Published in final edited form as:

J Mol Biol. 2011 October 21; 413(2): 416–429. doi:10.1016/j.jmb.2011.08.029.

Structural and Thermodynamic Comparison of the Catalytic Domain of AMSH and AMSH-LP: Nearly Identical Fold but Different Stability

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Abstract

AMSH plays a critical role in the endosomal sorting complexes required for transport (ESCRT) machinery, which facilitates the down-regulation and degradation of cell-surface receptors. It displays a high level of specificity towards cleavage of Lys63-linked polyubiquitin chain, the structural basis of which has been understood recently through the crystal structure of a highly related, but ESCRT-independent, protein AMSH-LP (AMSH-like protein). We have determined X-ray structure of two constructs representing the catalytic domain of AMSH: AMSH244, the JAMM-domain-containing polypeptide segment from residues 244 to 424 and AMSH219^{E280A}, an active-site mutant, Glu280 to Ala, of the segment from 219 to 424. In addition to confirming the expected zinc-coordination in the protein, the structures reveal that the catalytic domains of AMSH and AMSH-LP are nearly identical; however, guanidine hydrochloride-induced unfolding studies show that the catalytic domain of AMSH is thermodynamically less stable than that of AMSH-LP, indicating that the former is perhaps structurally more plastic. Much to our surprise, in the AMSH219^{E280A} structure, the catalytic zinc was still held in place, by the compensatory effect of an aspartate from a nearby loop moving into a position where it could coordinate with the zinc, once again suggesting the plasticity of AMSH. Additionally, a model of AMSH244 bound to Lys63-linked diubiquitin reveals a significantly different type of interface for the distal ubiquitin than seen in AMSH-LP. Altogether, we believe our data provides important insight into the structural difference between the two proteins that may translate into the difference in their biological function.

Introduction

AMSH, associated molecule with the SH3 domain (Src homology 3 domain) of STAM (signal transducing adaptor molecule), is a member of the JAMM (JAB1/MPN/MOV34) family of deubiquitinating enzymes (DUBs),¹ which catalyze the hydrolysis of isopeptide or

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ACCESSION NUMBERS:

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3RZU (AMSH244) and 3RZV (AMSH219^{E280A}).

peptide bonds between ubiquitin and target proteins, or between monomers in polymeric chains of ubiquitin. The JAMM family, one of the five classes of DUBs, are metalloproteases, the others being cysteine proteases.^{2; 3; 4; 5} These metalloproteases, although having very different sequences compared to the quintessential metalloprotease thermolysin, are generally thought to share similar features in their active site, and hence mechanism, with thermolysin. They have a Zn²⁺ ion in their active site coordinated by two histidines, an aspartate or a glutamate, and a water molecule, which in addition to the metal ion is held in its place by hydrogen bonding with a different glutamate residue. This water plays two critical roles: (1) it acts as the nucleophile during the hydrolysis of the peptide bond, and (2) in addition to the other coordinating side chains, it stabilizes the bound Zn²⁺ ion, which is responsible for polarizing the carbonyl group of the scissile peptide bond. However, of the fourteen JAMM proteins in the human genome, only seven contain a complete set of conserved residues needed for Zn²⁺ coordination,² six of which, AMSH, AMSH-LP (AMSH-like protein), BRCC36, RPN11 (POH1), MYSM1 and CSN5, have been shown to exhibit isopeptidase activity towards ubiquitin or ubiquitin-like proteins.^{2; 6; 7}

AMSH is one of the two DUBs, the other being the cysteine-protease DUB UBPY (also known as ubiquitin specific protease 8, USP8),⁸ known to be involved in receptor down-regulation and turnover mediated by the ESCRT (endosomal sorting complexes required for transport) machinery.⁹ The ESCRT machinery, composed of four distinct macromolecular assemblies, ESCRT-0, I, II and III, drives the internalization of ubiquitinated cell-surface receptors,¹⁰ which are shuttled from one ESCRT member to the next (for example, from ESCRT-0 to ESCRT-I, from ESCRT-I to II and so on) until they are finally degraded by the lysosome or recycled back to the membrane.¹¹ The initial (ESCRT-0) and the final (ESCRT-III) complexes are the two recognition points for both AMSH and UBPY. ESCRT-0 recruits the DUBs through the SH3 domain of STAM binding to the PX(V/I)(D/N)RXXKP (X is any amino acid) motif present on both DUBs, whereas ESCRT-III recruitment is facilitated by the MIT (microtubule interacting and transport) domain of the DUBs binding to CHMPs (chromatin modifying proteins) of ESCRT-III.⁹ The ability of DUBs to deubiquitinate endocytosed receptors at various points in the process, either before or after sequestration of the receptors into multivesicular bodies (MVBs) may have a role in altering receptor trafficking, regulating their turnover rate.¹²

The exact function of AMSH within the ESCRT machinery is still poorly understood. Studies have shown that AMSH has a role in the regulation of several receptors including: epidermal growth factor receptor (EGFR), calcium-sensing receptor, δ -opioid receptor, protease-activated receptor 2, and the chemokine receptor CXCR4.^{13; 14; 15; 16; 17; 18; 19; 20} Studies have also shown that AMSH serves to regulate endosomal trafficking by counteracting the ubiquitination of ESCRT-0 proteins themselves.¹² Not only does AMSH have a role in endocytosis of receptors, AMSH also plays a role in mitosis and cytokinesis.^{21; 22; 23; 24} During mitosis, AMSH deubiquitinates v-SNARE and VAMP8 in an ESCRT-dependent manner, while also being present in the early phases of cytokinesis, both implicating a potential role of AMSH as a regulator of proliferation and survival.^{21; 22; 23}

Even though AMSH has no clearly defined role to date, it is well characterized that AMSH has specificity towards Lys63-linked polymeric chains of ubiquitin, which have previously been shown to be the minimal requirement for directing receptors towards lysosomal degradation (in some cases, monoubiquitination instead of polyubiquitination is used as a signal for lysosomal degradation).¹³ The structural basis for Lys63-linked polyubiquitin chain cleavage has been elucidated with the crystal structure determination of the DUB (JAMM) domain of AMSH-LP.²⁵ AMSH and AMSH-LP share 54% identity and 75% sequence similarity in their JAMM domain.^{9; 25} It is known that both proteins act as regulators of free ubiquitin in the cell, bind clathrin, contain a putative NLS (nuclear

localization signal) and an MIT domain.^{9; 26; 27} Although perceived as functionally redundant, AMSH-LP lacks several key features when compared to AMSH. AMSH contains a SH3-binding motif, which facilitates interaction with STAM of ESCRT-0, while a functional SH3 binding motif is lost in AMSH-LP from primates, but present in chicken, cow, dog, frog, mouse, and snake.^{9; 26; 27} AMSH's MIT domain interacts with specific ESCRT-III proteins, CHMPs, while it was shown that none of the CHMP proteins interacted with AMSH-LP, suggesting a highly specific interaction with AMSH.²⁸ In the addition to these differences, it is possible that the biological difference between the two proteins may lie also in their catalytic domain, presumed to be very similar based on the similarity of primary structure.

The present study aims at providing a structural and thermodynamic comparison between the catalytic domains of AMSH and AMSH-LP. We have determined X-ray crystal structures of two constructs representing the catalytic domain of AMSH: AMSH244–424, the JAMM-domain-containing polypeptide segment from residues 244 to 424 (hereafter referred to simply as AMSH244) and AMSH219^{E280A}, an active-site mutant, Glu280 to Ala, of the segment 219–424, which, in addition to the JAMM domain, also features the SH3-domain-binding motif at its N-terminus. We see that while the overall folds of the two proteins are nearly identical, as expected, the catalytic domain of AMSH is thermodynamically less stable than of AMSH-LP under guanidine hydrochloride (GdHCl)-induced unfolding, based on which we propose that the catalytic domain of AMSH is structurally more plastic than the latter, a property that may endow specific advantage to AMSH enabling it to adapt to a variety of working environments within the context of ECSRT machinery.

Results

Structure of the catalytic domain of AMSH

AMSH244 crystallized in the *C*2 space group, with seven monomers in the asymmetric unit (Supplementary Fig. 1). The structure was solved by molecular replacement (MR) with the catalytic domain of AMSH-LP as the search model.²⁵ The MR search identified a model with seven subunits in the asymmetric unit. Rigid-body followed by restrained refinement of this model yielded a crystallographic R factor (R_{crys}) and free R factor (R_{free}) of 25.4 and 34.8 %, respectively. Electron density map at this stage was interpretable and showed clear density for the two Zn^{2+} ions where they should be located, indicating that the MR search was successful. Refinement using Phenix²⁹ after rounds of model building yielded a final R_{crys} and R_{free} of 19.3 and 22.9 %, respectively (Table 1). The structure of the polypeptide in different subunits is nearly identical except for the N-terminal residues, Asn244 to Asp252, which were ordered to different extent in different subunits.

The observation of seven monomers in the asymmetric unit prompted us to investigate the oligomeric state of the catalytic domain of AMSH in solution. AMSH244 was subjected to analytical ultracentrifugation (AUC) experiments, along with the catalytic domain of AMSH-LP for comparison. The AUC data indicate that in solution, the catalytic domains of both AMSH and AMSH-LP exist as predominantly globular, monomeric proteins with a sedimentation coefficient (s_{20}) value of 2.1 and 2.03, respectively (Fig. 1). However, this data does not rule out the possibility that the full-length protein can exist as a multimer in solution, through self-association of the part missing in our construct.

Like the DUB domain of AMSH-LP, the structure of AMSH244 consists of the JAMM core with two characteristic insertions, insertion 1 (Ins-1, residues 302–327) and insertion 2 (Ins-2, residues 381–403). The JAMM core comprises a mixed β -sheet in the shape of a partially unfurled β -barrel sandwiched by two α -helices, one on top (α 1) and one on the

bottom ($\alpha 3$) (Fig. 2a), a feature found in other JAMM domain proteins, such as AfJAMM and Prp8.^{30; 31; 32; 33} As seen in AMSH-LP, the catalytic site is lined mostly by residues from the JAMM core, from the loop between $\alpha 1$ and $\beta 2$, $\alpha 3$, $\beta 6$ and the loop following it (Fig. 2a). Residues from the β -hairpin turn segment in Ins-1 also contribute to the catalytic site. The catalytic zinc is coordinated by His335, His337 (located on $\beta 6$ and immediately following it), Asp348 (located on $\alpha 3$) and a water molecule that is hydrogen bonded to Glu280 (located on the loop following $\alpha 1$) (Fig. 2b). In addition to these zinc-coordinating residues, the oxyanion-stabilizing side chain from Ser345 (on $\alpha 3$) completes the set of catalytic residues required for peptide bond hydrolysis by a thermolysin-type, mechanistically speaking, zinc metalloprotease.^{34; 35} We confirmed the presence of a second zinc ion, 14Å away from the active site, by the presence of strong electron density in the 2Fo-Fc map in the area coordinated by the residues His350 from the JAMM core, and His396, His398, and Cys390 from Ins-2 (Fig. 2c), the same set of residues that coordinate the second zinc ion in AMSH-LP.²⁵

As seen in the structure of the DUB domain of AMSH-LP, the active-site of AMSH is in a closed configuration, covered by two side chains, that of an aspartate (Asp309) in Ins-1 and a phenylalanine (Phe395) in Ins-2, with their van der Waals surface touching each other (Fig. 2a). The side chains of these two residues have to move away from each other to let the scissile isopeptide bond on the substrate access the active site, suggesting that there is considerable flexibility either in these side chains or in the polypeptide backbone bearing them. Interestingly, in the structure of AMSH-LP DUB domain bound to Lys63-linked ubiquitin dimer, the corresponding residue pair is still seen in a similar position as the substrate-free form,²⁵ suggesting that these residues close the active site during catalysis as well, perhaps to help position the scissile peptide bond or stabilize the transition state or both. Both these residues are absolutely conserved in all AMSH sequences analyzed so far (data not shown) and in AMSH-LP as well suggesting a functional role.

In our efforts to structurally characterize AMSH's recruitment to the ESCRT-0 complex, we generated an N-terminally longer construct of the catalytic domain that has, in addition to AMSH's DUB domain, the peptide sequence which AMSH uses to bind to STAM's SH3 domain.⁹ An active-site mutant of this construct, AMSH219^{E280A}, in which Glu280 is mutated to alanine, crystallized in the tetragonal space group, $P4_32_12$, with a single molecule in the asymmetric unit, consistent with the monomeric state of AMSH244 found in solution. The structure of this mutant was solved by MR using the structure of AMSH244 as the search probe (Table 1). The overall fold of the two structures is very similar with a root mean square deviation (rmsd) between C α atoms of 0.84Å. The N-terminal SH3-binding peptide segment was not visualized in the structure perhaps because of disorder in the crystal, suggesting that this peptide segment is flexible and is easily accessible for binding to the SH3 domain of STAM. The catalytic activities of AMSH244 and AMSH219 are similar (Supplementary Fig. 2) suggesting that the SH3 domain-binding peptide segment does not fold onto the catalytic domain, at least not in way to influence diubiquitin binding. We speculate that the flexibility of this segment may allow it to serve as a part of a linker that connects the JAMM domain to the N-terminal portion of the protein. The structure of the mutant does confirm the presence of the E280A mutation; however, clear density in the vicinity of the active-site metal-coordinating residues was still observed, which was interpreted as the density corresponding to a Zn²⁺ ion (Fig. 3). Thus, despite having lost the catalytic glutamate, and consequently the water molecule bound to it, the active-site Zn²⁺ is still present. Further inspection reveals that the side chain of a nearby aspartate, Asp309, has swung by nearly 120 degrees from its position in the wild-type structure positioning itself such that one of its O δ atoms can coordinate to the zinc, thereby restoring the tetrahedral coordination around it (Fig. 3). As expected, the E280A mutant is still catalytically inactive despite having the zinc in place (Supplementary Fig. 2).

A Potential disulfide bond in the catalytic domain of AMSH

The overall structure of AMSH244 and AMSH219^{E280A} are nearly identical, however, further inspection during model building revealed the presence of a potential disulfide bond 7.4Å away from the catalytic zinc in the latter. Rounds of refinement showed positive density around Cys282 within which we modeled in three alternate conformations, one of which placed this cysteine side chain within disulfide-bonding distance from Cys311 (Sγ-Sγ distance of 2.0Å) (Supplementary Fig. 3). The presence of dithiothreitol (DTT) throughout purification and in the crystallization buffer presents a possible reason for the multiple conformations observed in the crystals, preventing the cysteine pair from achieving 100% occupancy in its oxidized form (Supplementary Fig. 3). It is possible that this disulfide may exist in AMSH244 as well, but its observation is precluded because of DTT, whose reducing effect is perhaps more in this construct than in AMSH219^{E280A}. Interestingly, sequence alignment analysis shows that these cysteines are conserved between AMSH and AMSH-LP;²⁵ however, we do not see the equivalent cysteine pair within disulfide-bonding distance in AMSH-LP (Sγ-Sγ distance is 4.2Å in this case). Cys282 is next to the active site, and in fact it makes a van der Waals contact with Leu73 of the distal ubiquitin in the structure of AMSH-LP DUB domain bound to Lys63-linked ubiquitin dimer.²⁵ In the disulfide orientation, however, as seen in the model of AMSH244 bound to Lys63-linked diubiquitin (please see below), the contact is lost. To probe if the oxidation state of Cys282 is important for the catalytic activity of the enzyme, we created a mutant that would lack the disulfide bond (the Cys282 to Ala mutant) and prepared the mutant and the wild type proteins avoiding DTT all throughout the purification procedure. The catalytic activity of the Cys282Ala mutant is close to the activity seen with the wild-type protein (the mutant retains ~80 % of the activity seen with the wild type) (Supplementary Fig. 2).

AMSH diubiquitin recognition

The overall three-dimensional fold of the catalytic domain of AMSH is very similar to the previously determined DUB domain of AMSH-LP (PDB code: 2ZNR, reference 25) with a rmsd of 1.49Å of Cα atoms as determined by the superposition program SuperPose.³⁶ The architecture of both the active site and the second zinc-binding site of both the proteins are nearly identical (Fig. 4). To gain insight into how AMSH would recognize Lys63-linked diubiquitin, a model of AMSH244 bound to Lys63-linked ubiquitin dimer was generated by superimposing AMSH244 onto the previously determined X-ray structure of the DUB domain of AMSH-LP^{E292A} bound to Lys63-linked ubiquitin dimer (PDB code: 2ZNV, reference 25) (Fig. 5). The overall structure of AMSH244 is similar to ubiquitin-bound DUB domain of AMSH-LP with an rmsd of 0.93Å of Cα atoms. This level of structural similarity suggests that the mode of diubiquitin binding seen in the model would be preserved in the actual structure of AMSH244 bound to Lys63-linked diubiquitin.

Proximal ubiquitin recognition—The residues that are involved in proximal ubiquitin recognition are completely conserved between AMSH and AMSH-LP (Fig. 6a) (in diubiquitin, a lysine residue of one ubiquitin, called the proximal ubiquitin, is linked by isopeptide bond to the C-terminal carboxylate group of another ubiquitin, called the distal ubiquitin). Figure 7 shows the superposition of AMSH244 with AMSH-LP's DUB domain, both in its free form and bound to Lys63-linked diubiquitin. The position and orientation of the conserved residues in the proximal site, Phe343, Phe395, and Thr341 of AMSH (Phe355, Phe407, and Thr353 in AMSH-LP) (Fig. 7), which are known to aid in aligning the isopeptide bond and making specific contacts with the two residues flanking Lys63 of proximal ubiquitin, Gln62 and Glu64, are very similar in both the enzymes, consistent with the Lys63-linkage specificity exhibited by both. The only difference seen from the modeling is the orientation of Ser346 (Ser358 in AMSH-LP). The Oγ atom of AMSH's Ser346 is pointing away from Glu64 of the proximal ubiquitin (Fig. 7), contrary to the equivalent

residue in both free and ubiquitin-bound AMSH-LP, which are at distances of 2.4 Å and 2.6 Å from proximal ubiquitin's Glu64, respectively.

Distal ubiquitin recognition—Inspection of the model representing AMSH244 bound to Lys63-linked diubiquitin reveals some significant differences from AMSH-LP in the residues that are supposed to be used for interactions with the distal ubiquitin. First, we see that Asp324 of AMSH-LP, which makes hydrogen-bonding and electrostatic interactions with Arg74 of the distal ubiquitin, is replaced by Asn312 in AMSH, which, though may be able to retain the hydrogen-bonding interaction, will lack the electrostatic component leading probably to weaker binding. While this change can be considered conservative to some extent (Fig. 8), the same cannot be said about the other two substitutions, AMSH's Thr313 and Glu316 in place of the equivalent Met325 and Val328, respectively, in AMSH-LP (Fig. 6b). Met325 and Val328 are making a number of important van der Waals contacts with hydrophobic residues on the C-terminus of the distal ubiquitin in AMSH-LP, particularly with residues Leu73, Val70 and Ile44 (Fig. 8). Threonine is significantly more polar than methionine, but can still provide van der Waals contact using its $\text{C}\gamma\text{H}_3$ group. Glutamate, however, cannot substitute readily for valine. On the other hand, Glu316 is appropriately positioned for electrostatic and polar interactions with Arg42 and Gln49 of the distal ubiquitin as seen in our model (Fig. 8). Crystal structure analysis of E280A mutant of AMSH244 bound to Lys63-linked diubiquitin should be sought to verify if such interactions actually occur.

In order to understand the consequences of these substitutions on the kinetic properties of AMSH, we determined the kinetic parameters of AMSH244 and compared them with those of the DUB domain of AMSH-LP. AMSH244 is catalytically less efficient than the DUB domain of AMSH-LP with Lys63-linked diubiquitin as the substrate, with most of the difference being contributed by a k_{cat} that is 7-fold less, but with a slightly better K_M (2-fold lower) (Table 2 and Supplementary Fig. 4). These data suggest that the substitutions in the distal site of AMSH may lead to weaker interactions with the substrate in the transition state of the reaction being catalyzed, whereas the interactions in the ground-state enzyme-substrate complex is relatively less affected.

Comparison of thermodynamic stability of the catalytic domains of AMSH and AMSH-LP

To explore further the similarity or the lack thereof between the catalytic domains of AMSH and AMSH-LP, we wondered if there would be any difference in thermodynamic stability between the two proteins. The unfolding of the catalytic constructs of both AMSH and AMSH-LP was induced by guanidine hydrochloride (GdHCl) and monitored at 220 nm using circular dichroism spectroscopy (CD). Both proteins reveal a loss in secondary structure with increasing concentration of GdHCl (Supplementary Fig. 5); however, it was observed that AMSH is less stable towards GdHCl than AMSH-LP, with a midpoint of transition at 2.7 and 3.5 M GdHCl for AMSH and AMSH-LP, respectively (Table 3). Using the linear extrapolation method described previously,³⁷ $\Delta G_{\text{H}_2\text{O}}$ of AMSH244 was found to be 1.2 kcal.mol⁻¹ less than that of the DUB domain of AMSH-LP, indicating the former is thermodynamically less stable than the latter (Fig. 9 and Table 3). We propose that this difference in stability implies a difference in flexibility between the catalytic domains of the two proteins. A more stable protein is likely to have better close packing of side chains making it more rigid, whereas a less stable protein, presumably because it has lesser amount of close packing, can tend to be more plastic, a property that may make AMSH more suitable for working in the context of a number different type of protein-protein complexes, such as ESCRT-0 and ESCRT-III. Whether this has anything to do with the biological differences between the two proteins is worth investigating in future.

Discussion

AMSH is one of the two DUBs that are employed to regulate the functionality of the ESCRT machinery by counteracting the ubiquitination of cell-surface receptors that are directed towards degradation by the lysosome. Studies have clearly indicated that AMSH is important for receptor turnover and for maintaining free ubiquitin pool within the cell; however, a clearly defined role is yet to be assigned. What is clearly known is that AMSH has specificity towards the Lys63-linked ubiquitin chains.^{9; 13} DUB specificity for one linkage over another was not understood until the structural basis for Lys63-linked polyubiquitin cleavage was revealed from the crystal structure of the DUB domain of AMSH-LP bound to Lys63-linked ubiquitin dimer.²⁵ The structure shows how both the proximal and distal ubiquitins are recognized; however, AMSH-LP's role in the cell is still unknown. Although it shares 54% identity and 75% similarity in its JAMM domain with AMSH,²⁶ AMSH-LP is not functionally redundant, based on its inability to bind to the ESCRT complex.^{9; 26} The study presented here seeks to compare the catalytic domains of AMSH and AMSH-LP structurally and thermodynamically to provide further insight into AMSH's sole specificity within the ESCRT machinery.

We have determined the structure of two constructs representing the catalytic domain of AMSH, AMSH244 and AMSH219^{E280A}. The structure of AMSH244 confirms that AMSH is indeed a zinc metalloprotease of the JAMM family of DUBs with two Zn²⁺ ions, one at active site and the other playing a structural role in supporting the placement of ubiquitin-binding residues in Ins-2 in a position appropriate for isopeptide bond recognition. The structure of AMSH219^{E280A} provided a surprising observation. We anticipated the loss of the active-site zinc due to the E280A mutation, because Glu280, according to the known mechanism of thermolysin-type zinc-dependent-metalloproteases, is responsible for holding a water molecule, the one that functions as the nucleophile, at a position such that it is able to serve as the fourth ligand in the coordination sphere of the catalytic zinc. Instead, we see clear density for the zinc. Asp309, on an adjacent loop (from Ins-1), has moved in place to become the fourth residue to coordinate the zinc (Fig. 3). The ability of the enzyme to counteract the loss of the glutamate by still retaining its zinc suggests a form of structural plasticity, allowing for conformational freedom. A similar mutation was incorporated in AMSH-LP for the purpose of co-crystallization with diubiquitin, and the authors stated that they do not see any electron density corresponding to the active site zinc.²⁵ Interestingly, Asp309 and its equivalent residue in AMSH-LP have to have a certain amount of flexibility; otherwise, the active site will not be accessible for the diubiquitin substrate. The question that if the observation pertaining to Asp309 in AMSH219^{E280A} is due to more flexibility of Ins-1 in AMSH than in AMSH-LP cannot be answered from the data presented here. It is possible that the same rearrangement of the equivalent aspartate in AMSH-LP might have been observed in the structure of ubiquitin-free AMSH^{E292A}.

Another interesting finding we see from the structure of AMSH219^{E280A} is the presence of a potential disulfide bond adjacent to the active site (Supplementary Fig. 3). It should be noted that a disulfide bond next to the putative active-site zinc was also observed in the structure the JAMM-domain protein AfJAMM, a cytosolic protein from *Archaeoglobus fulgidus*.³² The possibility that the cysteine pair in AMSH is capable of existing in reduced and oxidized forms, reduced when in cytosol and oxidized when associated with endosomes, cannot be ruled out. This feature may contribute to or result from the structural plasticity of the enzyme. However, we cannot say with any degree of certainty if this reversible disulfide bridge is unique to AMSH, and if it is, whether it contributes to the biological difference between AMSH and AMSH-LP, two very similar proteins. It is not clear why the DUB domain of AMSH not AMSH-LP should have a disulfide, or is it common to both of them (DTT was also present in the crystallization buffer and throughout purification in the case of

the AMSH-LP DUB domain). If it happens to be unique to AMSH, it could perhaps be linked to its association with endosomes, which have been shown to be oxidizing.³⁸

Moving forward, we sought to directly compare the catalytic domains of AMSH and AMSH-LP structurally and thermodynamically. It is expected that homologous proteins that share 75% sequence similarity to have a similar fold and overall architecture. On par with this expectation, the catalytic domains of AMSH and AMSH-LP have a nearly identical fold and superimposable zinc coordination sites (Fig. 4). Furthermore, AUC experiments confirmed that both the proteins are globular and monomeric in solution (Fig. 1). Thus, structurally, we cannot separate the two proteins from one another. Thermodynamically, however, we see a difference between the two. Chaotrope-induced unfolding with GdHCl indicates that the catalytic domain of AMSH is thermodynamically less stable than that of AMSH-LP (Fig. 9). Analysis of primary sequences of the proteins does reveal some differences in certain key positions, such as in the hydrophobic core (residues Val259, Val363, and Phe376 in AMSH are substituted by residues Leu271, Ile375, Ile388 in AMSH-LP, respectively), which could contribute to the difference in thermodynamic stability; however, this needs to be investigated in future.

We have sought to obtain structural insight into AMSH's ability to recognize Lys63-linked diubiquitin through modeling. Only recently was Lys63-linkage specificity understood from the structural analysis of the DUB domain of AMSH-LP bound Lys63-linked diubiquitin.²⁵ This structure reveals interactions between the enzyme and both the distal and proximal ubiquitins, but the basis for specificity arises from residues in the proximal site of the enzyme interacting with the proximal ubiquitin, despite a more extensive interaction with the distal ubiquitin (a significantly more accessible surface area is buried upon interaction with distal than proximal ubiquitin).²⁵ Overall, AMSH adopts very similar interactions when modeled according to the ubiquitin-bound structure of AMSH-LP. As indicated by our model, the proximal binding site is highly conserved between the two proteins (Fig. 6 and 7), consistent with Lys63-linkage specificity of AMSH as well. The only noticeable difference is the orientation of a critical serine residue, Ser346, which we propose will be induced to adopt an orientation seen in AMSH-LP upon binding to ubiquitin in the proximal site, consistent with the proposed plasticity of the enzyme.

Seeing that AMSH may have specific interactions with the proximal ubiquitin means that this enzyme will be unable to cleave a monoubiquitinated receptor because the last ubiquitin moiety is directly attached to the receptor, which is unlikely to have the same three-residue peptide sequence that occurs in ubiquitin and in the same conformation. Our analysis therefore seems to indicate that AMSH would only be a chain-modifying enzyme, having the ability to shorten a Lys63-linked polymeric chain, leaving the last ubiquitin still attached to the substrate. However, it has been proposed that AMSH acts to oppose E3-ligase activity on the receptor through deubiquitination, presumably by the complete removal of ubiquitin tag, at an early stage of endosomal sorting process thereby promoting recycling of the receptor.⁹ Consistent with this model, siRNA-mediated knockdown of AMSH leads to increased rate of EGFR degradation, presumably due to enhanced targeting to the lysosomal pathway.¹³ This implies that AMSH is able to completely deubiquitinate EGFR, since monoubiquitinated EGFR can still be targeted to lysosome.⁹ This apparent paradox can be resolved if we allow for a rearrangement of the active site of the enzyme, aided once again by its structural plasticity, brought about by its binding partners on the ESCRT machinery. Further studies are needed to confirm AMSH's ability to cleave a monoubiquitinated substrate to provide clarity to this hypothesis.

Our analysis reveals a difference in the distal site residues between the two enzymes. The rather non-polar face of the distal site in AMSH-LP is replaced by a polar one in AMSH

(Fig. 8). Considering the high level of overall similarity between the two proteins, this difference is striking. Our kinetic data appears to indicate that the distal-site differences may translate into a reduced affinity between the enzyme and the substrate in the transition-state of the reaction, rather than in the Michaelis complex, making the catalytic domain of AMSH less efficient than that of AMSH-LP. More work needs to be done in the future to see if the difference in kinetic properties we are observing here is related to the biological difference between AMSH and AMSH-LP. It should be noted that AMSH undergoes activation upon recruitment to the ECSRT complex,⁹ the functional significance of which has not been understood yet. If this activation is important for its biological function, one may speculate that the substitution seen in AMSH's distal site are 'designed' to keep the enzyme in a latent state.

In conclusion, we have determined the structure of the catalytic domain of the endosome-associated DUB AMSH and found that, though structurally nearly identical to the catalytic domain AMSH-LP, it possesses significantly less thermodynamically stability than the latter, an observation we propose implies greater structural plasticity for the catalytic domain of AMSH. This property may, in addition to having a functional SH3-binding motif and MIT domain, endow the enzyme with ability to take part in the ECSRT-mediated functions.

Materials and Methods

Cloning, Expression, and Purification

The DNA encoding AMSH244 was amplified by PCR using a plasmid that contained full-length DNA as the template (pGEX-6P1-AMSH, a kind gift from Sylvie Urbe, University of Liverpool, UK) 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 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).

AMSH219 was subcloned into a pGEX-6P1 expression vector as described above. The Glu280Ala mutation was introduced into the gene by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. DNA sequencing confirmed the presence of the mutation. AMSH219^{E280A} was purified using standard GST affinity followed by SEC (Superdex S200 column). The DUB domain of AMSH-LP (a kind gift from Prof. Shuya Fukai, The University of Tokyo, Japan) was purified as described in reference 25.

Crystallization and structure determination

Crystals of AMSH244 were grown at room temperature by the sitting drop vapor diffusion method from a mother liquor containing 0.2M sodium malonate pH 7.0, 20% PEG 3350, and 5% PEG 400. Crystals of AMSH219^{E280A} were grown at room temperature by the sitting drop vapor diffusion method from a mother liquor containing 0.1M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, 15% PEG 6000, and 5% 2-methyl-2,4-pentanediol (MPD). Crystals were briefly soaked in cryoprotectant solution (20% ethylene glycol) and then plunged into liquid nitrogen for flash cooling. X-ray diffraction data were collected at 100 K on a Mar300 CCD detector (Mar USA) at the beamline 23-ID-D at the Advanced Photon Source at Argonne National Laboratory. The data were processed using HKL2000.

The crystal structure of AMSH244 was solved by molecular replacement (MR) using a homology model of the catalytic domain of AMSH, built based on the crystal structure of the catalytic domain of AMSH-LP using SWISS-MODEL homology modeling server,³⁹ as the search model. Refinement and multiple rounds of model building were carried out using Phenix²⁹ and Coot,⁴⁰ respectively, yielding a final model with a crystallographic R factor (R_{crys}) of 19.3% and free R factor (R_{free}) of 22.9%. Residues Thr250 of chain C, Ile251 of chain D, Pro402 of chain E, and Glu314, Asn315, and Glu316 of chain G are within disallowed regions of the Ramachandran plot. All of these residues were in weak or no density at 1σ except Glu314 and Thr250. The structure of AMSH219^{E280A} was also determined by molecular replacement using AMSH244 structure as the search model. Refinement and model building were carried out in the same method as described above yielding R_{crys} of 18.0% and of R_{free} 20.5% (Table 1). Graphical analysis was carried out using PyMOL (DeLano Scientific).

Analytical ultracentrifugation

To characterize the oligomeric state of AMSH and AMSH-LP, sedimentation velocity experiments were conducted at 50,000 rpm using both the Beckman-Coulter XLI and XLA. (Beckman-Coulter, Fullerton, CA, USA). The samples were monitored by both absorbance and interference optics at 280 and 675 nm, respectively. The proteins were dialyzed in 50 mM Tris-HCl pH 7.6, 50 mM NaCl and 1 mM DTT. Three concentration series were conducted to evaluate the formation of higher order species, AMSH244 at 30, 60, and 120 μM , AMSH-LP at 25, 47, and 94 μM . The sedimentation coefficients and apparent molecular weights were calculated from size distribution analyses ($c(s)$) using SEDFIT v. 12.0^{41; 42}. The solvent density, viscosity and partial specific volume of the proteins were calculated using SEDNTERP v. 1.09 (<http://www.rasmb.bbri.org/rasmb/windows/sednterp-philos>).

Guanidine Melt using circular dichroism spectroscopy

The stability of the folded state of AMSH244 and AMSH-LP towards GdHCl was determined using 8M stock concentrations of GdHCl (Sigma). Varying concentrations of GdHCl were added to the protein (0.2 mg.mL⁻¹) 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 4 scans (50 nm.min⁻¹ scan speed, with a 8 second time constant) and corrected by subtraction of a spectrum of the buffer alone. Mean residue molar ellipticity values were calculated using the following equation:

$$[\theta] = \frac{\theta * 100 * M}{C * l * n}$$

Where θ is the ellipticity in degrees, l is the optical path in cm, C is the concentration in mg/ml, 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 deg.cm².dmol⁻¹. Unfolding curves were analyzed using a two-state unfolding model, using linear extrapolation to obtain the ΔG value in the absence of GdHCl³⁷.

Determination of kinetic parameters and DUB Assay

Human ubiquitin was subcloned into pGEX-6P1, 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 (reference 25). The reaction was incubated at 37° for 4 hours, and then 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) to obtain Lys63-diubiquitin.

The *in vitro* DUB assay was carried out by incubating AMSH244, wild-type AMSH219, wild-type AMSH219 expressed and purified in the absence of DTT (AMSH219^{-DTT}), AMSH219^{C282A} (expressed and purified in the absence of DTT), and AMSH219^{E280A} to a final enzyme concentration of 100 nM with 20 μM Lys63-diubiquitin in a total reaction volume of 20 μL. All reactions were carried out in reaction buffer (50 mM TRIS-HCl pH 7.0, 25 mM KCl, 5 mM MgCl₂, and 1 mM DTT) for one hour at 20°C, except AMSH219^{-DTT} and AMSH219^{C282A}, which were incubated in the same reaction buffer without DTT. The reaction was quenched by the addition of 5X SDS-PAGE sample buffer followed by boiling, and then analyzed by SDS-PAGE.

The kinetic parameters were determined by incubating the enzyme (100 nM of AMSH244 and 25 nM of the DUB domain of AMSH-LP) with four concentrations of diubiquitin ranging from 20–100 μM in reaction buffer (50 mM TRIS-HCl pH 7.0, 25 mM KCl, 5 mM MgCl₂, and 1 mM DTT). The reaction was carried out at 20°C for 15 minutes for initial velocity measurements. Reaction tubes were quenched by the addition of 5X 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 software (<http://rsb.info.nih.gov/ij/>). Ubiquitin standards ranging from 4–12.3 μg were used to draw a calibration plot, which was used to quantify the amount of ubiquitin produced. Kinetic parameters were calculated by fitting the data in SigmaPlot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AMSH	associated molecule with the SH3 domain of STAM
DUBs	deubiquitinating enzymes
AMSH-LP	AMSH-like protein
USP8	ubiquitin specific protease 8
EGFR	epidermal growth factor receptor
SH3	Src homology 3 domain
ESCRT	endosomal sorting complexes required for transport
MVB	multivesicular bodies
GdHCl	guanidine hydrochloride
AUC	analytical ultracentrifugation
CD	circular dichroism

DTT dithiothreitol
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

Acknowledgments

The authors acknowledge Sylvie Urbé (University of Liverpool) for her generous gift of the AMSH full-length plasmid from which we were able to carry out our studies. We thank Prof. Shuya Fukai (University of Tokyo) for providing GST-fused AMSH-LP. We thank Pavel Afonine for his helpful suggestions on refinement and Alex Tuttle for cloning GST-tagged ubiquitin. We also thank Venugopalan Nagarajan and Michael Becker at beam line 23-ID-D of Advanced Photon Source for assistance with data collection. The General Medicine and Cancer Institutes Collaborative Access Team has been funded in whole or in part with Federal funds from the National Cancer Institute (Y1-CO-1020) and the National Institute of General Medical Science (Y1-GM-1104). Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under Contract DE-AC02-06CH11357. Financial support from the National Institutes of Health (1R01RR026273) is gratefully acknowledged.

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Figure 1.

c(s) distribution plots of the DUB domains of AMSH244 and AMSH-LP as determined by AUC. (a) c(s) distribution plot of AMSH244 using interference optics shows that the majority discrete species at 2.1S is a globular, monomeric protein. The protein concentrations used were 30 μ M (large dashes), 60 μ M (solid line), and 120 μ M (small dashes). (b) c(s) distribution plot of AMSH-LP using absorbance at 280nm shows that the majority discrete species at 2.03S is a globular, monomeric protein. The protein concentrations used were 25 μ M (large dashes), 47 μ M (solid line), 94 μ M (small dashes).

Figure 2.

The structure of the catalytic domain of AMSH. (a) Ribbon representation of the X-ray structure of AMSH244. (b) Expanded view of the active-site zinc coordination. (c) Expanded view of the second zinc coordination site. The coordinating residues are shown as sticks, with carbon shown in green, oxygen in red, nitrogen in blue and sulfur in yellow. Zinc is shown as grey sphere.

Figure 3. Superposition of the active sites of AMSH244 and AMSH219^{E280A}. AMSH244 is shown in green and AMSH219^{E280A} in pink. The zinc-coordinating residues in AMSH219^{E280A} are outlined in electron density ($2F_o - F_c$) at 1σ .

Figure 4. Structural comparison of the catalytic domains of AMSH and AMSH-LP. (a) Backbone superposition of AMSH (shown in green) and AMSH-LP (shown in cyan). (b) Expanded view of the zinc-coordinating residues in the active site. (c) Expanded view of the residues coordinating the second zinc. The coordinating residues are shown as sticks, with carbon shown in green and cyan, oxygen in red, nitrogen in blue and sulfur in yellow. Zinc is shown as grey sphere.

Figure 5.

A model of AMSH244 bound to Lys63-linked ubiquitin dimer. Backbone superposition of AMSH244 (green), the DUB domain of AMSH-LP (cyan), and the DUB domain of AMSH-LP bound to Lys63-linked diubiquitin (gray). The proximal ubiquitin and distal ubiquitin are shown in magenta and yellow ribbon, respectively.

(a)

	341						346	395
AMSH_human	T	A	F	L	S	S		F
AMSH_Rat	T	A	F	L	S	S		F
AMSH_Mouse	T	A	F	L	S	S		F
AMSH_Pig	T	A	F	L	S	S		F
AMSH_Bovine	T	A	F	L	S	S		F
AMSH_Zebrafish	T	A	F	L	S	S		F
AMSH-LP_human	T	A	F	L	S	S		F
Rpn11_human	G	C	W	L	S	G		T

(b)

	312							320	358
AMSH_human	N	T	E	N	E	E	E	L	F
AMSH_Rat	H	T	E	N	E	E	E	I	F
AMSH_Mouse	H	T	E	N	E	E	E	I	F
AMSH_Pig	N	T	E	N	E	E	E	L	F
AMSH_Bovine	N	T	E	N	E	E	E	L	F
AMSH_Zebrafish	D	T	E	N	E	E	E	L	F
AMSH-LP_human	D	M	E	N	V	E	E	L	F
Rpn11_human	D	P	V	F	Q	A	K	M	L

Figure 6.

Sequence alignment of AMSH from different species including AMSH-LP and RPN11. (a)

Sequence alignment comparing the residues implicated in the proximal ubiquitin binding.

(b) Sequence alignment comparing the residues implicated in the distal ubiquitin binding.

Figure 7.

A view of the residues involved in the proximal ubiquitin recognition. Superposition of AMSH244 (green ribbon), the DUB domain of AMSH-LP (cyan ribbon) and the DUB domain of AMSH-LP bound to Lys63-linked diubiquitin (gray ribbon). Residues from the proximal ubiquitin making contact at the proximal site are shown in magenta.

Figure 8.

A view of the residues involved in the distal ubiquitin recognition. Superposition of AMSH244 (green ribbon) and the DUB domain of AMSH-LP bound to Lys63-linked diubiquitin (gray ribbon). The distal ubiquitin is shown in yellow. Hydrogen bonding interactions are shown as black dashes and van der Waals interactions are shown as blue dashes.

Figure 9.

Fraction unfolded curves comparing the stability of the catalytic domains of AMSH and AMSH-LP with increasing concentrations of GdHCl followed by circular dichroism spectroscopy (CD) at 220nm. Data for AMSH244 is shown in filled circles and that for the DUB domain of AMSH-LP is shown in open circles.

Table 1

Crystallographic Table

	AMSH244	AMSH219 ^{E280A}
Residues	244–424	219–424
Data Collection		
Space Group	C121	P4 ₃ 2 ₁ 2
Cell dimensions		
a, b, c (Å)	203.5, 67.4, 113.2	53.4, 53.4, 128.8
α, β, γ (°)	90, 112.4, 90	90, 90, 90
Resolution (Å)	50.0 – 2.50 (2.59 – 2.50)	50 – 1.68 (1.71 – 1.68)
[*] R_{sym}	8.5 (54.3)	7.1 (60.4)
I/σI	12.6 (2.04)	28.3 (3.7)
Unique reflections	48,879	22,375
Completeness (%)	99.5 (97.8)	99.6 (100.0)
Redundancy	3.5 (3.0)	12.1 (12.2)
Refinement		
Resolution	47.2 – 2.50	33.5 – 1.67
[†] R_{crys}/R_{free}	19.3/22.9	18.0/20.5
Number of atoms	9,560	1,398
Number of ions	14	2
rmsd		
Bond lengths (Å)	0.008	0.006
Bond angles (°)	1.4	0.99
Ramachandran Plot		
Preferred (%)	96.9	98.9
Allowed (%)	2.7	1.1
Disallowed (%)	0.4	0.0
B-factors (Å²)		
Protein	61.5	36.2
Solvent	49.9	45.4
Ions	54.9	23.5

^{*} $R_{\text{sym}} = \sum \sum |I_{\text{hkl}} - I_{\text{hkl}}(j)| / \sum I_{\text{hkl}}$, where $I_{\text{hkl}}(j)$ is the observed intensity and I_{hkl} is the final average intensity.

[†] $R_{\text{crys}} = \sum ||F_{\text{Obs}}| - |F_{\text{Calc}}|| / \sum |F_{\text{Obs}}|$ and $R_{\text{free}} = \sum ||F_{\text{Obs}}| - |F_{\text{Calc}}|| / \sum |F_{\text{Obs}}|$, where R_{free} and R_{crys} are calculated using a randomly selected test set of 5% of the data and all reflections excluding the 5% test data, respectively. Numbers in parentheses are for the high-resolution shell.

Table 2

Table of kinetic parameters

Enzyme	k_{cat} (s ⁻¹)	K_M (μM)	$k_{cat} / K_M \times 10^{-3}$ (μM ⁻¹ s ⁻¹)
AMSH244	1.4 ± 0.1	32.0 ± 5.3	43.8
AMSH-LP DUB	10.3 ± 0.3	66.2 ± 4.8	155.6
	0.860 ± 0.0654 *	71.8 ± 6.3 *	12.0 *

* Note: Kinetic parameters reported by Sato et al (reference 25). Differences in the values are presumably due to the difference in substrates used in the activity assay.

Table 3

Stability Data Table

	ΔG_{H2O} (kcal.mol ⁻¹)	[GdHCl] _{0.5} (M)	m (kcal.mol ⁻¹ .M ⁻¹)
AMSH244	3.7	2.7	-1.4
AMSH-LP DUB	4.9	3.5	-1.4