH with ubiquitin had 100 μM enzyme in the cell and 1 mM ubiquitin in the syringe. These experiments were conducted at 20 °C, in 18 total injections of 1.4 μL per injection, with 180 s between injections to allow for a return to baseline before the subsequent injection. The data were then baseline-corrected with NITPIC[36] and loaded into SEDPHAT[30−35] for global analysis and fitting using a 1:1 model. Figures were prepared using GUSSI. To determine the K_D for the AMSH−SH3 interaction, 50 μM AMSH was in the cell and 750 μM SH3 domain was in the syringe. The K_D of the AMSH−UIM-SH3 interaction was determined using 100 μM AMSH in the cell and 1 mM UIM-SH3 in the syringe. The AMSHK238T−UIM-SH3 experiment was conducted using 50 μM enzyme in the cell and 1 mM UIM-SH3 in the syringe. Characterization of the binding of ubiquitin to UIM-SH3 was conducted using 50 μM UIM-SH3 in the cell and 3.1 mM ubiquitin in the syringe. These data were fit to a two-site model. Confirmation of SH3−ubiquitin binding was achieved with 100 μM SH3 in the cell and 3.0 mM ubiquitin in the syringe. The UIM-SH3−Lys63-diubiquitin experiment had 50 μM UIM-SH3 in the cell and 750 μM diubiquitin in the syringe.

Guanidine Melt Using Circular Dichroism Spectroscopy. The stability of the folded state of the AMSH219T313I mutant (the DUB domain of AMSH from residues 219 to 424 bearing the MIC-CAP mutation Thr313Ile) toward GdHCl was determined using a GdHCl stock concentration of 8 M (Sigma). Varying concentrations of GdHCl were added to the protein (0.2 mg/mL) diluted in 100 mM phosphate buffer (pH 7.4) and allowed to sit at room temperature overnight to allow for complete equilibration. Changes in the folded state of the proteins were monitored using circular dichroism by following changes in ellipticity at 220 nm. CD spectra were recorded in a Jasco J-810 spectropolarimeter in the far-UV region (195−260 nm) in a 0.1 cm path-length cuvette. Each spectrum was averaged over four scans (scan speed of 50 nm/min, with a 8 s time constant) and corrected by subtraction of a spectrum of the buffer alone. Mean residue molar ellipticity values were calculated using the following equation:

$$[\theta] = \frac{\theta \times 100M}{Cln}$$

where θ is the ellipticity in degrees, l is the optical path in centimeters, C is the concentration in milligrams per milliliter, M is the molecular mass, and n is in the number of residues in the protein.

The mean residue molar ellipticity $[\theta]$ is given in degrees square centimeter per decimole. Unfolding curves were analyzed using a two-state unfolding model, using linear extrapolation to obtain the ΔG value in the absence of GdHCl.[37]

RESULTS

Mutational and Kinetic Analysis of the Catalytic Domain of AMSH. Previous modeling studies investigating the differences between the ability of AMSH and AMSH-LP to bind and cleave Lys63-linked diubiquitin revealed some interesting results. The active-site and proximal ubiquitin binding residues were identical between the two proteins; however, in the distal ubiquitin-binding site, AMSH has three residues different from those of AMSH-LP. On the basis of this analysis, we sought to conduct an extensive mutational and kinetic analysis of the putative residues in AMSH that might be involved in diubiquitin cleavage.

Active Site. The active site of AMSH consists of a Zn^{2+} ion, coordinated directly by three residues (Asp348, His335, and His337) and a water molecule that is hydrogen bonded to Glu280, and a putative oxyanion stabilizing residue (Ser345) (Figure 1). To probe the roles of Asp348 and Glu280, we generated two aspartate mutants (D348A and D348N) and a glutamate mutant (E280A) and, as expected, found that there was no detectable activity in these mutants (Table 1), most likely because of the loss of Zn^{2+} for the aspartate mutations and the loss of the water molecule in the glutamate mutation.

Next, we probed the function of the putative oxyanion hole-stabilizing residue, Ser345. Mutating serine to alanine resulted in a significantly impaired enzyme with a 1000-fold decrease in k_{cat} (Table 1). As described with other families of hydrolases, the oxyanion hole-stabilizing residue plays a critical role in donating a hydrogen bond to the negatively charged tetrahedral intermediate formed after the initial nucleophilic attack. A substantial reduction in k_{cat} alone upon mutation to alanine,

dx.doi.org/10.1021/bi401106b | *Biochemistry* 2013, 52, 7818−7829

Table 1. Kinetic Parameters of AMSH Mutants

site	protein	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)
	wild type	1400 ± 100	32 ± 5
active site	Glu280Ala	NA[a]	NA[a]
	Cys282Ala	230 ± 140	45 ± 7
	Ser345Ala	1.4 ± 0.1	38 ± 15
	Asp348Ala	NA[a]	NA[a]
	Asp348Asn	NA[a]	NA[a]
proximal	Thr341Ala	24 ± 1	15 ± 3
	Phe343Ala	5 ± 3	21 ± 4
	Ser346Ala	13 ± 3	23 ± 8
	Phe395Ala	22 ± 8	18 ± 9
distal	Asn312Ala	430 ± 60	19 ± 2
	Thr313Ala	2600 ± 600	82 ± 5
	Glu316Ala	19 ± 4	31 ± 9
	Glu317Ala	750 ± 200	19 ± 7
	Phe320Ala	370 ± 20	98 ± 15
MIC-CAP	Thr313Ile	225 ± 39	21 ± 5

[a]No observed activity up to 10 μM protein per 20 μL reaction volume over 4 h at 20 °C.

with K_M remaining nearly the same, is consistent with Ser345 playing the role as the oxyanion stabilizing residue in AMSH.

In our previous structural analysis, we noted a potential disulfide bridge between Cys282 and Cys311, 7.4 Å from the active-site Zn^{2+}.[25] Previous studies have shown that *N*-ethylmaleimide (NEM) inhibits AMSH activity (IC_{50} of 16.2 ± 3.2 μM),[21,38] presumably by modifying one of these two cysteines, perhaps Cys282 because it is proximal to the active-site cleft. Its modification might introduce some steric hindrance for substrate binding, thus explaining the inhibitory effect. We sought to determine if Cys282 has any role in the catalytic activity of the enzyme. Previously, when Cys282 was mutated to alanine, we noticed a loss of activity;[25] however, a more detailed kinetic analysis of this mutant revealed a more significant reduction in activity, a 6-fold decrease in k_{cat} (Table 1), which would suggest that Cys282 does indeed have a role in catalysis. Cys282 is seen making a van der Waals contact with Leu73 of the distal ubiquitin, which may explain the reduction in activity observed here.

Proximal Ubiquitin Site. Modeling of the catalytic domain of AMSH onto the structure of AMSH-LP bound to Lys63-linked diubiquitin revealed four residues within AMSH (Thr341, Phe343, Ser346, and Phe395) that could determine its specificity for Lys63-linked polyubiquitin chains by recognizing the Gln62-Lys63-Glu64 tripeptide sequence motif within the proximal ubiquitin, which encompasses the acceptor Lys63 and its two immediate flanking residues. Individual point mutants (to alanine) were generated, and kinetic analysis was performed to probe the functional significance of these residues. Overall, the four resides within AMSH showed a drastic reduction in k_{cat} with K_M values similar to those of the wild-type enzyme, confirming their utmost importance to the catalytic mechanism of the enzyme (Figure 2 and Table 1), especially during the rate-determining step of isopeptide bond hydrolysis.

Distal Ubiquitin Site. The distal site is where AMSH significantly differs from AMSH-LP in diubiquitin recognition. Three substitutions are found going from AMSH-LP to AMSH: aspartate to asparagine, methionine to threonine, and valine to glutamate. Two other important residues within the distal site are completely conserved between AMSH and AMSH-LP, a

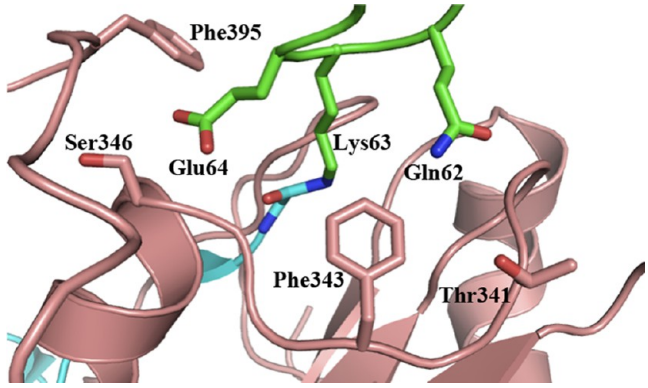


Figure 2. Residues involved in proximal ubiquitin recognition within the catalytic domain of AMSH. AMSH residues are shown as pink sticks, proximal ubiquitin residues as green sticks, and distal ubiquitin residues as cyan sticks.

phenylalanine (Phe320, AMSH numbering) and a glutamate (Glu317) (Figure 3). The conserved Phe320 when mutated to

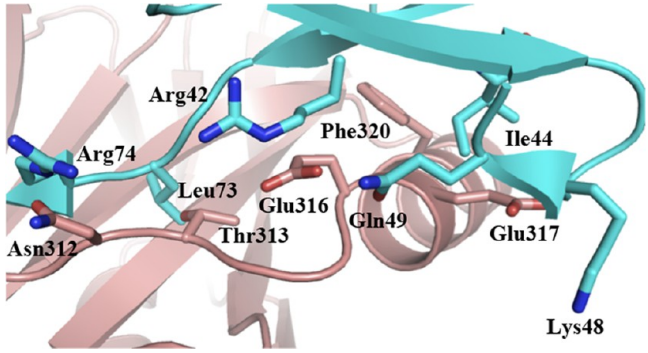


Figure 3. Residues involved in distal ubiquitin recognition within the catalytic domain of AMSH. AMSH residues are shown as pink sticks, proximal ubiquitin residues as green sticks, and distal ubiquitin residues as cyan sticks.

alanine exhibited a 4-fold reduction in k_{cat} and 3-fold increase in K_M, whereas the Glu317Ala mutant exhibits activity somewhat similar to that of the wild type, with a modest only 2-fold reduction in k_{cat} (Table 1).

Individual point mutations of the three substitutions between AMSH and AMSH-LP revealed some interesting results. Mutating Asn312 to alanine yielded only an approximate 3-fold reduction in k_{cat} (Table 1). Surprisingly, a qualitative diubiquitin cleavage assay revealed that Thr313Ala was apparently more active than the wild type (unpublished data); however, detailed kinetic analysis showed simply an approximate 2-fold increase in k_{cat}, with an ~3-fold decrease in K_M (Table 1).

Mutating Glu316 to alanine proved to cause the most significant change in enzymatic activity among the distal-site residues. Glu316Ala showed a substantial 74-fold reduction in k_{cat} (Table 1). This k_{cat} effect differs strikingly from the distal-site residues of AMSH-LP whose mutation to Ala showed a decrease primarily in K_M.[22] The effect of the glutamate mutation in AMSH mirrors that of residues from the proximal site. Inspection of the AMSH−diubiquitin model reveals that Glu316 is within hydrogen bonding distance of two distal ubiquitin residues: Arg42 within 3.1 Å (εN of Arg42 and εO of Glu316) and Gln49 within 2.8 Å, if the side chain is flipped by

dx.doi.org/10.1021/bi401106b | *Biochemistry* 2013, 52, 7818−7829

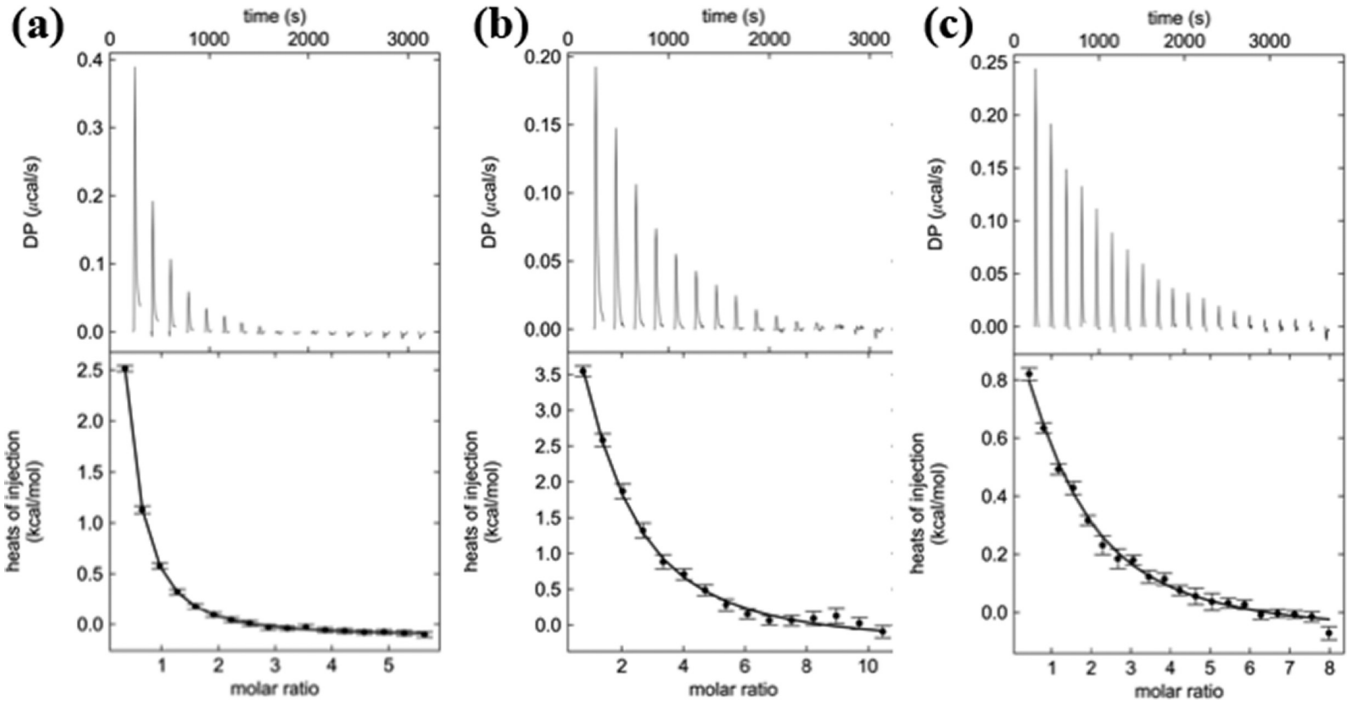


Figure 4. Isothermal titration calorimetry (ITC) thermograms of binding of ubiquitin to the catalytic domain of AMSH. (a) ITC thermogram of binding of ubiquitin to the catalytic domain of AMSH revealing a K_D of 19 ± 3 μM. (b) ITC thermogram of binding of Lys63-linked diubiquitin to the catalytic domain of AMSH revealing a K_D of 19 ± 4 μM. (c) ITC thermogram of binding of a Phe320Ala mutant of the catalytic domain of AMSH to ubiquitin revealing a K_D of 81 ± 15 μM.

Table 2. Thermodynamic Parameters Deduced from ITC Data

protein	titrant	K_D (μM)	ΔH (kcal/mol)	ΔS (cal mol^{-1} K^{-1})	N
AMSH	Ub	19 ± 3	19.3 ± 7.8	86.4	1
AMSH	DiUb	19 ± 4	13.7 ± 1.8	67.7	1
AMSHF320A	Ub	81 ± 15	2.6 ± 0.4	27.5	1
AMSH	SH3	1.4 ± 0.04	−15.1 ± 0.1	−23.7	1
AMSH	UIM-SH3	1.9 ± 0.1	−15.8 ± 0.1	−26.9	1
AMSHK238A	SH3	NA[a]	NA[a]	NA[a]	NA[a]
AMSHK238T	SH3	NA[a]	NA[a]	NA[a]	NA[a]
SH3	Ub	62 ± 7	−4.1 ± 0.2	5.3	1
UIM-SH3	Ub	273 ± 16	−18.2 ± 1.0	−44.7	2
UIM-SH3	DiUb	54 ± 21	−13.0 ± 6.6	−23.9	1

[a]No observed binding at 50 μM enzyme and 1 mM UIM-SH3.

180° (Figure 3). A significant decrease in k_{cat} but not in K_M is consistent with these hydrogen bonding interactions contributing to the stabilization of the transition state, perhaps by playing a role in orienting the scissile peptide bond for nucleophilic attack.

Kinetic and Thermodynamic Characterization of the Effect of the MIC-CAP-Associated Mutation, Thr313Ile. To improve our understanding of the molecular basis of MIC-CAP syndrome, the Thr313Ile (T313I) mutant was generated and analyzed both for its catalytic activity toward Lys63-diubiquitin and for its thermodynamic stability. In the absence of a structure, a modeled AMSH−diubiquitin structure suggests that the side chain hydroxyl from threonine is hydrogen bonded to the backbone NH group of Leu73 in the distal ubiquitin (Figure S1 of the Supporting Information). The T313I mutant was found to suffer a 6-fold decrease in k_{cat}, with a comparable K_M (Table 1). In terms of its thermodynamic stability, T313I was somewhat less stable than the wild type with a ΔG_{H_2O} of 2.9 kcal/mol compared to a value of 3.6 kcal/mol for the wild type (Figure S2 and Table 1 of the Supporting Information). This result indicates that the reduced catalytic activity of the mutant could lead to a loss of function of AMSH translating into the disease state.

Biophysical Characterization of Binding of Ubiquitin to the Catalytic Domain of AMSH. Mutational and kinetic analyses prompted us to seek a better understanding of the formation of the AMSH−ubiquitin complex in solution. Using isothermal titration calorimetry (ITC), we analyzed the binding of Lys63-linked diubiquitin and the catalytic domain of AMSH (AMSH 219−424^{E280A}, an inactive mutant to ensure diubiquitin is not hydrolyzed) and obtained an equilibrium dissociation constant (K_D) of 19 ± 4 μM (Figure 4b and Table 2). As a control, ubiquitin and the catalytic domain of AMSH were analyzed, and it was determined that it binds AMSH with similar affinity of 19 ± 3 μM (Figure 4a and Table 2). Both sedimentation velocity and sedimentation equilibrium experiments using analytical ultracentrifugation (AUC) confirmed the ITC results (Figures S3 and S4 and Table 2 of the Supporting

dx.doi.org/10.1021/bi401106b | Biochemistry 2013, 52, 7818−7829

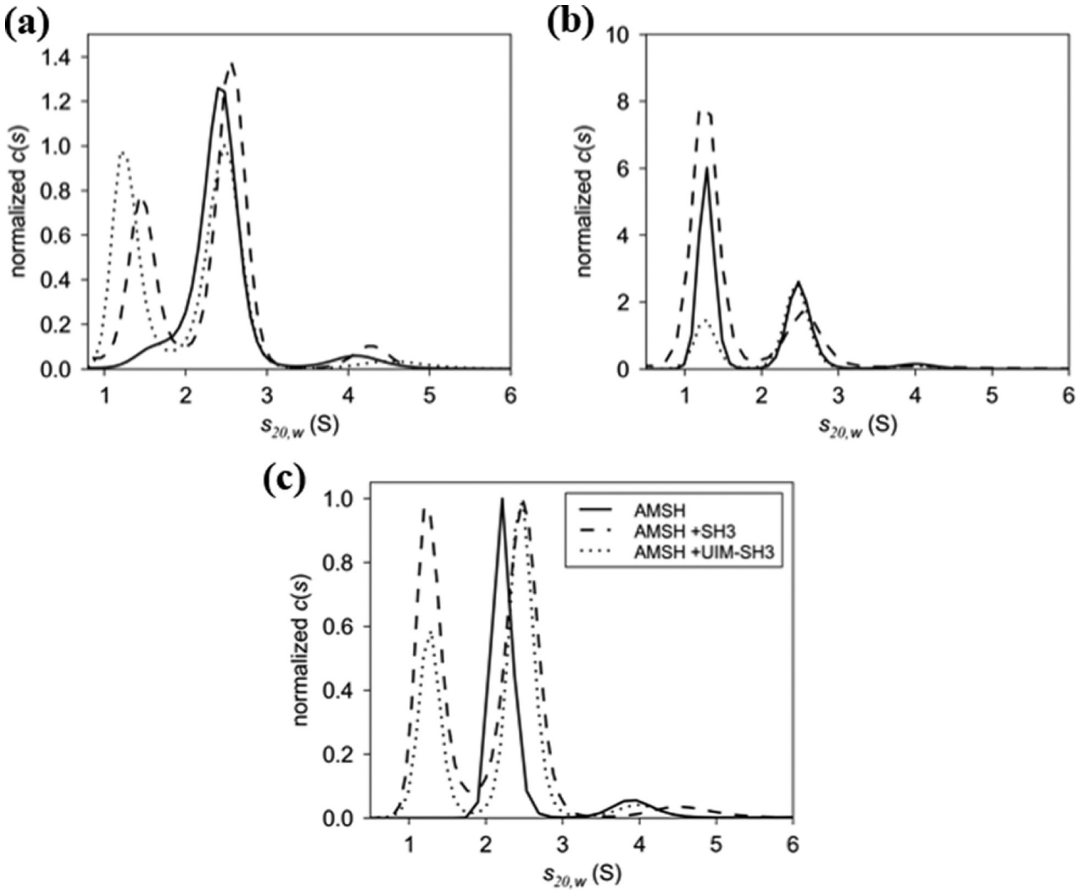


Figure 5. $c(s)$ distributions of the catalytic domain of AMSH binding to (a) the SH3 domain of STAM and (b) UIM-SH3. Three concentration series were used to assess the formation of the AMSH−SH3 and AMSH−UIM-SH3 complexes revealing 1:1 complexes at 2.5 and 2.6 S, respectively. Excess SH3 and UIM-SH3 are present at 1.3 S. The data for both $c(s)$ distributions were normalized to the peak area of the complexes. (C) Overlay of AMSH and the AMSH−SH3 and AMSH−UIM-SH3 complexes revealing changes in the s values of the AMSH−SH3 and AMSH−UIM-SH3 complexes compared to that of AMSH alone (2.2 S). The $c(s)$ distributions were normalized to the peak area of the complexes.

Information). Almost identical affinities of binding of diubiquitin and ubiquitin to the catalytic domain of AMSH suggest that there is only one binding site for ubiquitin. To probe which ubiquitin binding site is used, another ITC experiment was conducted with AMSH^{Phe320Ala} (Phe320 at the distal site is mutated to Ala) and ubiquitin. We observed an ∼4-fold decrease in affinity (K_D of 81 ± 15 μM) (Figure 4c and Table 2), consistent with what was observed from kinetics, suggesting that the distal ubiquitin makes the most significant contribution to diubiquitin binding, and the single binding site observed in our ITC experiments with ubiquitin corresponds to binding at the distal site. These data suggest that AMSH alone cannot discriminate between its polyubiquitin substrate and its ubiquitin product.

The Intact Minimal STAM Construct UIM-SH3 Is Necessary for AMSH Activation. The I44 patch, a hydrophobic surface centered on the Ile44 residue in ubiquitin, is ubiquitously used by proteins that specifically bind to ubiquitin, including DUBs. Inspection of our structural model representing the AMSH−diubiquitin complex reveals that the I44 patch of the distal ubiquitin is satisfied, with the Ile44 residue engaged in van der Waals interaction with Phe320; however, Ile44 of the proximal ubiquitin is unoccupied (Figure S5 of the Supporting Information). Looking at the domain structure of the ESCRT-0 member, STAM, one finds a UIM (ubiquitin-interacting motif) N-terminally adjacent to its SH3 domain (Figure S6 of the Supporting Information). We sought to understand if the UIM, separated from the SH3 domain by a short linker, could act as an adaptor to AMSH by interacting with the proximal ubiquitin while AMSH engages the distal one. To probe this, we used a combination of biophysical techniques and biochemical assays to assess three individual events: (1) recruitment of AMSH to STAM via the SH3 domain and a longer STAM segment in which the UIM is fused to the SH3 domain (UIM-SH3), (2) binding of ubiquitin to UIM-SH3, and (3) the ternary complex of the catalytic domain of AMSH, UIM-SH3, and Lys63-linked diubiquitin.

AMSH Binds to the SH3 Domain of STAM2. To confirm that we have the minimal domains required for the AMSH−STAM interaction, we conducted ITC and AUC experiments. We determined that the SH3 domain of STAM binds the catalytic domain of AMSH (AMSH219^{E280A}) with a K_D of 1.4 ± 0.04 μM (Table 2 and Figure S7 of the Supporting Information). Using the longer UIM-SH3 construct, we obtained an identical K_D of 1.9 ± 0.1 μM (Table 2 and Figure S7 of the Supporting Information), both of which are consistent with values from a previous ITC study, which showed that a peptide representing the SBM of AMSH binds the SH3 domain with an affinity of 7 μM.[14] Using an orthogonal and complementary technique, we confirmed complex formation by sedimentation velocity experiments using AUC and determined that the catalytic domain of

dx.doi.org/10.1021/bi401106b | Biochemistry 2013, 52, 7818−7829

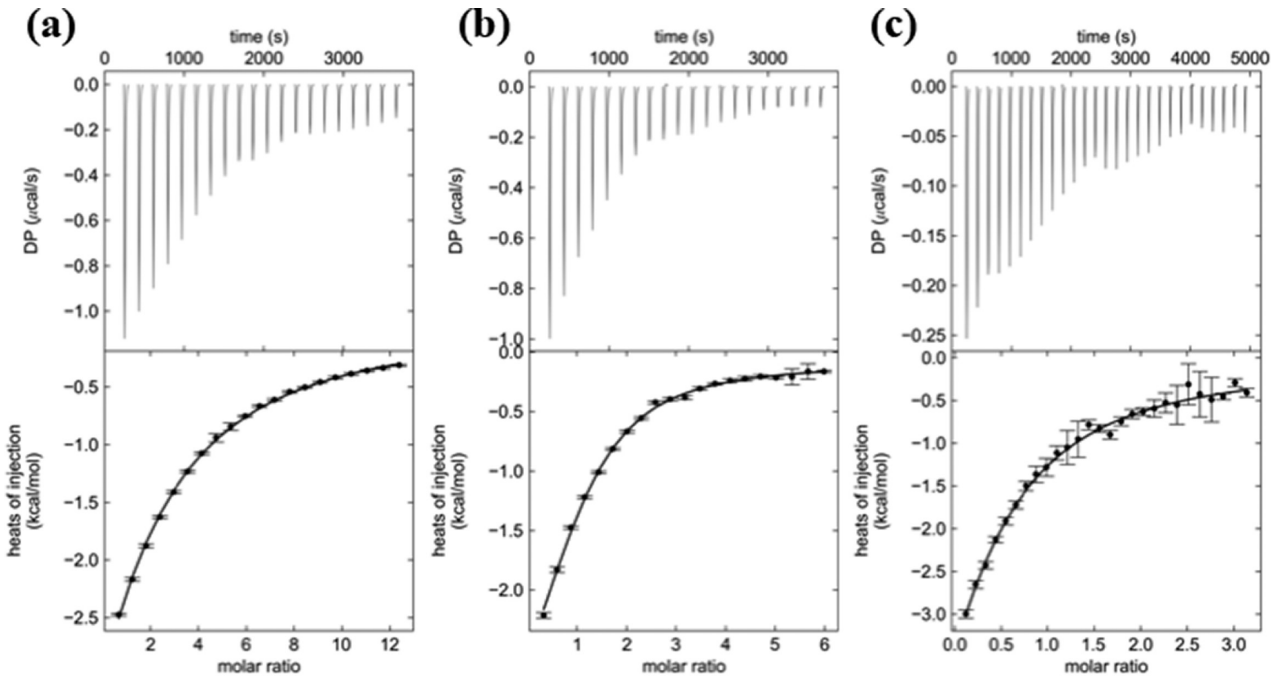


Figure 6. ITC thermograms of binding of ubiquitin to UIM-SH3 of STAM. (a) Thermogram of binding of ubiquitin to UIM-SH3 revealing a K_D of 273 ± 16 μM. (b) Thermogram of binding of ubiquitin to the SH3 domain of STAM revealing a K_D of 62 ± 7 μM. (c) Thermogram of binding of Lys63-linked diubiquitin to UIM-SH3 revealing a K_D of 54 ± 21 μM.

AMSH forms a 1:1 complex. The respective $s_{20,w}$ values of 2.5 and 2.6 S for the SH3 domain and UIM-SH3, respectively (Figure 5a,b), suggest that the UIM has no role in the recruitment of AMSH to STAM, as expected.

Both the SH3 Domain and UIM of STAM Bind Ubiquitin Independently. Second, we characterized binding of ubiquitin to the UIM of STAM. Because UIMs are only 30-residue domains, much too small for bacterial expression, we used UIM-SH3 to investigate UIM−ubiquitin binding by ITC. Somewhat surprisingly, we found that both the UIM and SH3 domains bind ubiquitin independently. This observation was based on two pieces of evidence. (1) The UIM-SH3 construct binds ubiquitin with a K_D of 273 ± 16 μM, in agreement with previous biosensor measurements of a STAM-derived UIM peptide binding to ubiquitin that provided a K_D of 182 μM.[39] (2) Interestingly, our measurement of SH3−ubiquitin binding by ITC resulted in a K_D of 62 ± 7 μM (Figure 6b and Table 2). It has been shown previously that a subset of SH3 domains bind ubiquitin,[40,41] and a recent study using nuclear magnetic resonance titration experiments showed that the SH3 domain of STAM does in fact bind ubiquitin and that this interaction can be competed off by USP8 binding to the SH3 domain of STAM.[42] Taken together, these data seem to indicate that both the SH3 domain and the UIM bind ubiquitin independently, with the former having a higher affinity than the latter, which would explain the overall K_D of 273 ± 15 μM obtained as the binding affinity of the UIM-SH3 construct for ubiquitin. It is possible that the two binding events corresponding to the two binding sites on UIM-SH3 have similar enthalpies of binding, and with binding affinities between them not being drastically different, the ITC experiment is unable to resolve them distinctly.

Alternatively, it is possible that the UIM and SH3 domain fold onto each other generating an interface for ubiquitin weaker than that with either of them alone. This seems unlikely because UIM-SH3 binds to Lys63-linked diubiquitin with a K_D of 54 ± 20 μM (Figure 6c and Table 2), an affinity higher than that of UIM-SH3 for ubiquitin. These results are consistent with the principle of avid binding of polyubiquitin chains at ESCRT-0, thus indicating that both the UIM and SH3 domain in UIM-SH3 are accessible for ubiquitin binding.

UIM and SH3 Domains Are Necessary for Stimulating the Activity of AMSH. Using the catalytic domain of AMSH, UIM-SH3, and Lys63-linked diubiquitin, we attempted to recapitulate the recruitment of AMSH to ESCRT-0 *in vitro*. We conducted a Lys63-diubiquitin DUB cleavage assay with the AMSH−UIM-SH3 complex. The initial experiment comparing the enzyme's activity alone and in the presence of the SH3 domain and then UIM-SH3 revealed a remarkable difference in the DUB activity of AMSH. In the presence of UIM-SH3, it turned over nearly all of the Lys63-diubiquitin to ubiquitin, whereas AMSH alone or in the presence of simply the SH3 domain had a significant amount of diubiquitin remaining, suggesting a stimulatory role for UIM-SH3 (Figure 7).

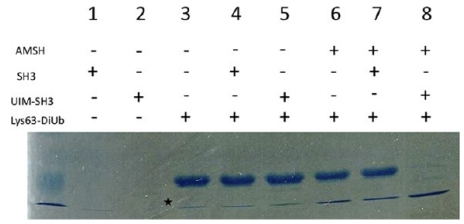


Figure 7. DUB activity assay conducted by monitoring diubiquitin cleavage. SDS−PAGE gel comparing the activity of the catalytic domain of AMSH alone and in the presence of the SH3 domain and UIM-SH3 of STAM. Only the lane with UIM-SH3 reveals activation. The asterisk indicates ubiquitin contamination in the diubiquitin purification.

dx.doi.org/10.1021/bi401106b | *Biochemistry* 2013, 52, 7818−7829

Diving deeper into the mechanism of activation, we performed a similar *in vitro* assay, this time using two SBM mutant versions and one UIM mutant version of the UIM-SH3 construct (Figure 8). Two individual point mutations within

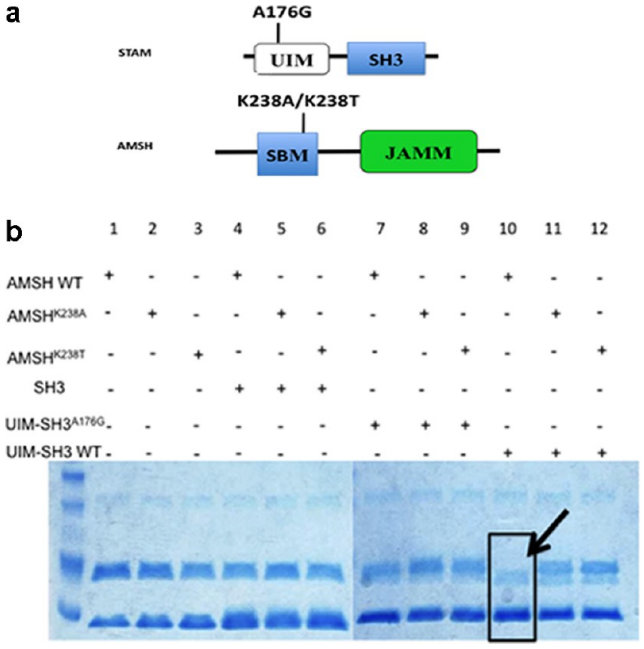


Figure 8. Catalytic activation of AMSH in the presence of UIM-SH3. (a) Domain diagram of the minimal AMSH and STAM proteins, indicating the locations of the introduced mutations. (b) SDS–PAGE investigating the effects of mutants on the catalytic activation of AMSH. Only in the presence of the wild-type enzyme and wild-type UIM-SH3 is the activity of AMSH enhanced, indicated by the complete disappearance of the diubiquitin substrate (black arrow). All lanes contained Lys63-linked diubiquitin.

the SBM of AMSH were introduced (Lys238Ala and Lys238Thr) to obliterate the SH3–SBM interaction. Lys238 is a completely conserved residue in the canonical SBM motif known to bind SH3 domains, and mutating this to threonine made AMSH look like AMSH-LP in terms of its SBM. AMSH-LP has the conserved set of residues within its SBM, except the critical Lys is replaced with Thr. We confirmed that there was no binding between the AMSH SBM mutants and the SH3 domain using ITC (Figure S8 of the Supporting Information). Second, we introduced a mutation within the UIM of UIM-SH3 (Ala176Gly) to interrupt ubiquitin binding (Figure 8). The Ala to Gly mutation has been shown previously to cause a significant decrease in the level of ubiquitin binding.[39] The diubiquitin cleavage reactions were performed at 37 °C for 15 min using 1 μM enzyme, 20 μM Lys63-linked diubiquitin as the substrate, and 5 μM STAM binding partner (SH3, UIM^{A176G}-SH3, or UIM-SH3). SDS–PAGE analysis revealed that only in the presence of the wild-type enzyme and UIM-SH3 is diubiquitin completely hydrolyzed to ubiquitin; hence, an intact SBM–SH3 interaction and a functional UIM are necessary for AMSH activation (Figure 8).

Furthermore, we wanted to understand this activation phenomenon in more detail, in terms of k_{cat} and K_M. To this end, we conducted another kinetic assay in which the catalytic domain of AMSH was preincubated in the presence of a 20-fold excess of UIM-SH3 to ensure that equilibrium favors the formation of the AMSH–UIM-SH3 complex. We saw 6-fold activation in AMSH in the presence of UIM-SH3, contributed by a somewhat greater change in k_{cat} than in K_M (Table 3 of the Supporting Information). The k_{cat} effect is not entirely surprising because the UIM interacts with the proximal ubiquitin, and as we have shown in our mutational and kinetic analysis, the proximal site plays a significant role in properly aligning the isopeptide bond within the active site of AMSH, as determined by the significant decrease in k_{cat} upon mutation of the residues involved in binding.

DISCUSSION

AMSH is one of the two DUBs recruited to the human ESCRT machinery to regulate the endosomal–lysosomal degradation pathway.[9] It is a JAMM family DUB,[1] having exquisite specificity for recognizing and cleaving Lys63-linked polyubiquitin chains,[9] which serve as signals for ESCRT-mediated sorting to the lysosome. However, a clearly defined role for AMSH has yet to be elucidated. A homologous protein, AMSH-LP, with a 54% identical and 73% similar sequence,[9,23] of the same family of DUBs has the same ubiquitin linkage specificity and, from our previous work, is structurally almost identical in its catalytic domain to AMSH.[25] Even though the catalytic domains of AMSH and AMSH-LP are structurally nearly identical, we showed that AMSH is significantly less stable than AMSH-LP, and consequently perhaps conformationally more plastic, and the enzymes differ in their ubiquitin recognition.[25] As a result of these differences, we sought to further investigate AMSH kinetically and biophysically to advance our understanding of the enzyme with a view of understanding its role in the context of the ESCRT machinery.

Our kinetic analysis using site-directed mutagenesis of conserved residues in the proximal ubiquitin binding site has shown that the specificity of AMSH for Lys63-linked polyubiquitin chains arises from its recognition of the proximal ubiquitin, similar to the case of AMSH-LP.[22] Lys63-linked chain specificity for AMSH plays a significant role in understanding its function as an ESCRT-DUB. Because Lys63-linked polyubiquitin chains are targeting signals for ESCRT-mediated degradation, the specific DUB activity of AMSH may play a central role in the persistent functionality of the ESCRT machinery. Mutation of the proximal ubiquitin binding residues causes a drastic reduction in k_{cat}, suggesting that recognition of the Gln62-Lys63-Glu64 tripeptide sequence motif within the proximal ubiquitin plays a significant role in the ability of AMSH to cleave Lys63-linked polyubiquitin chains. Recognition of the Lys63 isopeptide bond and its two flanking residues in the proximal ubiquitin would mean that AMSH could only efficiently hydrolyze bonds between successive ubiquitins in a polymeric chain, and not the last ubiquitin directly attached to a protein receptor (the cargo). This impediment toward completely deubiquitinating a ubiquitinated receptor could have multiple functional implications (discussed below). At the outset, it calls into question the functional role of AMSH when it is recruited to ESCRT-III, where complete deconjugation of a ubiquitinated cargo is the absolute desire, because ubiquitin will otherwise end up in intraluminal vesicles (ILVs) attached to the cargo and will be subsequently degraded in the lysosome. It seems unlikely that AMSH can have a significant catalytic role with respect to hydrolyzing the last ubiquitin attached to the cargo, yet AMSH binds to ESCRT-III component CHMP3 with relatively high affinity.

Our data from mutational analysis of the distal ubiquitin binding residues offer some interesting insights. Of the three residues different between AMSH and AMSH-LP, two of them, Glu316 and Thr313, contribute significantly to catalysis, playing roles different from those of the corresponding residues in AMSH-LP. The AMSH residue Glu316 contributes to stabilization of the transition state as indicated by the largely k_{cat} effect when it is mutated to Ala, in contrast to a mostly K_M effect observed when the corresponding Val residue in AMSH-LP is mutated to Ala.[22] Thr313 in our AMSH−diubiquitin model is seen making hydrogen bonding contact with the backbone NH group of Leu73 of the distal ubiquitin using its side chain hydroxyl group, and its methyl group is engaged in van der Waals contact with the aliphatic side groups of Leu73 of ubiquitin. Its substitution with Ile as seen in children with MIC-CAP syndrome is expected to preserve the van der Waals contact but weaken the hydrogen bond. Our data show that the substitution of the Thr with an Ile has a minimal effect on protein folding and stability but results in a significantly reduced catalytic efficiency. The Thr residue is AMSH is replaced with a Met in AMSH-LP, which could contribute only van der Waals interaction with the substrate. Thus, it appears that the hydrogen bonding interaction of Thr in AMSH has a unique role whose loss leads to a dramatic effect resulting in a loss of function substantial enough to cause the disease. Overall, these results indicate that subtle differences between very similar enzymes can have profound functional effects.

We found the minimal domain of STAM that is required to stimulate the activity of AMSH. Previous work has shown that STAM has a role in AMSH activation toward Lys63 polymeric chains;[13,14] however, these studies were not able to fully elucidate the mechanism of activation. Our study begins to divulge the mechanism underlying activation. This work suggests a simple model invoking simultaneous recognition of two ubiquitin groups in a polyubiquitin chain by AMSH, and the UIM of STAM could explain the catalytic activation of the DUB. The UIM of STAM, separated from the SH3 domain by a short linker, could act as an adaptor for AMSH by interacting with the proximal ubiquitin, while AMSH engages the distal one. Such an arrangement would create a more extensive binding interface for diubiquitin in the AMSH−STAM complex than in the enzyme alone, causing catalytic activation. It appears that such activation is necessary because, as our ITC data show, AMSH has no preference for binding to Lys63-linked diubiquitin and, therefore, by extrapolation for Lys63-linked polyubiquitin chains over the ubiquitin product.

Prior to activation, AMSH is in a more latent state, but when it is recruited to STAM, its full activity is unveiled. AMSH is known to have diverse subcellular localization profiles. Perhaps the free form of the enzyme needs to be in a less active state so as not to hydrolyze the Lys63 chains that are present in the cytosol other than endosomes. Once it is recruited to the endosomes, its true activity comes alive, as seen by the 6-fold enhancement in activity upon binding to the STAM-derived UIM-SH3 construct. A significant implication of this mechanism of activation is that the activation will not occur when AMSH is trying to cleave the last ubiquitin attached to the cargo. While efficiently cleaving between two ubiquitin groups in a Lys63-linked polyubiquitin chain, AMSH might show a severe impediment in hydrolyzing the last ubiquitin attached directly to a cargo, because of two factors: (1) its high specificity for the Lys63-linked chain between two ubiquitins, which in turn would make it a poor enzyme when ubiquitin is attached to a nonubiquitin protein, the cargo, and (2) the lack of an activation effect when cleaving ubiquitin attached to a nonubiquitin moiety.

Finally, bringing all our data together, we can envision a mechanism for the recruitment and activation of AMSH that will ultimately define a function for the enzyme. ESCRT-0 has the defined function of ubiquitinated cargo clustering, capable of harboring up to eight ubiquitin moieties at a time,[43,44] which now, with the addition of the SH3 domain, could be 10 ubiquitins. Our ITC data show that the SH3 domain can actually bind ubiquitin tighter than the UIM. Subsequently, AMSH is recruited to STAM. The AMSH−SH3 binding affinity is stronger than the SH3−ubiquitin binding affinity, making it possible for AMSH to effectively displace ubiquitin from the SH3 domain (the binding interfaces on the SH3 domains for the two proteins show substantial overlap),[42] leading to its recruitment to ESCRT-0. With the UIM from STAM acting as an adaptor to the enzyme, facilitating enzyme activity enhancement, AMSH begins to efficiently disassemble the polyubiquitin chain attached to the cargo. Deubiquitination of the chain will continue until the last ubiquitin directly attached to the cargo. Thus, the recruitment of AMSH at ESCRT-0 will lead to substantial chain trimming but not complete deconjugation of ubiquitin from the cargo. As discussed in the next paragraph, this would promote the passage of cargo from ESCRT-0 to ESCRT-I and subsequent complexes.

Our proposed mechanism defines AMSH as the DUB that facilitates the passage of cargo from ESCRT-0 to the next complex. This idea is supported by previous data that show that avidly bound ubiquitin chains have a binding affinity of ∼20 μM[45] whereas the ESCRT-I subunit, UBAP1, binds ubiquitin anywhere from 70 to 140 μM.[46] The binding affinity of ubiquitin at ESCRT-0 needs to be reduced at least 5−10-fold for cargo destined for lysosomal degradation to be transferred to ESCRT-I. With the transition from Lys63-linked tetraubiquitin to diubiquitin, ESCRT-0 has an ∼6-fold reduced affinity and a remarkable 46-fold reduction in its affinity for ubiquitin.[45] Therefore, we presume that because of the specificity and activation of AMSH, this would be the enzyme that would be most suited to the promotion of the passage of cargo, all in support of an idea proposed previously.[47] By occupying the binding site on ESCRT-0, AMSH will serve to keep USP8 off the initial ESCRT complex. The recruitment of USP8 at ESCRT-0 would be detrimental to the passage of the cargo to the lysosome because USP8 has no hindrance in complete deconjugation. We suggest that the role of USP8 is specifically at the ESCRT-III level where complete deconjugation is desired.

The main conclusion from our study implies that loss of function mutations in AMSH would lead to the impairment of ubiquitin-dependent sorting to the lysosome via the ESCRT pathway. One possible outcome of this impairment is the accumulation of ubiquitinated proteins (cargo). Indeed, patient cell lines with the AMSH mutation show accumulation of aggregated ubiquitin−protein conjugates.[16] Furthermore, impairment of ESCRT-mediated endocytic sorting to the lysosome is expected to cause hyperactivation of signaling across the lipid bilayer. As suggested by the authors of the paper describing MIC-CAP mutations, the capillary abnormalities associated with the syndrome could be a consequence of hyperactive RAS-MAPK signaling induced in humans by impaired AMSH function.[16] However, because AMSH has

several roles within the cell, it would be difficult to assign loss of function in one pathway as the exclusive molecular basis of MIC-CAP syndrome.

In summary, using a combination of biochemical and biophysical studies, guided by a structural model, we were able to learn about many important aspects of AMSH. (1) The T313I mutation underlying MIC-CAP syndrome leads to a significant loss of catalytic activity because of the loss of a hydrogen bonding interaction with ubiquitin. (2) The recognition of proximal ubiquitin contributes significantly to catalysis. (3) The activation of AMSH is allowed by facile, simultaneous binding to two ubiquitin groups in a polyubiquitin substrate, one by the catalytic domain of the DUB (binding to the distal ubiquitin) and the other (the proximal ubiquitin) by the UIM from STAM. (4) Taken together, these three points strongly indicate that AMSH will suffer a severe loss of catalytic efficiency when cleaving the last ubiquitin attached to cargo compared to a Lys63-linked polyubiquitin chain substrate. These studies provide biochemical and biophysical evidence that supports a hypothesis that postulates that AMSH is recruited to the initial ESCRT complex to facilitate the transfer of cargo from one ESCRT member to the next, but not to completely deubiquitinate it.[9] Its recruitment therefore would facilitate cargo shuttling rather than release from ESCRT and subsequent recycling back to the plasma membrane.

■ ASSOCIATED CONTENT

Supporting Information

Cartoon representations of the MIC-CAP-associated mutation and a model of AMSH bound to Lys63-linked diubiquitin, a table of the fraction unfolded GdHCl melting curve and stability data, ITC thermograms of binding of AMSH to SH3, UIM-SH3, and AMSHK238T with UIM-SH3, sedimentation velocity and equilibrium data, AMSH and STAM domain diagram, and table of AMSH activation data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: cdas@purdue.edu. Phone: (765) 494-5478. Fax: (765) 494-0239.

Author Contributions

C.W.D. and L.N.P. contributed equally to this work.

Funding

This work was funded by National Institutes of Health Grant 1R01RR026273 to C.D. C.W.D. thanks the American Heart Association for Predoctoral Fellowship 12PRE12060249.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Dr. Myung-il Kim for his help in optimization of the Lys63-linked diubiquitin chain synthesis.

■ ABBREVIATIONS

AMSH, associated molecule with a Src homology 3 domain of STAM; AMSH-LP, AMSH-like protein; AUC, analytical ultracentrifugation; CD, circular dichroism; DUBs, deubiquitinating enzymes; ESCRT, endosomal sorting complexes required for transport; ITC, isothermal titration calorimetry; STAM, signal-transducing adaptor molecule; MIC-CAP, microcephaly capillary malformation; UBPY/USP8, ubiquitin specific protease 8.

■ REFERENCES

(1) Maytal-Kivity, V., Reis, N., Hofmann, K., and Glickman, M. H. (2002) MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem. 3*, 28.

(2) Komander, D. (2010) Mechanism, specificity and structure of the deubiquitinases. *Subcell. Biochem. 54*, 69−87.

(3) Komander, D., Clague, M. J., and Urbe, S. (2009) Breaking the chains: Structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol. 10*, 550−563.

(4) Wilkinson, K. D. (2009) DUBs at a glance. *J. Cell Sci. 122*, 2325−2329.

(5) Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell 123*, 773−786.

(6) Zhu, P., Zhou, W., Wang, J., Puc, J., Ohgi, K. A., Erdjument-Bromage, H., Tempst, P., Glass, C. K., and Rosenfeld, M. G. (2007) A histone H2A deubiquitinase complex coordinating histone acetylation and H1 dissociation in transcriptional regulation. *Mol. Cell 27*, 609−621.

(7) Sobhian, B., Shao, G., Lilli, D. R., Culhane, A. C., Moreau, L. A., Xia, B., Livingston, D. M., and Greenberg, R. A. (2007) RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science 316*, 1198−1202.

(8) Avvakumov, G. V., Walker, J. R., Xue, S., Finerty, P. J., Jr., Mackenzie, F., Newman, E. M., and Dhe-Paganon, S. (2006) Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8). *J. Biol. Chem. 281*, 38061−38070.

(9) Clague, M. J., and Urbe, S. (2006) Endocytosis: The DUB version. *Trends Cell Biol. 16*, 551−559.

(10) Wollert, T., Wunder, C., Lippincott-Schwartz, J., and Hurley, J. H. (2009) Membrane scission by the ESCRT-III complex. *Nature 458*, 172−177.

(11) Saksena, S., Sun, J., Chu, T., and Emr, S. D. (2007) ESCRTing proteins in the endocytic pathway. *Trends Biochem. Sci. 32*, 561−573.

(12) Roxrud, I., Stenmark, H., and Malerod, L. (2010) ESCRT & Co. *Biol. Cell 102*, 293−318.

(13) McCullough, J., Row, P. E., Lorenzo, O., Doherty, M., Beynon, R., Clague, M. J., and Urbe, S. (2006) Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery. *Curr. Biol. 16*, 160−165.

(14) Kim, M. S., Kim, J. A., Song, H. K., and Jeon, H. (2006) STAM-AMSH interaction facilitates the deubiquitination activity in the C-terminal AMSH. *Biochem. Biophys. Res. Commun. 351*, 612−618.

(15) Solomons, J., Sabin, C., Poudevigne, E., Usami, Y., Hulsik, D. L., Macheboeuf, P., Hartlieb, B., Gottlinger, H., and Weissenhorn, W. (2011) Structural basis for ESCRT-III CHMP3 recruitment of AMSH. *Structure 19*, 1149−1159.

(16) McDonell, L. M., Mirzaa, G. M., Alcantara, D., Schwartzentruber, J., Carter, M. T., Lee, L. J., Clericuzio, C. L., Graham, J. M., Jr., Morris-Rosendahl, D. J., Polster, T., Acsadi, G., Townshend, S., Williams, S., Halbert, A., Isidor, B., David, A., Smyser, C. D., Paciorkowski, A. R., Willing, M., Woulfe, J., Das, S., Beaulieu, C. L., Marcadier, J., Geraghty, M. T., Frey, B. J., Majewski, J., Bulman, D. E., Dobyns, W. B., O'Driscoll, M., and Boycott, K. M. (2013) Mutations in STAMBP, encoding a deubiquitinating enzyme, cause microcephaly-capillary malformation syndrome. *Nat. Genet. 45*, 556−562.

(17) Carter, M. T., and Boycott, K. M. (2011) Microcephaly-capillary malformation syndrome: A story of rapid emergence of a new recognizable entity. *Am. J. Med. Genet., Part A 155A*, 2078−2079.

(18) Mirzaa, G. M., Paciorkowski, A. R., Smyser, C. D., Willing, M. C., Lind, A. C., and Dobyns, W. B. (2011) The microcephaly-capillary malformation syndrome. *Am. J. Med. Genet., Part A 155A*, 2080−2087.

(19) Isidor, B., Barbarot, S., Beneteau, C., Le Caignec, C., and David, A. (2011) Multiple capillary skin malformations, epilepsy, microcephaly, mental retardation, hypoplasia of the distal phalanges: Report of a new case and further delineation of a new syndrome. *Am. J. Med. Genet., Part A 155A*, 1458−1460.
(20) Suzuki, S., Tamai, K., Watanabe, M., Kyuuma, M., Ono, M., Sugamura, K., and Tanaka, N. (2011) AMSH is required to degrade ubiquitinated proteins in the central nervous system. *Biochem. Biophys. Res. Commun. 408*, 582−588.
(21) McCullough, J., Clague, M. J., and Urbe, S. (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *J. Cell Biol. 166*, 487−492.
(22) Sato, Y., Yoshikawa, A., Yamagata, A., Mimura, H., Yamashita, M., Ookata, K., Nureki, O., Iwai, K., Komada, M., and Fukai, S. (2008) Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature 455*, 358−362.
(23) Kikuchi, K., Ishii, N., Asao, H., and Sugamura, K. (2003) Identification of AMSH-LP containing a Jab1/MPN domain metalloenzyme motif. *Biochem. Biophys. Res. Commun. 306*, 637−643.
(24) Agromayor, M., and Martin-Serrano, J. (2006) Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo. *J. Biol. Chem. 281*, 23083−23091.
(25) Davies, C. W., Paul, L. N., Kim, M. I., and Das, C. (2011) Structural and thermodynamic comparison of the catalytic domain of AMSH and AMSH-LP: Nearly identical fold but different stability. *J. Mol. Biol. 413*, 416−429.
(26) Nakamura, M., Tanaka, N., Kitamura, N., and Komada, M. (2006) Clathrin anchors deubiquitinating enzymes, AMSH and AMSH-like protein, on early endosomes. *Genes Cells 11*, 593−606.
(27) Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, pp 90−125, Royal Society of Chemistry, Cambridge, U.K.
(28) Brown, P. H., and Schuck, P. (2006) Macromolecular size-and-shape distributions by sedimentation velocity analytical ultracentrifugation. *Biophys. J. 90*, 4651−4661.
(29) Schuck, P. (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys. J. 78*, 1606−1619.
(30) Balbo, A., Minor, K. H., Velikovsky, C. A., Mariuzza, R. A., Peterson, C. B., and Schuck, P. (2005) Studying multiprotein complexes by multisignal sedimentation velocity analytical ultracentrifugation. *Proc. Natl. Acad. Sci. U.S.A. 102*, 81−86.
(31) Dam, J., and Schuck, P. (2005) Sedimentation velocity analysis of heterogeneous protein-protein interactions: Sedimentation coefficient distributions c(s) and asymptotic boundary profiles from Gilbert-Jenkins theory. *Biophys. J. 89*, 651−666.
(32) Dam, J., Velikovsky, C. A., Mariuzza, R. A., Urbanke, C., and Schuck, P. (2005) Sedimentation velocity analysis of heterogeneous protein-protein interactions: Lamm equation modeling and sedimentation coefficient distributions c(s). *Biophys. J. 89*, 619−634.
(33) Houtman, J. C., Brown, P. H., Bowden, B., Yamaguchi, H., Appella, E., Samelson, L. E., and Schuck, P. (2007) Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: Application to adaptor protein complexes in cell signaling. *Protein Sci. 16*, 30−42.
(34) Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R. A., Rouault, T. A., and Schuck, P. (2004) Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. *Anal. Biochem. 326*, 234−256.
(35) Schuck, P. (2003) On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Anal. Biochem. 320*, 104−124.
(36) Keller, S., Vargas, C., Zhao, H., Piszczek, G., Brautigam, C. A., and Schuck, P. (2012) High-precision isothermal titration calorimetry with automated peak-shape analysis. *Anal. Chem. 84*, 5066−5073.
(37) Pace, C. N. (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol. 131*, 266−280.
(38) Arnst, J. L., Davies, C. W., Raja, S. M., Das, C., and Natarajan, A. (2013) High-throughput compatible fluorescence resonance energy transfer-based assay to identify small molecule inhibitors of AMSH deubiquitinase activity. *Anal. Biochem. 440*, 71−77.
(39) Fisher, R. D., Wang, B., Alam, S. L., Higginson, D. S., Robinson, H., Sundquist, W. I., and Hill, C. P. (2003) Structure and ubiquitin binding of the ubiquitin-interacting motif. *J. Biol. Chem. 278*, 28976−28984.
(40) He, Y., Hicke, L., and Radhakrishnan, I. (2007) Structural basis for ubiquitin recognition by SH3 domains. *J. Mol. Biol. 373*, 190−196.
(41) Stamenova, S. D., French, M. E., He, Y., Francis, S. A., Kramer, Z. B., and Hicke, L. (2007) Ubiquitin binds to and regulates a subset of SH3 domains. *Mol. Cell 25*, 273−284.
(42) Lange, A., Ismail, M. B., Riviere, G., Hologne, M., Lacabanne, D., Guilliere, F., Lancelin, J. M., Krimm, I., and Walker, O. (2012) Competitive binding of UBPY and ubiquitin to the STAM2 SH3 domain revealed by NMR. *FEBS Lett. 586*, 3379−3384.
(43) Mayers, J. R., Fyfe, I., Schuh, A. L., Chapman, E. R., Edwardson, J. M., and Audhya, A. (2011) ESCRT-0 assembles as a heterotetrameric complex on membranes and binds multiple ubiquitinylated cargoes simultaneously. *J. Biol. Chem. 286*, 9636−9645.
(44) Wollert, T., and Hurley, J. H. (2010) Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature 464*, 864−869.
(45) Ren, X., and Hurley, J. H. (2010) VHS domains of ESCRT-0 cooperate in high-avidity binding to polyubiquitinated cargo. *EMBO J. 29*, 1045−1054.
(46) Agromayor, M., Soler, N., Caballe, A., Kueck, T., Freund, S. M., Allen, M. D., Bycroft, M., Perisic, O., Ye, Y., McDonald, B., Scheel, H., Hofmann, K., Neil, S. J., Martin-Serrano, J., and Williams, R. L. (2012) The UBAP1 subunit of ESCRT-I interacts with ubiquitin via a SOUBA domain. *Structure 20*, 414−428.
(47) Hurley, J. H. (2011) Nipped in the bud: How the AMSH MIT domain helps deubiquitinate lysosome-bound cargo. *Structure 19*, 1033−1035.